INFLUENCES OF DIETARY POLYUNSATURATED FATTY ACIDS ON TISSUE FATTY ACID COMPOSITION AND EICOSANOID PRODUCTION IN ATLANTIC SALMON *(Salmo salar)*

Thesis presented for the degree of Doctor of Philosophy

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by

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This thesis was composed by myself and the results presented are a product of my own work.

TO

MY FAMILY

Contents

ABSTRACT

1. The literature has been reviewed with respect to the dietary intake and subsequent metabolism of polyunsaturated fatty acids (PUFA), of both the n-6 and n-3 series, in teleost fish. Particular emphasis has been made to the physiological roles of PUFA with respect to cell membrane function and eicosanoid production.

2. Atlantic salmon post-smolts were fed practical-type diets, based on fish meal, in three separate dietary experiments of 10-16 weeks duration. The first trial compared dietary lipid supplied either as fish oil (FO) or as sunflower oil (SO) with the diets having an n-3/n-6 PUFA ratio of 9.4 and 0.2 respectively. The second trial used diets formulated with blends of FO, SO, grape seed oil and safflower oil to provide linoleic acid at 10, 25 and 45% of total dietary fatty acids. The third trial was similar to the first but with an additional diet in which the lipid component was supplied by linseed oil (LO). All diets satisfied the nutritional requirements of salmonid fish for n-3 PUFA. There were no statisticallly significant differences in final weights between dietary treatments in the third trial. However, in the second trial fish fed the intermediate level of linoleic acid (25%) attained a significantly higher final weight compared to both other treatments while fish fed the highest level of linoleic acid (45%) had significantly lower final weights compared to both other treatments. In the first trial the effect of diet on growth (weight gain) could not be ascertained as the initial weights of the fish were significantly different.

3. A number of fish fed SO developed severe cardiac lesions which caused thinning of the ventricular wall and heart muscle necrosis. In addition the fish fed diets containing SO were susceptible to a transportation-induced shock syndrome that resulted in 30% mortality.

4. Incorporation of linoleic acid (18:2n-6) into membrane phospholipids increased in response to dietary intake with fish fed SO having increased levels of 18:2n-6 (up to 15-fold), 20:2n-6 (up to 12-fold), 20:3n-6 (up to 25-fold) and arachidonic acid (AA;

20:4n-6) (up to 3-fold), and decreased levels of eicosapentaenoic acid (EPA; 20:5n-3) (up to 3-fold). The ratio of n-3/n-6 PUFA was decreased (up to 4-fold) and the 20:4n-6/20:5n-3 ratio increased (up to 9-fold) in membrane phospholipids from fish fed SO compared to those fed fish oil. While the tissue phospholipids from fish fed LO had increased levels of 18:2n-6, 20:2n-6 and 20:3n-6, the levels of AA, 22:4n-6 and 22:5n-6 were similar to or significantly reduced compared to fish fed FO. Membrane phospholipids from fish fed LO also had increased 18:3n-3 and 20:4n-3 compared to both other treatments while in some tissues and phospholipid classes EPA was increased compared to fish fed FO.

5. These dietary induced changes in phospholipid eicosanoid precursor ratio were reflected in altered eicosanoid production. In gill cells, stimulated with the calcium ionophore A23187, 12-hydroxy-8, 10, 14, 17-eicosapentaenoic acid (12-HEPE) was the major 12-lipoxygenase product in fish fed FO. In stimulated gill cells from fish fed SO and LO, 12-HEPE, 12-hydroxy-5, 8, 10, 14-eicosatetraenoic acid (12-HETE), 14-hydroxy-4, 7, 10, 13, 16, 19-docosahexaenoic acid (14-HDHE) and thromboxane B_2 (TXB_2) were all decreased compared to fish fed FO. However, the ratio of 12-HETE/12-HEPE was significantly elevated in stimulated gill cells from SO-fed fish compared to both other treatments. In stimulated blood leucocytes leukotriene B_4 (LTB_4), 12-HETE and TXB_2 were significantly increased while LTB_5 and 12-HEPE were significantly decreased in fish fed SO compared to those fed FO. Blood leucocytes from fish fed LO produced less TXB_2 compared to fish fed SO and prostaglandin E_2 was reduced compared to both other treatments. In isolated cardiac myocytes stimulated with A23187, TXB_2 production was increased in SO-fed fish compared to those fed FO.

6. The activity of cardiac sarcoplasmic reticulum Ca^{2+}-Mg^{2+}ATPase was not affected by dietary treatment.

7. An established cell line derived from chum salmon heart (CHH-1) was utilised to

study PUFA metabolism. The CHH-1 cells exhibited considerable Δ6 desaturase activity but showed no preference towards n-3 over n-6 PUFA. CHH-1 cells did exhibit significant Δ5 desaturase activity which showed a preference towards n-3 PUFA. No Δ4 desaturation activity was observed. Elongation of C_{20} PUFA was especially active in CHH-1 cells with C_{22} PUFA being specifically incorporated into phosphatidylethanolamine (PE) and phosphatidylserine (PS). CHH-1 cells supplemented with 20:3n-6 showed reduced growth rate, cell death and unusual pycnotic appearance, compared to those supplemented with other PUFA.

8. The lipid compositions of hearts and livers from wild and farmed parr and pre-smolts were analysed and compared. The fatty acid compositions of triacylglycerols (TAG) and phospholipids from both farmed parr and pre-smolts contained greater amounts of monoenoic fatty acids compared to their wild counterparts. TAG, phosphatidylcholine (PC) and PE from heart and liver of wild fish contained more 18:2n-6 and AA compared to farmed fish. Linolenic acid, EPA and 22:5n-3 were increased in hearts and livers of wild fish compared to farmed. Docosahexaenoic acid (DHA; 22:6n-3) levels were higher in heart and liver of farmed fish, particularly in heart PC, PS and TAG. The n-3/n-6 PUFA ratio was generally lower in wild compared to farmed fish, largely due to higher n-6 PUFA, in particular AA, in wild fish.

9. The results are discussed with respect to the competitive interactions between PUFA of the n-6 and n-3 series which determine the fatty acid compositions of membrane phospholipids in salmon. The ratio of n-3/n-6 PUFA in membrane phospholipids, and in particular the ratio of AA/EPA, appears important in terms of membrane physiology and biochemistry, eicosanoid production and the development of cardiac histopathological lesions.

ABBREVIATIONS

The following abbreviations were used throughout the text:

AA, arachidonic acid

BHT, butylated hydroxytoluene

BSA, bovine serum albumin

CL, cardiolipin

DHA, docosahexaenoic acid

EFA, essential fatty acid

EPA, eicosapentaenoic acid

FCS, fetal calf serum

FO, fish oil

FSH, follicle stimulating hormone

GRH, gonadotrophin-releasing hormone

HDHE, hydroxy-4, 7, 10, 13, 16, 19-docosahexaenoic acid

HEPE, hydroxy-5, 8, 10, 14, 17-eicosapentaenoic acid

HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)

HETE, hydroxy-5, 8, 10, 14-eicosatetraenoic acid

HPLC, high-performance liquid chromatography

HPTLC, high-performance thin-layer chromatography

HUFA, highly unsaturated fatty acid

LA, linoleic acid

LH, luteinising hormone

LO, linseed oil

LTB_4, leukotriene B_4

LTB_5, leukotriene B_5

NL, neutral lipid

PA, phosphatidic acid

PC, phosphatidylcholine

PD, pancreas disease

PE, phosphatidylethanolamine

PG, prostaglandin

PGA, prostaglandin A

PGD, prostaglandin D

PGE_2, prostaglandin E_2

$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$

$PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$

PGI_2, prostacyclin

PI, phosphatidylinositol

PL, polar lipid

PLA, phospholipase A

PLC, phospholipase C

PS, phosphatidylserine

PUFA, polyunsaturated fatty acid

SD, standard deviation

SM, sphingomyelin

SR, sarcoplasmic reticulum

SO, sunflower oil

TAG, triacylglycerol

TXA_2, thromboxane

TXB_2, thromboxane B_2

TLC, thin-layer chromatography

UV, ultraviolet

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Section 1

GENERAL INTRODUCTION

Section 1: Introduction

1:1 Lipid classes

The term 'lipid' generally describes a group of biological substances distinct from proteins, carbohydrates and nucleic acids, which are not water soluble but are soluble in organic solvents such as chloroform, hexane, diethylether etc. More specifically the term describes compounds fulfilling the aforementioned solubility characteristics and which contain esterified fatty acids. Lipids so defined can be divided into a) neutral lipids, including triacylglycerols (TAG), partial acyl glycerols, alkyldiacylglycerols, wax esters and sterol esters, and b) polar lipids, including glycerophospholipids, sphingolipids and glyceroglycolipids. The neutral lipids are generally involved in lipid storage whereas the polar lipids provide the structural components of cell membranes. Among these structural lipids the glycerophospholipids are quantitatively the most important in animal cell membranes and upon hydrolysis yield fatty acids, glycerol and a polar head group. The head group contains phosphate simultaneously esterified to a small molecule e.g. an amine, an amino acid or an alcohol esterified to the *sn*-3 position of glycerol, while the fatty acids are esterified at the *sn*-1 and *sn*-2 positions of glycerol (Gurr and Harwood, 1991). The major glycerophospholipid components of animal cell membranes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and cardiolipin (CL).

1:2 Fatty acid structure and nomenclature

The area of fatty acid nomenclature can be something of a minefield for the uninitiated in that a variety of trivial, systematic and shorthand forms are all commonly used. For example, palmitic acid is a saturated fatty acid containing 16 carbon atoms in a straight chain (Fig. 1.2.1) and is given the shorthand nomenclature 16:0. The number before the colon gives the carbon number while

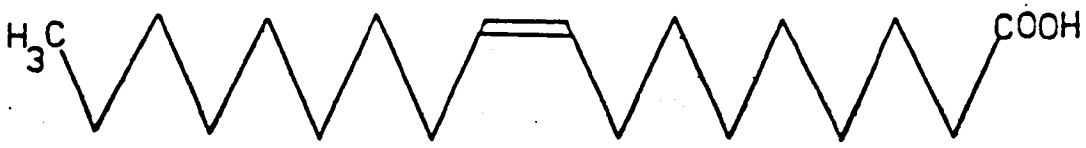
Fig. 1:2:1 FATTY ACID CLASSES AND NOMENCLATURE

SATURATED

e.g. 16:0; hexadecanoic acid; palmitic acid

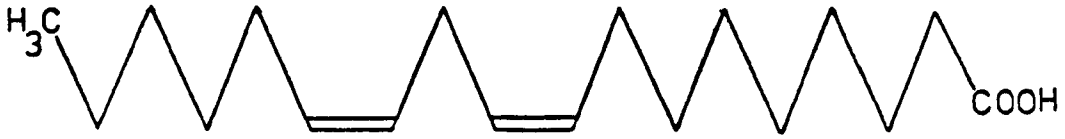
MONOUNSATURATED

e.g. 18:1n-9; cis-9-octadecenoic acid; oleic acid

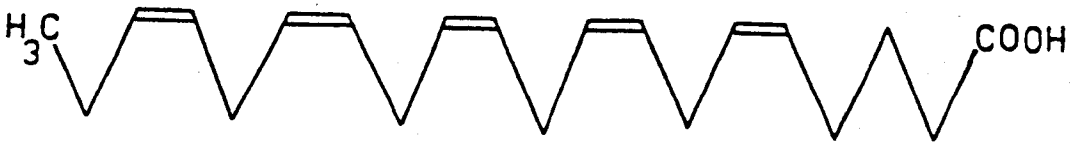


POLYUNSATURATED

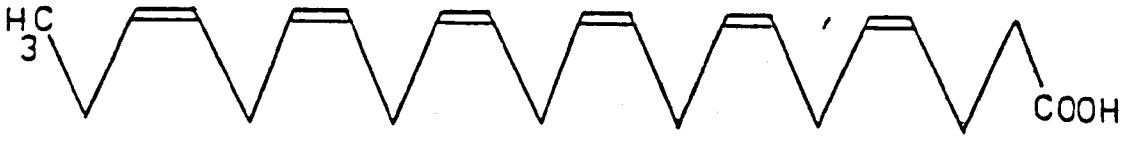
e.g. 18:2n-6; Δ 9, 12-octadecadienoic acid; linoleic acid



e.g. 20:5n-3; Δ 5,8,11,14,17-eicosapentaenoic acid



e.g. 22:6n-3; Δ 4,7,10,13,16,19-docosahexaenoic acid



the number after the colon signifies the number of double bonds. In the present study double bonds have the *cis* nomenclature and where two or more double bonds are present they are methylene interrupted, unless otherwise stated (Christie, 1982). The monounsaturated fatty acid, oleic acid, is given in shorthand form as 18:1n-9 where 9 is the position of the double bond in the chain while n (or sometimes ω) shows that numbering is from the methyl end. The polyunsaturated fatty acid (PUFA) linoleic acid is given the shorthand form 18:2n-6 where the n-6 identifies the position of the first double bond from the methyl end. An alternative nomenclature using the Δ notation identifies the position of the first double bond counting from the carboxyl end. Thus oleic acid is 18:1Δ9 and linoleic acid is 18:2 Δ 9, 12. The long-chain highly unsaturated fatty acids (HUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are shown in Fig. 1.2.1. Throughout this study the n-x shorthand nomenclature has been used when describing fatty acids. In general, PUFA of the n-3 series tend to predominate in the marine environment whereas PUFA of the n-6 series are the dominant species in terrestrial organisms (Sargent and Henderson, 1986; Wood, 1988).

1:3 Biosynthesis and metabolism of PUFA

1:3:1 Introduction

Lipids are probably the most intensively studied of the biochemical components in aquatic organisms. Much interest has been generated by the high levels and wide ranges of lipids in many aquatic organisms and also by them containing, by comparison to most terrestrial organisms, high levels and a wide range of the n-3 series polyunsaturated fatty acids (PUFA). Fish lipids in particular are characteristically rich in n-3 PUFA (Henderson and Tocher, 1987; Ackman, 1980), although considerable differences exist between freshwater and marine species. For example, in fish from northern latitudes, freshwater species generally have higher levels of C_{18} PUFA and lower levels of C_{20} and C_{22} PUFA compared to

marine species (Sargent *et al.* 1989) .

1:3:2 Fatty acid synthetase

Although the fatty acid synthetase enzyme complex has not been fully characterised in fish, it is generally agreed that fatty acid synthesis in fish proceeds using pathways broadly similar to those which occur in mammals (Wakil *et al.* 1983). Thus 2C acetyl-CoA units derived from the tricarboxylic acid cycle are carboxylated to malonyl-CoA which is subsequently converted to fatty acids by the fatty acid synthetase complex via a series of condensation and reduction reactions utilizing NADPH (Wakil *et al.,* 1983). Fish possess a similar cytosolic fatty acid synthetase complex to that found in other vertebrates (fatty acid synthetase type I) (Sargent *et al.* 1989) although the chain lengths of the saturated fatty acid products (mainly palmitic and stearic acids in fish) may differ in range and proportion to those found in mammals. A detailed description of the enzyme pathways involved in *de novo* fatty acid synthesis is given in a number of general biochemistry textbooks (Lehninger, 1975; Zubay, 1988) or textbooks devoted to lipid metabolism (Harwood and Russell, 1984; Gurr and Harwood,1991). A recent review of lipid biosynthesis in fish can be found in Sargent *et al.* (1989).

1:3:3 Fatty acid elongation

By comparison to the *de novo* synthesis of fatty acids by the fatty synthetase type I enzyme, the lengthening of preformed or dietary-derived fatty acids is performed by type III synthetases or elongases. While this system has been little studied in fish, it is presumed to operate by similar mechanisms to those found in other vertebrates. These elongation reactions occur in the membrane fraction of the endoplasmic reticulum and which can be isolated as the microsomal fraction by differential ultracentifugation. The reaction involves fatty acyl-CoAs as primers with malonyl-CoA being the donor of 2C units and requires NADPH as reducing co-enzyme.

Details of these reactions can be found in Gurr and Harwood (1991). In general, the elongases are not all that well characterised in mammals and could be very complex. There appear to be four component reactions, which require fatty acid CoA derivatives, and can utilise both NADH and NADPH, involving both cytochrome b_5 and cytochrome P450. The enzyme complex resides on the cytoplasmic surface of the endoplasmic reticulum and appears to function in concert with desaturases. The complex was identified in all mammalian tissues investigated, except heart (Cinti *et al.* 1992).

1:3:4 Fatty acid desaturation

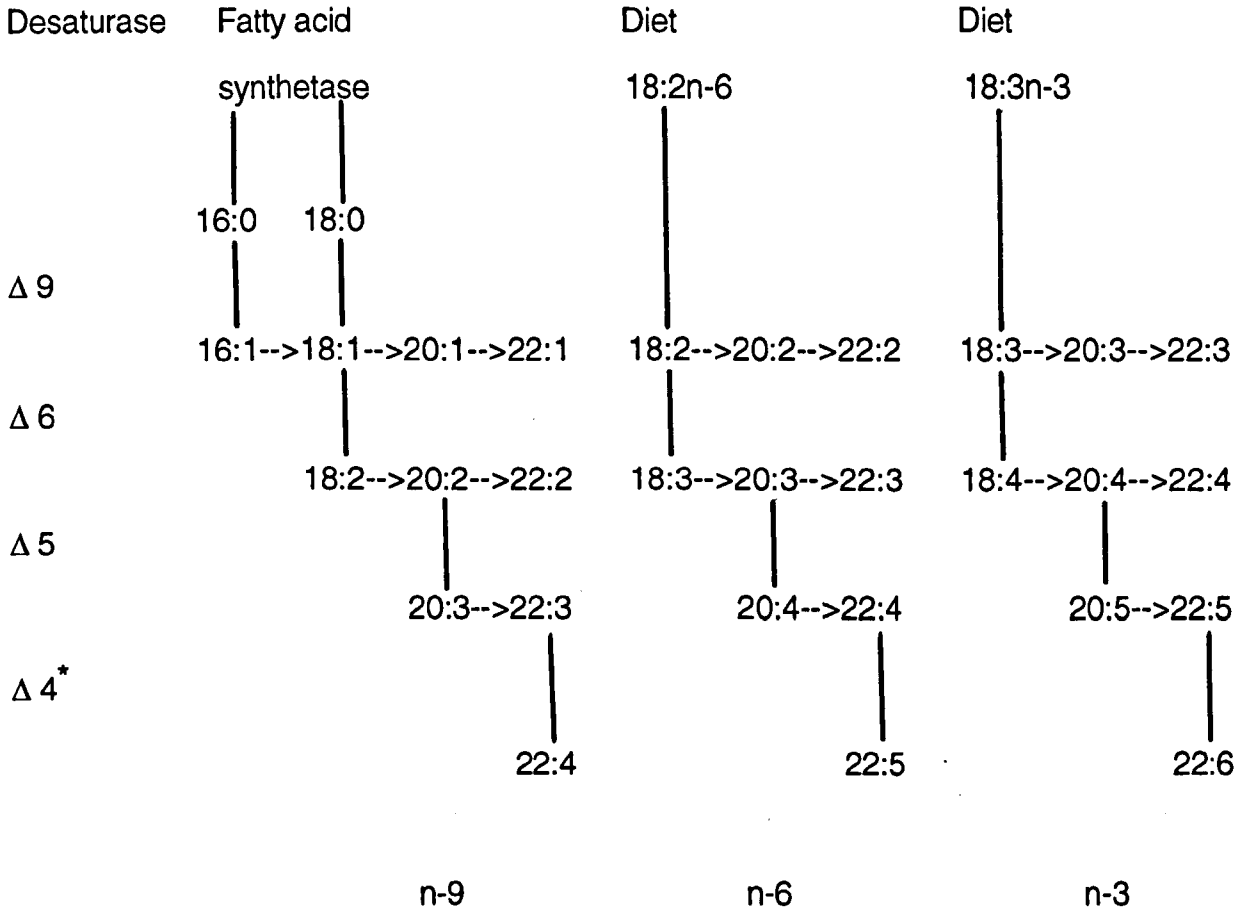
The utilisation of acetyl units for both elongation reactions and *de novo* fatty acid synthesis has been demonstrated in carp and eels injected with ^{14}C-acetate (Farkas and Csenger, 1976; Henderson, 1995). These studies also observed the presence of radioactivity in 16:1 and 18:1n-9 indicating that endogenously synthesised fatty acids had undergone Δ 9 desaturation reactions. However, as with other vertebrates, fish do not possess the Δ 12 and Δ 15 desaturase enzymes which are required for the synthesis of 18:2n-6 and 18:3n-3, and consequently these fatty acids or their longer chain metabolites must be obtained from dietary sources. Thus, 18:2n-6 and 18:3n-3, linoleic and a-linolenic acids respectively, are regarded as essential fatty acids (EFA) and must be supplied by the diet. The ultimate source material for both n-3 and n-6 PUFA in both mammals and fish are photosynthetic organisms, i.e. higher plants in the terrestrial environment and phytoplankton in the marine environment (Harwood, 1988). In photosynthetic organisms the synthesis of both n-3 and n-6 PUFA occurs initially by conversion of biosynthesised 18:0 to 18:1n-9 (or similarly 16:0 to 16:1n-7) utilising Δ 9 desaturase (Harwood, 1988). Plants can further desaturate 18:1n-9 to 18:2n-6 utilising a Δ 12 desaturase and finally produce 18:3n-3 by Δ 15 desaturation. These enzymatic processes which insert double bonds into a monounsaturated fatty acid between the existing double

bond and the methyl end of the molecule are the source of all n-3 and n-6 PUFA. The reactions occur within the thylakoid membranes of eukaryotic chloroplasts.

Animals, unlike plants, are not able to insert double bonds between existing double bonds and the methyl terminus of any fatty acid i.e. animals do not possess either Δ12 or Δ15 desaturase. However, the desaturase enzymes present in animals and some plants can insert additional double bonds between existing double bonds and the carboxyl end of the molecule. These latter reactions can occur with both 18:2n-6 and 18:3n-3, both before and after chain elongation, and utilise Δ 6, Δ 5 and Δ 4 desaturases (Fig. 1:3:1). The Δ4 desaturation reaction is now believed to occur, in rats, by elongation of 20:5n-3 to 24:5n-3 followed by Δ6 desaturation to give 24:6n-3 which is converted to 22:6n-3 following peroxisomal b oxidation (Voss *et al.* 1991) (See Figure 1:3:2, page 15). Thus, animals can convert 18:2n-6 to 22:5n-6 and 18:3n-3 to 22:6n-3, although different species may contain varying complements of desaturase enzymes and therefore different end products. In plants the Δ12 and Δ15 desaturases mainly occur in the glycolipids of the thylakoid membranes, for the production of 18:2n-6 (Δ12) and 18:3n-3 (Δ12 and Δ15), whereas in animals the further conversion of these fatty acids to 22:5n-6, 22:5n-3 and 22:6n-3 are associated with the phospholipids of the endoplasmic reticulum where the Δ6 and Δ5 desaturation reactions occur. Plants also possess Δ6 desaturase since many produce oils rich in 18:3n-6 and 18:4n-3 (Cook, 1985). Members of the phytoplankton produce 20:5n-3 and 22:6n-3 and therefore have Δ6, Δ5 and Δ4 desaturase activities. However, whether the species concerned are true plants is very debatable. Most of the species concerned are phototrophic but many can be simultaneously heterotrophic, especially those species rich in 22:6n-3 which are generally flagellates and include the dinoflagellates. The distinction between 'plants' and 'animals' in single celled eukaryotes is confused and tends to be avoided in modern classifications based on non-symbiotic functions of protists

Figure 1:3:1 PATHWAYS OF DESATURATION AND ELONGATION

(Horizontal lines represent elongation by addition of acetyl units. Vertical lines represent desaturation steps).



*The recent work of Voss *et al.* (1991) has established that Δ4 desaturation actually proceeds via elongation to 24:5n-3 (or 24:4n-6) and subsequent Δ6 desaturation producing 24:6n-3 (or 24:5n-6) which is then converted to 22:6n-3 (or 22:5n-6) via peroxisomal β - oxidation.

(Margulis, 1993). In line with this the precise amounts of 16, 18, 20 and 22C in phytoplankton and algae are dependent on both the species and the developmental cycle of the cell (Wood, 1988).

Although relatively few fish species have been studied in detail there are general trends regarding desaturation and elongation of fatty acids which have traditionally been related to whether the fish inhabit either the freshwater or marine environment. Experiments involving administration of ^{14}C-18:3n-3 by injection or feeding, and nutritional studies feeding 18:2n-6 and 18:3n-3, have confirmed that various freshwater fish including rainbow trout, ayu and eel are able to elongate and desaturate 18:3n-3 to 22:6n-3 (reviewed by Henderson and Tocher, 1987). However, a considerable variation in the conversion rate of 18:3n-3 to 22:6n-3 between different freshwater species has been observed (Kanazawa, 1985). Marine species, including the turbot (*Scophthalmus maximus)*, appear unable to synthesise long-chain HUFA from 18C precursors supplied in the diet (Owen *et al.* 1975) although recent work with turbot cells in culture has suggested they actively convert 18:2n-6 to 18:3n-6 and 18:3n-3 to 18:4n-3 via Δ 6 desaturase (Tocher *et al.* 1989). It appears that marine fish, including turbot, striped jack, gilthead bream and grey mullet, are deficient in or have a very low Δ 5 desaturase activity and consequently require a dietary supply of 20:5n-3, 22:6n-3 and probably also 20:4n-6 (Bell *et al.* 1985; Takeuchi *et al.* 1992; Kalogeropoulos *et al.* 1992; Argyropoulou *et al.* 1992). (The ability of marine fish to convert 20:5n-3 to 22:6n-3 at a rate sufficient to satisfy their requirement for the latter PUFA is discussed in detail on pages 13 & 14). In studies with an anadromous fish, the Atlantic salmon, the parr stage appears to possess similar desaturation and elongation capability to freshwater species but while undergoing smoltification the ability to elongate and desaturate 18:2n-6 may be reduced or even absent (Ackman and Takeuchi, 1986). This effect during smoltification may reflect less of an inherent inability to metabolise

18:2n-6 but more a strong preference to metabolise 18:3n-3 to 22:6n-3 because the latter HUFA is known to increase in anadromous fish undergoing the transition to sea water. This phenomenon in salmon will be returned to in the discussion of this thesis.

1:4 Competitive interactions between PUFA

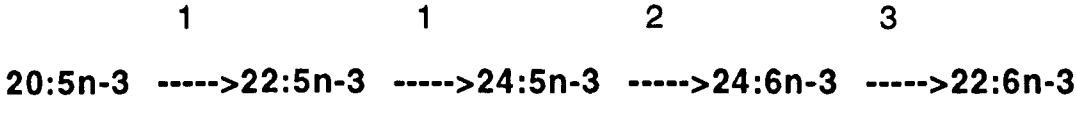
When undertaking dietary studies utilising oils of varying PUFA composition, it is important to bear in mind that the presence of one type of fatty acid can influence the metabolism of others (Garcia and Holman, 1965). In mammals it is generally accepted that the substrate preference for the Δ 6 desaturase is 18:3n-3 > 18:2n-6 > 18:1n-9 and that competitive interactions exist between these three substrates (Sprecher, 1989). The same substrate affinity pattern exists in fish and, although an excess of dietary 18:2n-6 may inhibit metabolism of 18:3n-3, it is generally accepted that 18:3n-3 is more effective in inhibiting the metabolism of 18:2n-6 than vice versa (Yu and Sinhuber, 1976, 1979). In addition, the Δ 6 desaturase can be subjected to feedback inhibition by long-chain HUFA such as 20:5n-3 and 22:6n-3 which therefore prevent desaturation of both 18:2n-6 and 18:3n-3 (Leger *et al.* 1981). As a consequence of these competitive interactions, 18:1n-9 is only utilised as a substrate for Δ 6 desaturase when both 18:2n-6 and 18:3n-3 are absent. Thus, trout given diets lacking both 18:2n-6 and 18:3n-3 tend to accumulate 20:3n-9, originating from the Δ 6 desaturation of 18:1n-9, in their tissue phospholipids (Castell *et al.* 1972) (See Fig. 1.3.1). Moreover, recent studies have established that Δ 4 desaturation can occur, in rats, as a result of Δ 6 desaturase acting on 24:5n-3 which results in 22:6n-3 after chain shortening of 24:6n-3 (Voss *et al.* 1991; see Fig. 1:3:2). If Δ 4 desaturation proceeds extensively by this mechanism, as now seems likely, a further consequence of the competitive inhibitions between dietary PUFA concerns whether 20:5n-3 can be elongated and desaturated to 22:6n-3, at a rate

sufficient to satisfy requirements. This may be especially important in marine fish consuming diets which are rich in 20:5n-3 but relatively poor in 22:6n-3. Evidence suggests that the conversion 20:5n-3 to 22:6n-3 is slow (Sargent *et al.* 1989) and may be influenced by the presence of additional Δ6 desaturase substrates in the diet. Recent studies utilising cultured cells from the turbot have confirmed that this marine fish can convert 20:5n-3 to 22:6n-3, but at a relatively slow rate (Tocher and McKinlay, 1990; Tocher and Sargent, 1990). Whether this reaction rate is quantitatively sufficient to satisfy 22:6n-3 requirement or if additional dietary 22:6n-3 is required has been difficult to establish.

A recent study using isolated hepatocytes from trout and rats established that Δ 6 desaturase activities were similar in both species at 12^{o} and 37^{o}C respectively, and that 18:3n-3 was preferred as substrate compared to 18:2n-6 in both species (Hagve *et al.* 1986). However, Δ 5 desaturase, measured with 20:3n-6 as substrate, was four times greater in rat than in trout hepatocytes although the difference between species was less marked when 18:2n-6 and 18:3n-3 were initial substrates for Δ 5 desaturation. In contrast, Δ 5 desaturase activity measured with 20:4n-3 as substrate was almost twice as great in trout hepatocytes compared to rats. Moreover, considerably more of the radioactive substrate fatty acids were incorporated into 20:2n-6, 20:3n-3 and 22:3n-3 in trout hepatocytes compared to rat. These fatty acids are considered 'dead end' products not normally subject to further metabolism and are generally stored in triglycerides until they are subsequently released and retroconverted to be utilised as substates for more conventional pathways of desaturation and elongation (Sellner and Hazel, 1982). However, while retroconversion of 22:6n-3 to 20:5n-3 occurs in rats, (Schlenk *et al.* 1969) there is some evidence that rainbow trout are not capable of performing the same reaction (Yu and Sinnhuber, 1972). The balance between chain shortening activity and chain elongating activity is a very important factor in ultimately determining the

PUFA composition of membrane lipids. There is evidence from mammalian experiments which suggests that the balance between elongation and shortening of n-6 PUFA is such that 20:4n-6 rather than 22:5n-6 is the end product in membrane lipids. However, for n-3 PUFA, 22:6n-3 is the prefered end product incorporated in membranes in preference to 20:5n-3 (Rosenthal *et al.* 1991). The way in which this balance operates in fish is of obvious importance in fish nutrition. However, because of the complexities of predicting the competitive interactions involved in desaturation and elongation, it has proved extremely difficult to envisage which PUFA will be incorporated into tissues of fish, and other animals, from PUFA compositions given in the diet (Lands, 1991). The recent confirmation that Δ 4 desaturase may actually be a Δ 6 desaturase which operates on 24:5n-3 (following elongation of 22:5n-3) producing 24:6n-3 which is then converted to 22:6n-3 after undergoing peroxisomal β - oxidation (Voss *et al.* 1991), would consolidate the preferential conversion of 18:3n-3 rather than 18:2n-6 by the Δ 6 desaturase (See Fig. 1:3:2). Thus, the apparent inability to metabolise 18:2n-6 during smoltification of salmon parr may reflect the increased requirement for 22:6n-3 and the consequent increased competition for the Δ 6 desaturase.

Figure 1:3:2 PATHWAY FOR CONVERSION OF 20:5n-3 TO 22:6n-3 (Voss *et al.* 1991)



Where 1 represents a microsomal fatty acid elongase, 2 is the microsomal Δ 6 desaturase and 3 is the peroxisomal β-oxidation chain shortening system. The same pathway will also operate to convert 20:4n-6 to 22:5n-6.

1:5 Functional roles of PUFA

1:5:1 Membrane Structure and Function

1:5:1:1 Homeoviscous adaptation to environmental temperature

PUFA are an integral component of phospholipids which are in turn the building blocks which form the bilayer structure of cellular membranes in all animal cells, including fish. Since fish are poikilothermic organisms, the ability to undergo homeoviscous adaptation to environmental temperature changes is a particularly important role for membrane lipids. To function correctly the fluid state of cell membranes must be controlled and this is governed largely by the fatty acid compositions of the membrane phospholipids (Hazel and Williams, 1990). The phase transition temperatures (melting points) of fatty acids are determined by the number and position of double bonds in the carbon chain and all PUFA commonly found in fish membranes have transition temperatures far below $0^{o}C$. (18:2n-6, -$8^{o}C$; 18:3n-3, -$10^{o}C$; 20:4n-6, -$49.5^{o}C$; 20:5n-3, -$54.4^{o}C$; 22:6n-3, -$44.5^{o}C$; Fasman, 1975). The balance of PUFA with saturated and monounsaturated fatty acids maintains the biomembrane in a fluid state (Hazel, 1984). During cold adaptation the amount of total phospholipid does not alter but the relative proportions of phospholipids, the fatty acid composition of phospholipids and the distribution of fatty acids within phospholipids are all altered. Thus, in trout transferred from 20^{o} to $5^{o}C$ PE increases and PC decreases in both liver and gills (Hazel, 1979). Similar changes in the PE:PC ratio have been observed during cold acclimation in goldfish intestine and carp muscle (Miller *et al.* 1976; Wodke, 1981). During cold adaptation, the proportions of PUFA and especially the n-3 PUFA increase in the phospholipids of trout liver, while saturated fatty acids decrease (Hazel, 1979). Such changes in PUFA composition of phospholipids are brought

about by a combination of increased desaturase activity and increased incorporation of long-chain PUFA into phospholipids (Ninno *et al.* 1974). However, studies by Cossins (1977) suggest that the major fatty acid compositional alterations in membrane phospholipids involve the monounsaturated fatty acids with much smaller changes occuring in saturated and polyunsaturated fatty acids. This result suggests that the activity of the Δ9-fatty acid desaturase may be of major importance in determining the adaptive changes in phospholipid fatty acid compositions which occur as a result of temperature acclimation.

1:5:1:2 Neural tissues

Fish cell membranes are characteristically rich in n-3 PUFA, particularly 20:5n-3 and 22:6n-3. The brain, retina and reproductive tissue in mammals are all markedly rich in n-3 PUFA relative to other body tissues and, likewise, the brain and retina of fish are particularly rich in these PUFA, especially DHA (Sastry, 1985; Bell and Dick, 1991). It has now been established that DHA is essential for proper development of neural including visual membranes in mammals and that deficiency during embryonic development in primates and *Homo sapiens* leads to visual and mental subnormalities (Neuringer *et al.* 1988; Bazan, 1990; Hoffman *et al.* 1993). Until recently, no direct evidence of neural or visual impairment has been observed in fish but a study by Bell *et al.* (1995) has shown that a dietary deficiency of DHA, in larval herring (*Clupea harengus)*, results in impaired vision at low light intensities. Recent studies involving larval turbot have established that DHA is initially deficient in intensively reared fish but is rapidly taken up and incorporated into brain phospholipids when larvae are weaned onto particulate feeds (Mourente *et al.* 1991; Mourente and Tocher, 1992). The larval turbot were fed a diet of brine shrimp (*Artemia)*, which had been enriched using fish oil, for the first 51 days after hatching. After 51 days the larvae were weaned onto a dry pelleted diet which contained 13 times more DHA than the enriched *Artemia*. Upon weaning DHA was

rapidly and specifically incorporated into brain phospholipids. Clearly DHA levels in developing larvae are extremely important and the low levels of DHA in the brains of *Artemia* -fed larvae may be one factor underlying the high mortalities encountered in larvae during first feeding.

Clearly the accumulation of DHA into neural tissues must have an important functional significance possibly related to the physical properties of the DHA molecule. Increasing the number of double bonds in a long-chain fatty acid molecule results in an increasingly compact conformation. In DHA this results in the minimum energy conformation of a compact helix or angle iron having a length shorter than that of 18:0 (Applegate and Glomset, 1986). In fish neural tissues DHA is largely found in PE and PS where a very large percentage (ca. 70%) is in the form of the di-22:6n-3 molecular species (Bell and Tocher, 1989; Bell and Dick, 1991). It has been suggested from physico-chemical studies with mammalian rod outer segment membranes that the abundance of di-22:6n-3 PE maintains the required membrane balance between fluidity and rigidity which is necessary to allow the rapid conformational changes undergone by the retinal chromophore of rhodopsin (Dratz and Deese, 1986). A model for rhodopsin function describing the conformational transition between metarhodopsin I (MI) and metarhodopsin II (MII) as the fundemental triggering event in the visual process has been described by Brown (1994). This study details the use of flash photolysis to determine the (MII)/(MI) ratio for rhodopsin in various recombinant membranes and allows investigation of the role of phospholipid head groups and fatty acid composition on rhodopsin function. To achieve normal photochemical function the membrane must comprise PC, PE and PS in combination with DHA and the MI-MII transition appears favoured by relatively small head groups, e.g. PE, which produce a condensed bilayer surface together with bulky acyl chains like DHA which have a large cross-sectional area. The result is a force imbalance across the bilayer giving a curvature

stress at the lipid/water interface and the relatively unstable membrane composition required for rhodopsin transition and triggering of the visual process (Brown, 1994).

1:5:1:3 Membrane-bound proteins

Cell membranes are not only composed of a phospholipid bilayer but also contain proteins which perform various critical physiological functions including signal reception, transport of ions and molecules, and enzymic reactions. The fatty acid composition of the membrane phospholipids, which can be influenced by dietary lipid input, can directly affect the function of membrane-associated proteins (Spector and Yorek, 1985). The effects of changes in membrane fatty acid composition may be extremely complex in that different tissues or cell types may respond very differently, in terms of protein functionality, to the same dietary induced compositional changes. For example, Nalbone *et al.* (1990) observed that cardiomyocytes supplemented with a high n-6/n-3 PUFA ratio had higher phospholipase A activity compared to those supplemented with a low n-6/n-3 PUFA ratio. In contrast, rats given a fish oil diet, rich in n-3 PUFA, had an increased gastric phospholipase A activity compared to rats given a corn oil diet rich in n-6 PUFA (Grataroli *et al.* 1988). Functional modifications of this type may be attributed to changes in membrane fluidity such that the lipid micro-environment adjacent to the enzyme active site influences substrate access, and thus enzymic activity (Stubbs and Smith, 1984). It would appear that the composition of both acyl chains and phospholipid head group are fundamental in the control of overall membrane bilayer energetics and that the mechanism described for rhodopsin (Brown, 1994) may be a marked example of a general phenomenon related to protein conformational change.

1:5:2 Eicosanoid production

The term eicosanoid was first used by Corey *et al.* (1980) to describe a group of biologically active compounds derived from eicosapolyenoic acids, particularly

arachidonic acid (AA), but also 20:3n-6 (di-homo γ- linolenic acid) and 20:5n-3. The two major di-oxygenase enzymes involved in eicosanoid production are cyclooxygenase and lipoxygenase. Cyclooxygenase converts AA into prostaglandin H (PGH) which can be further metabolised by isomerases to produce a range of compounds collectively known as prostanoids, including prostaglandins (PG), prostacyclins (PGI) and thromboxanes (TX). The lipoxygenases initially produce hydroperoxy fatty acids which are further metabolised into a number of derivatives including hydroxy fatty acids, leukotrienes (LT) and lipoxins. A summary of the pathways involved in eicosanoid production and the various classes of eicosanoids produced is presented in Figure 1:5:1. The eicosanoid precursors 20:3n-6, 20:4n-6 and 20:5n-3 can be converted, after reaction with cyclooxygenese, to prostaglandins of the 1-, 2- and 3-series respectively. Alternatively 20:3n-6, 20:4n-6 and 20:5n-3 can be metabolised by a number of lipoxygenase enzymes to yield hydroxyeicosatrienoic, hydroxytetraenoic and hydroxypentaenoic acids (HETES and HEPES) respectively. Initial reaction with a 5-lipoxygenase can be followed by further hydroxylation to produce di-HETE/HEPE products, some of which are known as leukotrienes, and are designated 3-, 4- and 5-series when derived from 20:3n-6, 20:4n-6 and 20:5n-3 respectively.

Eicosanoids are synthesised and released locally in response to stimuli which can be physiological, pharmacological or pathological in origin. The processes involved in eicosanoid production in response to an extracellular stimulus are often described as "the arachidonic acid cascade", largely because AA is the major precursor for eicosanoid production in mammals. The precursor fatty acids for eicosanoid production are obtained from membrane phospholipids, although specifically which ones is still a matter of some controversy, which are hydrolysed after activation of cell surface receptors. These cellular receptors are linked to G proteins (guanine nucleotide regulatory proteins) which are activated upon binding

of ligand. The activated G proteins then act directly on other membrane proteins e.g. adenylate cyclase, ion channels, protein kinases etc, which ultimately leads to activation of phospholipase A_2 (PLA_2) (Blackwell *et al.* 1978; Smith, 1989). The activated phospholipase A_2 enzyme then acts to release AA and other eicosanoid precursors from the *sn*-2 position of membrane phospholipids (Wolfe, 1982). The phospholipase A enzymes are ubiquitous in eukaryotic cells and may be cytosolic but migrate to the membrane on activation. They may be associated with plasma, Golgi and mitochondrial membranes and are often stimulated by calcium ions (van den Bosch, 1980; Dennis *et al.* 1995). An alternative supply of eicosanoid precursors may arise from the sequential action of phospholipase C and diacylglycerol lipase (Irvine, 1982), which form part of the phosphoinositide cycle, or from the combined action of both PLC and PLA (Rittenhouse-Simmons, 1979). Eicosanoids synthesised by the above mechanisms can themselves activate other receptors and G proteins to generate, *inter alia,* other and more eicosanoids (Smith, 1989).

1:5:3 Eicosanoids in fish

Prostanoids have been identified in a large range of freshwater fish, including carp, tilapia, Asian catfish (Bandyopadhyay *et al.* 1982), eel, (Srivastava and Mustafa, 1984) and rainbow trout (Kayama *et al.* 1986), and in marine fish such as plaice (Anderson *et al.* 1981), turbot (Henderson *et al.* 1985), flounder and black and red sea breams (Matsumoto *et al.* 1989). Cyclooxygenase activity has been demonstrated in virtually all fish tissues studied to date, in general following addition of exogenous precursors. However synthesis of prostanoids from endogenous fatty acids has been established in a number of tissues including gills (Christ and van Dorp, 1972), leucocytes (Pettitt *et al.* 1989), ovarian tissues (Ogata *et al.* 1979) and heart, liver, intestine, brain, testes, kidney and air sac (Nomura and Ogata, 1976). Production of lipoxygenase metabolites has also been established in

both freshwater and marine fish (Tocher, 1995). Tissues identified as having significant lipoxygenase activity include gills, skin, blood and brain (German *et al.* 1986; German *et al.* 1985; Pettitt and Rowley, 1991; Tocher *et al.* 1991).

The prostaglandins PGE_2, $PGF_{2\alpha}$ and PGD_2 have been identified in numerous fish tissues (Rowley *et al.* 1987; Henderson and Tocher, 1987) and TXB_2, the stable metabolite of thromboxane, is produced by thrombocytes from rainbow trout (Kayama *et al.* 1985) and by dogfish leucocytes (Rowley *et al.* 1987). 12-Lipoxygenase has been identified in gills and skin from fourteen species of fish (Tocher, 1995) and has also been measured in rainbow trout head kidney macrophages and brain (Pettitt *et al.* 1991; Tocher *et al.* 1991). The 15- and 5-lipoxygenase enzymes have also been identified in a number of fish species and tissues including, gills, brain, neutrophils and head kidney macrophages (German and Creveling, 1990; Tocher *et al.* 1991; Tocher and Sargent, 1987; Pettitt *et al.* 1991). Leukotriene B (LTB) appears the most commonly occuring leukotriene in fish having been reported in trout macrophages and peripheral blood leucocytes (Pettitt *et al.* 1991; Pettitt and Rowley, 1990) as well as plaice neutrophils and rainbow trout brain cells (Tocher and Sargent, 1987; Tocher *et al.* 1991). The peptido-leukotrienes LTC, LTD and LTE have been identified in American eel gills (Piomelli, 1985). In general fish appear capable of synthesising a similar varied range of eicosanoids to those occuring in mammals.

1:5:4 Functions of eicosanoids

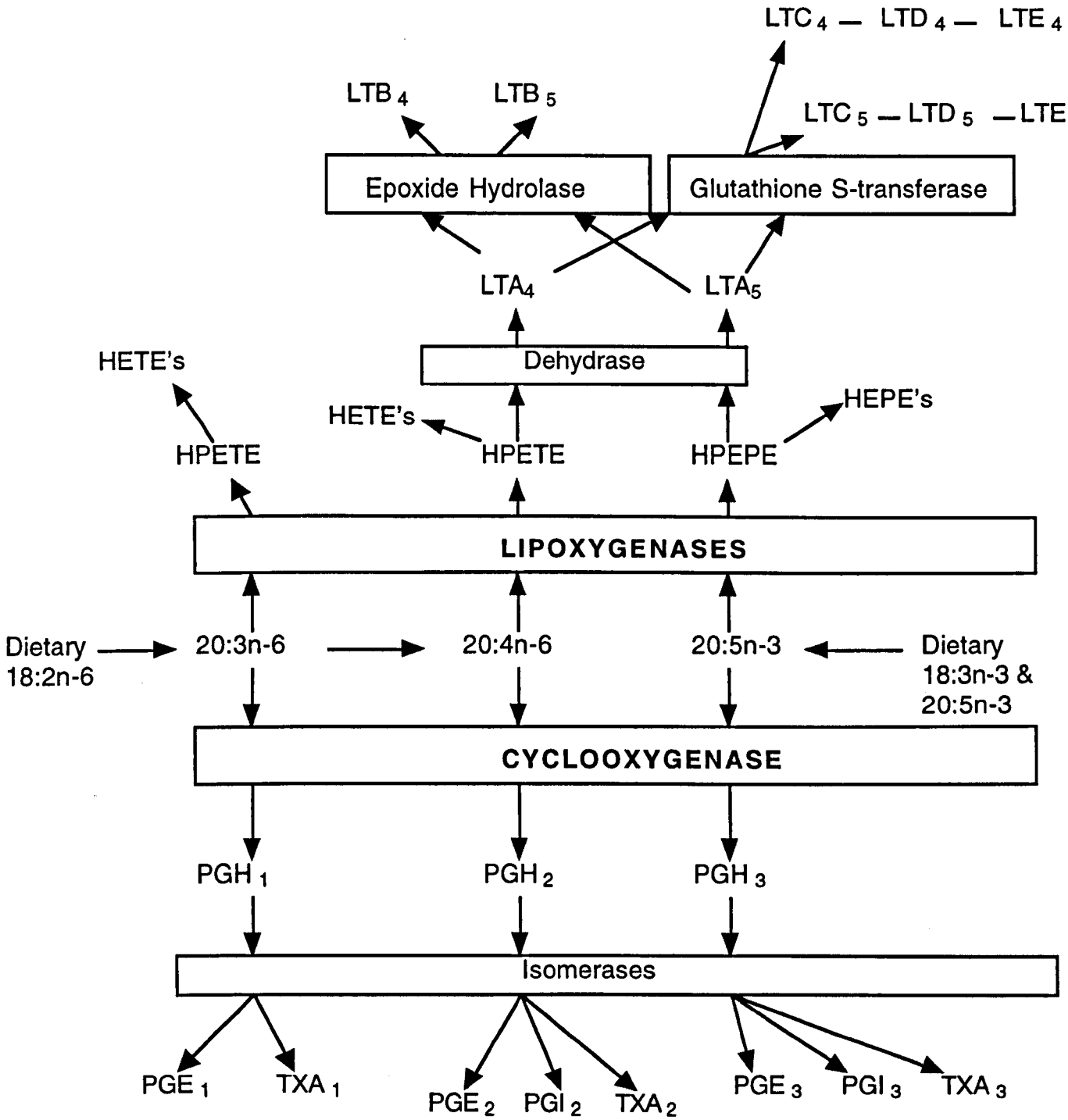
It is currently accepted that mammalian tissues can synthesise up to thirty different eicosanoids each possessing a different biological activity. Individual tissues can synthesise a wide range of eicosanoids which may have complementary or opposing physiological activities in the same tissue. Since tissue and species variations exist in mammalian systems it is difficult, if not impossible, to extrapolate the roles of cyclooxygenase and lipoxygenase metabolites from mammalian

studies, to their roles in fish. Prostaglandins in mammals are involved in a range of physiological functions including contraction of smooth muscle, control of blood pressure, nerve transmission, development of inflammatory response, electrolyte balance, blood clotting, thermoregulation and reproductive mechanisms. The leukotrienes and hydroxy acids also have a role in smooth muscle contraction as well as vascular permeability, chemotaxis and chemokinesis towards leucocytes and enzyme secretion. Although eicosanoid function has been less well studied in fish their involvement in a number of physiological processes have been established.

1:5:4:1 Control of fluid and electrolyte fluxes

In humans and other mammals, the prostaglandins PGE, PGA and PGI have natriuretic activity i.e. they increase urine volume and urinary output of Na^+ and Cl^- ions (Bolger *et al.* 1978; Smith, 1989). In fish also, prostaglandins appear to regulate fluid and electrolyte balance, either directly, or in conjunction with catecholamines, cortisol, prolactin, growth hormone or vasopressin. In rainbow trout PGE_2 injection resulted in antiduiresis resulting from decreased glomerular filtration rate and increased renal tubule water reabsorption (Brown and Bucknall, 1986). The antiduiretic action of PGE_2 may reflect a direct effect on renal tubule adenylate cyclase resulting in increased water permeability. Rainbow trout injected with increasing amounts of PGE_1 showed a dose-dependent increase in circulating cortisol which was diminished on administration of indomethacin, an inhibitor of prostaglandin production (Gupta *et al.* 1985). This suggests that electrolyte balance in fish could be regulated jointly by the mineralcorticoid cortisol and prostaglandins. The hormone prolactin is also involved in osmoregulation by reducing influx of water and efflux of Na^+ and Cl^- at the gill and the studies of Horseman and Meier (1978) indicate that the osmoregulatory actions of prolactin also operate in conjunction with prostaglandins.

Figure 1:5:1 Major Pathways of Eicosanoid Synthesis



The eicosanoid precursors 20:3n-6, 20:4n-6 and 20:5n-3 can be converted, after reaction with cyclooxygenase, to prostaglandins of the 1-, 2- and 3-series respectively. Alternatively 20:3n-6, 20:4n-6 and 20:5n-3 can be metabolised by a number of lipoxygenase enzymes to yield hydroxyeicosatrienoic, hydroxytetraenoic and hydroxypentaenoic acids (HETES and HEPES), respectively. Initial reaction with a 5-lipoxygenase can be followed by further hydroxylation to produce di-HETE/HEPE products, which are known as leukotrienes, and are designated 3-, 4- and 5-series when derived from 20:3n-6, 20:4n-6 and 20:5n-3 respectively.

1:5:4:2 Eicosanoids and the cardiovascular system

In mammals the role of prostaglandins and leukotrienes in the cardiovascular system has been extensively studied. In general PGE_2 and PGI_2 exhibit vasodilatory activity while $PGF_{2\alpha}$, TXA_2 and the leukotrienes normally have vasoconstrictor activity (Wolfe, 1982). The role of eicosanoids in the cardiovascular system of non-mammalian animals has been little studied. However, cyclooxygenase activity has been identified in hearts from goldfish, eel and flounder, (Cagen *et al.* 1983; Herman *et al.* 1984; Srivastava and Mustafa, 1984) and in blood cells of toadfish, rainbow trout and dogfish (Cagen *et al.* 1983; Kayama *et al.* 1986; Rowley *et al.* 1987). In all cardiovascular tissues studied, PGE_2 was formed and, as in mammals, this prostaglandin was found to have hypotensive activity in rainbow trout (Brown and Bucknall, 1986). In carp, intravenous injections of PGE_2 caused bradycardia as a result of vagal inhibition and tachycardia, which also occurs after PGE_2 injection in mammals (Peyraud-Waitzenegger *et al.* 1976).

1:5:4:3 Reproductive functions of eicosanoids

Arachidonic acid-derived prostaglandins are involved in all aspects of the mammalian female reproductive system from release of gonadotrophic hormones to parturition and lactation. The order of importance of the cyclooxygenase metabolites in reproductive function is as follows; $PGE_2 > PGF_1 > PGF_2 > PGI_2 > PGD_2$ (Mustafa and Srivastava, 1989). These prostaglandins may act directly on the reproductive organs or in conjunction with reproductive hormones and cAMP. Most studies on the function of prostaglandins in teleost fish reproduction are concerned with females, although fish testes and sperm also produce considerable amounts of prostaglandins (Nomura *et al.* 1973).

In mammals the gonadotrophic hormones luteinizing hormone (LH) and follicle

stimulating hormone (FSH) are released from the anterior pituitary following stimulation by the peptide gonadotrophin-releasing hormone (GRH). In rats PGE_2 or PGE_1 can trigger FSH and LH release from the pituitary, (Spies and Norman, 1973) while treatment with indomethacin can decrease plasma levels of gonadotrophic hormones (Sato *et al.* 1975). In carp (*Cyprinus carpio)* a single injection of indomethacin blocked ovulation for up to 12 days whereas controls ovulated spontaneously in 24 hours (Kapoor and Toor, 1979). Injection of clomiphene citrate, which stimulates GRH release in carp (Breton *et al.* 1975), restored ovulation in indomethacin treated carp and suggested that the cyclooxygenase inhibitor was blocking GRH release (Stacey and Goetz, 1982). Similar effects of PGE_2 and $PGF_{2\alpha}$ on GRH release have also been described in goldfish and catfish (*Heteropneustes fossilis)* (Peter and Billard, 1976; Singh and Singh, 1976). Inhibition of cyclooxygenase can prevent the pre-ovulatory surge of GRH release from the pituitary in both rats and fish. However, in both rats and goldfish, ovulation is not restored by LH injection, suggesting that indomethacin also blocks the action of gonadotrophic hormones on the ovary (Armstrong and Grimwich, 1972; Stacey *et al.* 1979). Thus, in rats it appears that prostaglandins synthesised in the ovary, as a response to LH stimulus, cause contraction of smooth muscle in follicle cells resulting in rupture of the follicle and release of the ovum. In rainbow trout mature follicles could be induced to ovulate in response to $PGF_{2\alpha}$ acting directly on the smooth muscle cells of the theca (Jalabert and Szollosi, 1975). $PGF_{2\alpha}$ also induced ovulation in brook trout (*Salvelinus fontinalis)* , whereas PGE inhibited ovulation (Goetz *et al.* 1982). In contrast PGE_2, PGE_1 and PGD_2 were all equally effective in inducing ovulation in goldfish. In a further study utilising postpartum follicles and vitellogenic oocytes from guppy (*Poecilia reticulata)* , the synthesis of PGE and PGF was demonstrated *in vitro* utilising both endogenous and exogenous precursors

(Tan *et al.* 1987). This confirms the ability of locally synthesised prostaglandins to participate in ovulation.

1:5:4:4 Eicosanoids in the nervous system

In mammals eicosanoids have been implicated in many physiological processes which are controlled by the nervous system (Horrobin, 1978). In teleost fish prostaglandins, and in particular $PGF_{2\alpha}$, have been shown to induce spawning behaviour in goldfish (Stacey and Liley, 1974). Non-ovulating goldfish developed spawning behavior when injected with ovulated oocytes, although this could be inhibited by simultaneous injection of indomethacin, and restored by injection of $PGF_{2\alpha}$ (Stacey, 1976). Injection of $PGF_{2\alpha}$ directly into goldfish brain was the most effective site of injection for stimulation of spawning behavior and thus it seems likely that $PGF_{2\alpha}$ released from the ovary acts on the central nervous system to induce spawning behaviour (Stacey, 1981). Stimulation of spawning behaviour by prostaglandins has also been demonstrated in a number of other fish species including Pacific herring (*Clupea hallengus pallasi)*, rainbow trout (*Salmo gairdneri)* and three-spined stickleback (*Gasterosteus aculeaus)* (Stacey and Goetz, 1982). In some fish species sexually active males are thought to secrete a pheromone-like substance which induces ovulation and spawning behavior in females. Similarly females may secrete a substance which induces spermiation in males. Although not positively identified the pheromone-like substance was thought to be a prostanoid (Crow and Liley, 1979). However, more recent evidence suggests the pheromone may be a hydroxylated progesterone derivative (Stacey *et al.* 1994).

Although prostaglandins produced in the brain do not regulate normal body temperature in mammals, there is evidence that prostaglandins regulate the increase in body temperature arising from bacterial infection (Milton, 1982). The

crawfish (*Camborus bartoni),* a poikilothermic crustacean, injected with a suspension of killed bacteria (*Aeromonas hydrophila)* showed a $2^{o}C$ increase in body temperature, while crawfish injected with doses of PGE_1 from 50-500 mg/animal showed increases in body temperature from 1^{o} to $3.5^{o}C$ (Casterlin and Reynolds, 1978). Similar results were recorded in blue gill sunfish (*Lepomis machrochirus)* and largemouth bass (*Micropterus salmonoides)* (Reynolds *et al.*, 1976).

1:6 Dietary n-3/n-6 PUFA ratio

There is now a wealth of evidence arising from epidemiological studies and clinical trials that many of the pathophysiological conditions which are widespread in Western society are associated with a low dietary n-3/n-6 PUFA ratio which is in turn due to an excessive intake of vegetable oil-derived n-6 PUFA (Lands,1986). In human populations consuming a "Western"-type diet, arachidonic acid (AA) is by far the most abundant precursor fatty acid available for eicosanoid production (Horrobin, 1983). In the preceding pages I have described many of the physiological processes which are controlled by eicosanoids. If these latter are "switched on" excessively then many of the disease conditions arising from an over-active arachidonic acid cascade can result. These disorders include atherothrombotic conditions including coronary heart disease, stroke and hypertension, and auto-immune and inflammatory conditions such as asthma, psoriasis, rheumatism and arthritis (Weber, 1990). Eskimo and other populations consuming large quantities of fish have a relatively high dietary ratio of n-3/n-6 PUFA and suffer a much lower incidence of coronary heart disease than most western populations (Lands, 1986). At present considerable research effort is directed towards identifying the optimal ratio of n-3/n-6 PUFA in human diets and likely estimates between 1:4 and 1:1 would seem to be beneficial, depending on

developmental age (Crawford, 1987; Anon., 1992). Although fish and fish oils are considered the best way to bring about a desirable alteration in dietary n-3/n-6 PUFA ratio (Simopoulos *et al.* 1991) the importance of green vegetables and some plant oils rich in 18:3n-3 should not be overlooked (Okuyama, 1992), but this, of course, depends on the rate or extent of conversion of C_{18} PUFA to C_{20} and C_{22} PUFA.

The mechanisms by which increased dietary intake of n-3 PUFA act to overcome the undesirable results of excessive n-6 PUFA intake can probably be attributed to two separate, although related, biochemical effects. First, there is the direct effect which increased n-3 PUFA in membrane phospholipids can have on membrane-bound enzymes. Second, there is the ability of n-3 PUFA to modify the arachidonic acid cascade.

The increased incorporation of n-3 PUFA, especially DHA, into membrane phospholipids may be particularly important in the physiological function of cardiac tissue (Kinsella, 1990). Fish oil consumption protects heart muscle against ischemic damage in dogs and can reduce isoprenaline-induced arrhythmias in rats (Culp *et al.* 1980; Charnock *et al.* 1985). The beneficial effects may have resulted from increased uptake of calcium by the sarcoplasmic reticulum (SR). The uptake and release of Ca^{2+} from the SR requires an ATP-dependent calcium pump which is situated in the SR membrane bilayer. The activity of this pump controls cytosolic calcium concentration in cardiac myocytes and is thus important in the regulation of the contraction and relaxation cycle of heart muscle. Since the Ca^{2+}-Mg^{2+} ATPase is located within the membrane, the fatty acid composition of the surrounding phospholipids may regulate its activity. In mice fed menhaden oil, the cardiac Ca^{2+}-Mg^{2+} ATPase had a specific activity *in vitro* over six times less than in mice consuming corn oil. The reduction in activity following menhaden oil intake was

related not to fatty acid unsaturation index but to DHA levels in SR phospholipids, especially PC and PE (Swanson *et al.* 1989). The effect of DHA may reflect changes in membrane fluidity or thickness causing altered accessibility of enzyme substrates (Froud *et al.* 1986). Other membrane-associated enzymes whose activities can be altered by dietary n-3/n-6 PUFA ratio include adenyl cyclase, which generates the important second messenger cAMP, and 5' nucleotidase which produces adenosine, a blood vessel dilator and attenuator of b-adrenergic responses (Gudbjarsson *et al.* 1978; Alam *et al.* 1988).

There are essentially three main mechanisms identified in mammals by which an increased dietary n-3/n-6 PUFA ratio can 'down regulate' the arachidonic acid cascade: first, by intake of n-3 PUFA decreasing tissue levels of AA; second, by EPA and DHA competitively inhibiting cyclooxygenase and lipoxygenase activity; third, by EPA-derived eicosanoids competing with AA-derived homologues for receptor binding sites (Kinsella, 1990). Ingestion of dietary fish oil results in enhanced incorporation of EPA and DHA into cellular lipids causing a replacement and redistribution of AA in membrane phospholipids (Garg *et al.* 1990). In addition, competitive effects of n-3 PUFA from both fish oil and linseed oil can reduce activity of microsomal Δ6 and Δ5 desaturases resulting in depressed production of cellular AA (Garg *et al.* 1988a, 1988b). In both cases the result is a reduction in the phospholipid pool of AA which is available for eicosanoid production. Once incorporated in cell membranes, both AA and EPA can subsequently be released by phospholipase A_2 and are available as substrates for cyclooxygenase and lipoxygenase. However, mammalian studies indicate that EPA-derived eicosanoids tend to be of lower biological efficacy compared to their AA-derived homologues (Terano *et al.* 1986; Bruckner *et al.* 1984). Thus, thromboxane A_3 (TXA_3) formed in small concentrations by n-3 PUFA- enriched platelets has only negligible vasoconstrictor and aggregatory activity compared to TXA_2 which is also reduced

considerably by increasing the dietary n-3/n-6 PUFA ratio (Weber, 1990). The EPA-derived leukotriene B_5 produced by rat neutrophils and macrophages has around 30 times less chemotactic activity than LTB_4 which is in turn reduced by prolonged intake of n-3PUFA (Strasser *et al.* 1985). However, EPA-derived PGI_3, which can account for 50% of total prostacyclin production in n-3 PUFA-supplemented individuals, is as biologically active as AA-derived PGI_2 in inhibiting platelet aggregation and reducing vascular tone (von Shacky *et al.* 1985).

Although phospholipids in wild fish tend to have an excess of EPA over AA (Henderson and Tocher, 1987; Ackman, 1980), fish tissues still produce considerable amounts of AA-derived eicosanoids (Mustafa and Srivastava, 1989). Indeed AA appears to be the preferred substrate for cyclooxygenase in plaice skin and neutrophils and in turbot gills (Anderson *et al.* 1981; Tocher and Sargent, 1987; Henderson *et al.* 1985). As with humans and farmed mammals, intensively reared fish like Atlantic salmon are susceptible to a wide range of disease conditions, many of which can be induced or exacerbated by periods of stress. Commercial salmon feeds often contain some plant-derived lipids, either as a result of using components such as brewing by-products or soybean meals, or by addition of vegetable oils. The result is that farmed salmon generally contain around five times the amount of n-6 PUFA compared to their wild counterparts. The alteration in cellular fatty acid composition which would arise from consuming diets with a reduced n-3/n-6 PUFA ratio could potentially upset the normal spectrum of eicosanoids produced by the fish. It is possible therefore that increased production of AA-derived eicosanoids in farmed Atlantic salmon could increase occurrence and severity of some pathophysiological conditions occurring in this species.

1:7 Aims of this study

In the relatively few fish species studied to date it is generally accepted that while

freshwater fish can elongate and desaturate 18C PUFA to 22C HUFA, marine fish require 20C and probably 22C HUFA to be provided in the diet. In anadromous fish like Atlantic salmon, which start their life cycle in freshwater before migrating to seawater after undergoing smoltification, the situation is less clear. While freshwater parr are thought to express the full complement of desaturase enzymes there has been evidence suggesting a loss of some desaturating capacity during smoltification (Ackman and Takeuchi, 1986). In addition, lipid metabolism in post-smolt Atlantic salmon has been little studied. The aim of this study was to utilise the Atlantic salmon as an n-3 PUFA-rich model system in which to study PUFA metabolism and to advance basic knowledge of PUFA interactions and eicosanoid metabolism in an n-3 PUFA-rich organism. PUFA metabolism would be followed by measuring the fatty acid composition of membrane phospholipids in a range of tissues after feeding diets with varying n-3/n-6 PUFA ratios. In addition, by feeding these diets it was predicted that membrane fatty acid compositions would be altered so as to affect eicosanoid precursor supply and elicit changes in the spectrum of both cyclooxygenase and lipoxygenase products. Gill and white blood cells were chosen to study eicosanoid production because of their established activity in this respect. During the course of the study the development of any gross or histological pathologies arising from feeding diets with varying n-3/n-6 PUFA ratio was routinely recorded.

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Section 2

MATERIALS AND METHODS

page number

Section 2

MATERIALS AND METHODS continued

Section 2. Materials and methods

2:1 Materials

TLC plates (20 x 20 cm) and HPTLC plates (10 x 10 cm), pre-coated with silica gel 60 (0.25mm), were obtained from Merck (Darmstadt, Germany).

All solvents were of HPLC grade and were obtained from Rathburn Chemicals (Walkerburn, U.K.).

Leukotriene B_5 (LTB_5), 12-hydroxyeicosatetraenoic acid (12(R,S)-HETE) and 12-hydroxyeicosapentaenoic acid (12(S)-HEPE were purchased from Cascade Biochemicals Ltd. (Reading, U.K.).

Leukotriene B_4 (LTB_4), calcium ionophore A23187 (free acid), disodium ATP, collagenase (type IV), all PUFA (approx. 99% pure), fatty acid-free bovine serum albumin (BSA), 'Trizma' and 'Sigmacote' were from Sigma Chemical Co. Ltd. (Poole, U.K.).

15-Hydroxyeicosatetraenoic acid (15(S)-HETE) and 5-hydroxyeicosatetraenoic acid (5(R,S)-HETE) were purchased from Novabiochem Ltd. (Nottingham, U.K.).

[1-^{14}C]-DHA was obtained from Du Pont (U.K.) Ltd., Stevenage.

Lymphocyte separation medium, Hanks balanced salt solution, tryptose phosphate broth, 10 x concentrated non-essential amino acids, sodium bicarbonate, antibiotics, HCl and phosphate buffered saline were obtained from Flow Ltd. (Rickmansworth, U.K.).

Eagles' Minimum Essential Medium (EMEM), Dulbeccos modification of Eagles medium (DMEM), trypsin-EDTA and foetal calf serum (FCS) were obtained from Northumbria Biologicals Ltd. (Cramlington, U.K.).

ODS 'Sep-Pak' mini-columns were purchased from Millipore (U.K.) Ltd., (Watford).

2:2:1 Experimental animals and husbandry

Atlantic salmon S1 smolts (salmon undergoing transition to seawater in one year) were obtained from the S.O.A.E.F.D. Fish Cultivation Unit (Aultbea, Wester Ross, Scotland) or from Seafarm Kerry Ltd. (Gairloch, Scotland) and were used in three dietary trials in successive years. The fish weighed between 36 and 85g, depending on length of time in seawater after smoltification. In all three experiments fish were less than two months post-seawater transfer. In trials B and C, fish were distributed randomly into circular tanks of 1m diameter containing 500 L of seawater (100 fish per tank) which were supplied with seawater, subjected to sub-sand filtration, at a rate of 10 L/min. In trial A fish were placed into larger tanks of 2000 L capacity which were supplied with seawater at a rate of 26 L/min. In trial C fish were transferred to the 2000 L tanks after 8 weeks in the smaller (500 L) tanks. (See following "Results" section for details of each dietary trial). In all three dietary trials one tank of fish only was allotted to each dietary treatment. The tanks were subjected to natural photoperiod and the water temperature over the experimental periods (May-November) varied from 9-15^{o}C. The diets were supplied by automatic feeders which were activated for a few seconds every 15 min during daylight hours and were adjusted to provide between 20 and 28g/kg biomass each day. Fish were weighed individually at the start and finish of each trial and were bulk weighed every 28 days during the intervening period and the ration adjusted accordingly. During bulk weighing batches of 50 fish were placed in a tared dustbin containing approximately 50 litres of water and the final weight recorded. After weighing fish were returned to the same tank from which they came. The experiment was conducted in accordance with British Home Office guidelines regarding research on experimental animals.

2:2:2 Wild Atlantic salmon

Samples of wild Atlantic salmon parr were obtained by electrofishing in the river

Gairn, a tributary of the river Dee in Grampian, Scotland. Samples of smolts were obtained from smolt traps placed in the Dinnet burn, another tributary of the river Dee. Fish were transported alive to the S.O.A.E.F.D. Marine Laboratory, Aberdeen where the fish were killed, tissues dissected and stored at -80^{o}C until analysed.

2.3 Diet formulation and preparation

The practical-type diets, utilising fish meal as protein source, were formulated to meet the nutritional requirements of salmonid fish (U.S. National Research Council, 1981). Diets containing 47% crude protein and 17% lipid were prepared in 5 kg batches. The composition of the basal diet is shown in Table 2:3:1. All diets satisfied the nutritional requirements of salmonid fish for n-3 PUFA, which were supplied by the dietary fishmeal. The fatty acid composition of the LT 94 fish meal, which contained 11% lipid by weight, is shown in Table 2:3:2. The dry dietary components were mixed in 5 kg batches using a 'Hobart' commercial food mixer for at least 5 minutes. The appropriate oil containing added antioxidant mix (see page 35) was then added slowly, with continual mixing for a further 2 minutes, to the dry ingredients. Immediately before pelleting water (10ml/100g diet) and choline chloride (40% w/v) were added and the diet mixed thoroughly for five minutes. The diet was pelleted using a 'California Pellet Mill' producing pellets of 2 or 3 mm diameter. The pellets were then spread on ventilated trays and placed in a drying cabinet supplied with a continual flow of warm air (approx. 25^{o}C) for a period of 12-16 h after which the diets were sealed in dark plastic bags. The fatty acid compositions of the diets were determined immediately after drying and again ca. 6 weeks after pelleting. There were no significant differences in the fatty acid compositions after this storage period.

TABLE 2:3:1. Composition of basal diet

Ingredient	g/kg
LT-94 Fishmeal[1]	650
Starch[2]	150
Oil	100
Vitamin mix[3]	10
Mineral mix[4]	24
α-cellulose	61.6
Antioxidant mix[5]	0.4
Choline chloride	4

[1]LT-94 Norwegian low temperature herring meal, Ewos Ltd., Bathgate, Scotland.

[2]Passelli WA4 pre-cooked potato starch, Avebe (U.K.) Ltd., Ulceby, England.

[3]Supplied (mg/kg diet): all-rac -α-tocopheryl acetate, 40; menadione, 10; ascorbic acid, 1000; thiamin hydrochloride, 10; riboflavin, 20; pyridoxine hydrochloride, 12; calcium pantothenate, 44; nicotinic acid, 150; biotin, 1; folic acid, 5; cyanocobalamin, 0.02; myo-inositol, 400; retinyl acetate 7.3; cholecalciferol, 0.06.

[4]Supplied (per kg diet): KH_2PO_4, 22g; $FeSO_4.7H_2O$, 1.0g; $ZnSO_4.7H_2O$, 0.13g; $MnSO_4.4H_2O$, 52.8mg; $CuSO_4.5H_2O$, 12mg; $CoSO_4.7H_2O$, 2mg; KI, 2mg.

[5]Dissolved in propylene glycol and contained (g/L): butylated hydroxyanisole, 60; propyl gallate, 60; citric acid, 40.

Table 2:3:2. Fatty acid composition of LT 94 fishmeal. Values are weight % of total fatty acids.

Fatty acid	LT 94 fish meal
14:0	4.8
16:0	14.8
18:0	1.6
Total saturates[1]	21.8
16:1n-7	7.6
18:1n-9	10.7
18:1n-7	3.0
20:1n-9	13.0
22:1n-11	10.4
22:1n-9	1.2
24:1	1.3
Total monoenes[2]	47.6
18:2n-6	1.3
18:3n-6	0.1
20:2n-6	0.2
20:4n-6	0.5
22:5n-6	0.2
Total n-6	2.3
18:3n-3	0.7
18:4n-3	2.1
20:3n-3	0.1
20:4n-3	0.4
20:5n-3	7.9
22:5n-3	0.8
22:6n-3	11.9
Total n-3	23.9
Total PUFA	26.2
n-3/n-6	10.4

[1]Includes 15:0 and 17:0. [2]Includes 20:1n-7.

2:4 Lipid extraction and analysis

2:4:1 Extraction of diets and tissues

Samples of diets were collected after pelleting and drying, and again approximately 6 weeks later, and stored at -20°C until extracted. Tissue samples were dissected, after fish had been killed by a blow to the head or decapitation, and immediately placed in liquid nitrogen. Samples were stored at -80°C until extracted. Total lipid was extracted from tissues and diets by the method of Folch *et al.* (1957). Samples were homogenised in 10 volumes of ice-cold chloroform:methanol (2:1, v/v) using either a glass-teflon homogeniser for soft tissues e.g. liver, kidney, brain, leucocytes and retina or a 'Polytron' tissue disrupter for tougher material e.g. heart, muscle, gill and diets. Homogenates were filtered through filter paper pre-washed with choroform:methanol (2:1 v/v) and the filtrate mixed with 0.2 volumes of 0.88% KCl. The extract was then mixed vigourously in a stoppered test-tube using a vortex mixer. After the layers had separated the lower chloroform layer was removed using a pasteur pipette, placed in a pre-weighed tube and dried under a stream of nitrogen. The lipid extract was then dried for at least one hour in a vacuum dessicator containing KOH before re-weighing. The lipid extracts were dissolved in chloroform:methanol (2:1, v/v) containing 0.05% butylated hydroxytoluene at a concentration of 10 mg/ml and stored at -20°C.

2:4:2 Analysis of lipid class composition

Total lipid (10-20 µg) was applied as a 2 mm streak to a 10 x 10 cm high-performance thin-layer chromatography (HPTLC) plate which had been pre-run in hexane:diethyl ether (1:1, v/v) and activated in an oven at 110°C for 30 min. The plates were developed to 6 cm in methyl acetate:isopropanol:chloroform:methanol:0.25% aqueous KCl (25:25:25:10:9 by volume) to separate polar lipid classes with neutral lipids running at the solvent front

(Vitiello and Zanetta, 1978). After drying the plates were then developed fully in hexane:diethyl ether:acetic acid (85:15:1.5, v/v/v) to separate neutral lipids and cholesterol. Lipid classes were visualised by charring at 160^{o}C for 15 min after spraying with 3% copper acetate (w/v) in 8% phosphoric acid (v/v) and identified by comparison with commercially available standards (Sigma Chemical Co. Ltd., Poole, U.K.) . Lipid classes were quantified by scanning densitometry using a Shimadzu CS-930 dual wavelength TLC scanner and a DR-2 recording integrator.

2:4:3 Lipid class separation and fatty acid transmethylation

Total lipid extracts were separated into phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) fractions by TLC. Samples were loaded onto 20 x 20 cm TLC plates (1 mg/cm) and the plate developed with methyl acetate:isopropanol:chloroform:methanol:0.25% aqueous KCl (25:25:25:10:9 by volume). When amounts of total lipid available were less than 2 mg the phospholipid fractions were separated on 10 x 10 cm HPTLC plates loaded at 0.75 mg/cm and developed using the solvents described above. The phospholipid bands were visualised by spraying the plate lightly with 0.1% 2', 7',-dichlorofluorescein in 97% methanol containing 0.05% BHT and viewing under UV light. The phospholipid bands were marked in pencil and the areas of silica scraped into stoppered test tubes containing 2 ml of 1% H_2SO_4 in methanol. The tubes were flushed with nitrogen, stoppered and placed in a heated hot block at 50^{o}C. Acid-catalysed transmethylation was carried out for 16 h as described by Christie (1982). Triacylglycerol fraction (TAG) was separated from the total lipid extract by TLC using the solvent system hexane:diethyl ether:acetic acid (70:30:1 v/v/v). Loading and identification of lipid classes were carried in the same way as for phospholipid classes. Acid-catalysed transmethylation of TAG samples were carried out in 2 ml of

1% H_2SO_4 in methanol and 1 ml of toluene at 50°C for 16h. After transmethylation, the fatty acid methyl esters (FAME) from phospholipids and TAG were extracted after first neutralising the acid with 2 ml of 2% $KHCO_3$ (w/v) followed by succesive extractions with 5 ml of hexane followed by 5 ml of hexane:diethyl ether (1:1, v/v). The two solvent extracts were combined and dried under a stream of nitrogen. The dry FAME were dissolved in hexane containing 0.02% BHT at a concentration of 2 mg/ml and stored at -20°C prior to analysis.

2:4:4 Separation and identification of fatty acid methyl esters

The fatty acid methyl esters were separated and quantified by gas-liquid chromatography performed with a Carlo Erba Vega series 2 model 6180 gas chromatograph (Fisons Ltd., Crawley, U.K.). The capillary columns utilised were coated with the moderately polar phases, CP-Wax 51 (Chrompak (U.K.) Ltd., London) or FFAP (free fatty acid phase, S.G.E. Ltd., Milton Keynes, U.K.). Both columns were 50m long and 0.32mm and 0.22mm internal diameter respectively. Injection of samples was 'on-column' and hydrogen was used as carrier gas. Separation of fatty acid methyl esters was achieved using a two-stage thermal gradient from 50°C to 225°C. Fatty acids were separated in terms of carbon number with the most saturated eluted first followed by the monounsaturated and then the di-unsaturated etc in the same series. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman, 1980; Bell *et al.* 1983).

2:5 Isolation of leucocytes

Blood was collected, from the caudal vessel, into heparinised syringes from six fish per dietary treatment and was diluted with 3 volumes of Hanks balanced salt solution containing 20 i.u./ml of heparin. Four ml of diluted blood was layered onto 5

ml of lymphocyte separation medium and centrifuged at 400 x g for 45 min at 4°C. The leucocytes located around the interface of the separation medium and the salt solution were harvested and washed twice in phosphate buffered saline. If erythrocyte contamination was greater than 2% the gradient separation was repeated. The leucocytes obtained from six fish per dietary treatment were pooled and the lipids extracted by the method of Folch *et al.* (1957).

2:6:1 Preparation of isolated gill cells (1)

Fish were killed by a blow to the head, the pericardium opened and the bulbus arteriosus was perfused with 20 ml of ice-cold 0.9% NaCl (w/v) to remove blood from the gills. The gill arches were then removed and placed in ice-cold calcium free modified Hanks medium. This is a medium specially formulated for use with salmonid fish (Moon *et al.* 1985). The composition was: 10.29 g/L NaCl, 0.4 g/L KCl, 0.2 g/L $MgSO_4.7H_2O$, 0.06 g/L KH_2PO_4, 0.13 g/L $Na_2HPO_4.12H_2O$, 0.5 g/L $NaHCO_3$, and 2.38 g/L (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid (HEPES). The medium was adjusted to pH 7.4 with KOH. The gill arches were then blotted on tissue and the epithelial layer scraped with a blunt scalpel blade to remove cells. The cells were then suspended in ice-cold calcium-free Hanks medium, gently separated by stirring with a magnetic stirrer and filtered through a 100 µm nylon gauze. The cells were collected by centrifugation at 1000 x g for 5 min.

2:6:2 Preparation of isolated gill cells (2)

Fish were killed and the gills perfused as described above. The intact gill arches were dissected out and placed in ice-cold medium. The gill filaments were dissected from the arches and chopped finely with scissors before addition of 25 ml of medium containing 0.1 % (w/v) collagenase (type IV). This suspension was incubated for 45 min at room temperature and was stirred constantly using a magnetic stirrer. The suspension was finally filtered through two grades of nylon

gauze (190 and 100 μm) and the filtered cells collected by centrifugation at 1000 x g for 5 min. The cells were washed twice in Hanks medium and finally resuspended in 1 ml of the same medium containing 1 mM $CaCl_2$, 50 μl was retained for protein determination.

2:7 Challenge of whole blood and isolated gill cells with calcium ionophore

Glass test tubes used in the incubations were pre-coated with the silanizing reagent, 'Sigmacote', prior to use to prevent cell adhesion. One ml of whole blood was mixed with an equal volume of modified Hanks medium containing 1 mM $CaCl_2$ and both blood and gill cells were incubated in a shaking water bath at 18°C for 10 min. Calcium ionophore A23187 was added in up to 5 μl of dimethyl sulphoxide at a final concentration of 20 μM and the incubation continued for a further 20 min. The cells were removed by centrifugation (12 000 x g, 2 min) and the supernatant removed for eicosanoid extraction.

2:8 Eicosanoid extraction

Eicosanoids were extracted from incubation media using ODS reverse-phase 'Sep-Pak' mini-columns largely using the methods described by Powell (1982). Samples were loaded onto 'Sep-Pak' columns which had been pre-washed with 5 ml methanol and 10 ml distilled water. The sample was then washed with 5 ml of 15% (v/v) ethanol, 10 ml of distilled water, 5 ml of hexane:chloroform (65:35 v/v) before prostaglandins were eluted with 10 ml ethyl acetate. When measuring lipoxgenase metabolites e.g. leukotrienes and hydroxyfatty acids the wash with hexane:chloroform was omitted.

2:9 HPLC separation of lipoxygenase products

The profiles of lipoxygenase products extracted from gill and blood supernatants were determined using reverse phase high-performance liquid chromatography

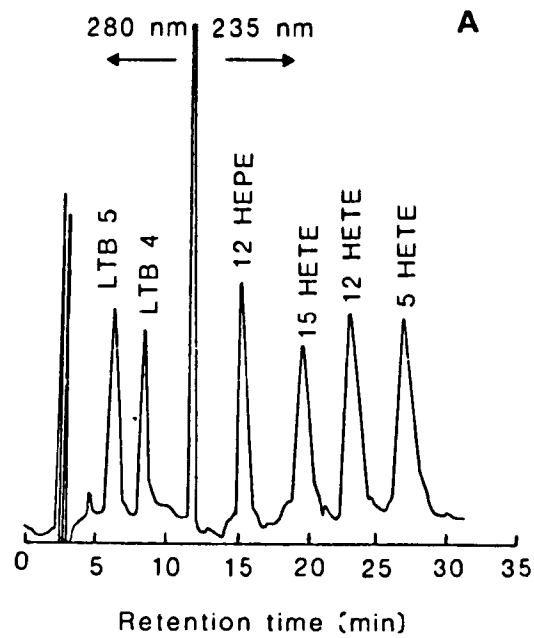
(HPLC) using a 'Spherisorb' 5 μm octadecylsilane (ODS2) column (25 cm x 4.6 mm) supplied by Scotlab Ltd., Bellshill, Scotland. The chromatographic system was equipped with Waters Model M-45 pumps and 680 Automated Gradient Controller and the effluent was monitored at 280 nm and 235 nm to detect leukotrienes and hydroxy fatty acids, respectively, using a Pye-Unicam LC-UV detector. Two isocratic solvent systems were utilised at a flow rate of 1 ml/min. To separate the monohydroxy fatty acids a solvent system containing methanol/water/acetic acid (80:20:0.05 v/v/v) (Solvent 1) was used. To separate leukotrienes and monohydroxy fatty acids a solvent system containing acetonitrile/methanol/water/acetic acid/phosphoric acid/ammonia (40:29:30:0.5:0.3:0.2 by vol.) (Solvent 2) was used. Quantitations were based on external standards of 12-HEPE, 12-HETE, LTB_5 and LTB_4. Chromatograms representing separation of leukotriene and hydroxy fatty acid standards and lipoxygenase products obtained after stimulation of whole blood with A23187, are shown in Figure 2:9:1.

2:10 Preparation and chromatography of hydroxy fatty acid methyl esters

The peak eluting at a retention time similar to that of standard 12-HEPE on HPLC separation of gill lipoxygenase products was collected and concentrated. Methyl esters of standard 12-HEPE and the concentrate were prepared using diazomethane as described by Schwartz and Bright (1974). The methyl esters were purified using HPLC (Solvent 1) and analysed by gas-liquid chromatography (Philipis PU 4500 chromatograph) using a CP-Sil 5 column (50 m x 0.32 mm i.d.; Chrompak, Middleberg, The Netherlands). Hydrogen was used as carrier gas and the temperature was programmed from 50°C to 260°C at 4°C/min.

2:11 Labelling of gill cells with [1-14C]-22:6n-3

Isolated gill cells produced as described previously were resuspended in 2 ml



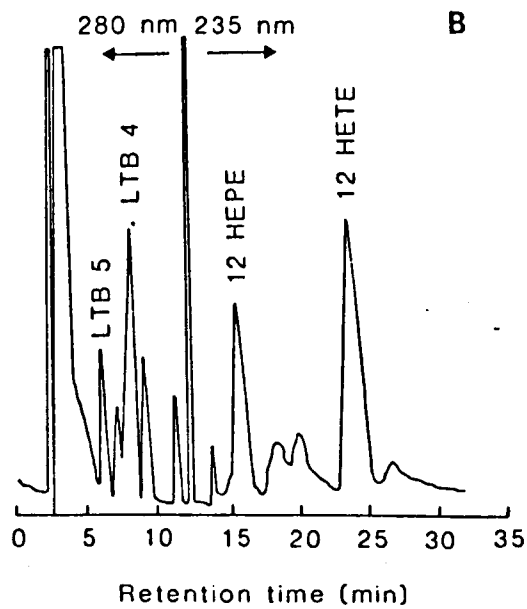


Fig. 2:9:1 A typical HPLC chromatogram of leukotriene and hydroxy acid standards separated using solvent system described in Materials and Methods (A). A typical HPLC chromatogram of lipoxygenase products obtained after stimulation of whole blood with A23187 (20 μM) (B).

Dulbeccos modification of Eagles medium (DMEM). After 10 min at 18°C in a shaking water bath, 0.5 μCi radiolabelled DHA (in 5 μl ethanol) was added and the incubation continued for 5 h. The cells were sedimented by centrifugation (400 x g, 5 min) and washed in 5 ml PBS containing 1% (w/v) bovine serum albumin (fatty acid free) to remove non-incorporated radiolabel. The cells were resuspended in 2 ml of fresh DMEM and incubated in a shaking water bath for 10 min, before addition of A23187, as described previously. Eicosanoids were extracted from the cell supernatant after centrifugation as described above. The eicosanoid extract was dissolved finally in 0.2 ml HPLC solvent B and 0.1 ml was injected onto the HPLC column and eluted as described previously. Fractions of 1 ml were collected and counted in a scintillation counter after addition of 5 ml 'Ecoscint A' (BS and S Ltd., Edinburgh, Scotland).

2:12 Determination of 6-keto prostaglandin $F_{1\alpha}$ and thromboxane B_2

6-keto $PGF_{1\alpha}$ and TXB_2, the stable metabolites of prostacyclin (PGI_2) and TXA_2 respectively, were measured by radioimmunoassay using the tritium isotope kits supplied by Amersham (U.K.) Ltd. The assay is based on the competition between unlabelled prostaglandin and a fixed quantity of the tritium labelled compound for binding to a protein with high specificity and affinity for the prostaglandin. The amounts of antibody and radioactive prostaglandin remain constant and the amount of radioactivity bound by the antibody is inversely proportional to the concentration of added non-radioactive prostaglandin. Measuring the amount of antibody-bound radioactivity enables the amount of unlabelled prostaglandin in the sample to be calculated. The unbound prostaglandin is separated from the antibody-bound prostaglandin by absorption of the free prostaglandin on to dextran-coated charcoal, followed by centrifugation. By measuring the amount of radioactivity in the supernatant the amount of labelled prostaglandin bound by the antibody can be

calculated. The concentration of unlabelled prostaglandin in the sample is then determined from the linear standard curve.

2:13 Isolation of cardiac sarcoplasmic reticulum

The procedure used was largely that described by Swanson *et al.* (1989). Hearts from two fish were pooled (300-500 mg wet weight) and homogenised in 20 volumes of ice-cold 20 mM Tris-HCl (pH 7.4) buffer containing 30 mM L-histidine and 250 mM sucrose using a 'Polytron' tissue homogeniser. The homogenate was centrifuged at 1600 x g for 10 min at 4°C to remove nuclei and cell debris. The supernatant was retained and the pellet re-homogenised and centrifuged as described above. The combined supernatants were then centrifuged at 14000 x g for 15 min at 4°C to remove mitochondria and lysosomes. The pellet was discarded and the supernatant centrifuged at 45000 x g for 50 min at 4°C. The pellet was resuspended in 20 ml homogenisation buffer and re-centrifuged at 48000 x g for 50 min at 4°C. The final sarcoplasmic reticulum pellet was resuspended in 0.25 ml of homogenisation buffer prior to assaying Ca^{2+}-Mg^{2+} ATPase activity.

2:14 Ca^{2+}-Mg^{2+} ATPase assay

The assay was performed largely as described by Bonis *et al.* (1985). Sarcoplasmic reticulum vesicles, containing between 25-50 µg protein, were incubated in a 2.0 ml reaction medium comprising 50 mM Tris-HCl (pH 7.1), 5 mM potassium oxalate, 60 µM $CaCl_2$, 30 mM KCl and 5 mM $MgCl_2$ for 5 min at 18°C. The reaction was initiated by addition of 2 mM disodium-ATP and the incubation was continued for a further 45 min before the reaction was terminated by addition of 1.0 ml of 5% (w/v) trichloroacetic acid. Total ATPase activity was determined by measurement of inorganic phosphorus released from ATP by the method of Stanton (1968), using a diagnostic kit (Sigma Chemical Co., Poole, U.K.). Calcium-

dependent ATPase activity was determined by subtracting the basal value obtained in the presence of 0.1 mM [ethylenebis (oxyethylene nitrilo)] tetra acetic acid and in the absence of calcium from values obtained with 60 μM $CaCl_2$.

2:15 Isolation of cardiac myocytes and Ca^{2+}-ionophore stimulation

The procedure for enzymatic isolation of cardiac myocytes is based on the method of Powell *et al.* (1980). Hearts from two salmon were cannulated via the bulbus arteriosus and perfused with modified Hank's medium (Moon *et al.* 1985) which was gassed continuously with 95% O_2, 5% CO_2. After 15 min, the system was switched to recirculation and the medium changed to modified Hanks containing 0.6% (w/v) collagenase. The perfusion was continued for a further 45 min, after which the partially digested hearts were removed and chopped finely in perfusion medium. This material was filtered through two grades of nylon mesh (190 and 100 μm) and the myocytes collected by centrifugation (1000 x g, 2 min). The myocytes were washed twice in Hank's medium (without collagenase) and finally resuspended in 1.0 ml of the same medium containing 1 mM CaCl2. The suspensions routinely contained 60-85% of the total cells present as rod-shaped, cardiac myocytes. Fifty microlitres of the suspension were retained for protein determination. The myocyte suspensions were incubated in a shaking water bath at 18°C for 10 min before addition of calcium ionophore (A23187, final concentration 20 μM in 5 μl dimethyl sulphoxide). The incubation was continued for 20 min, after which the cells were sedimented by centrifugation and the prostanoids extracted from the supernatant as described previously.

2:16 Chum salmon heart cells and medium

The chum salmon (*Oncorhynchus keta*) established heart (CHH-1) cell line was obtained from Dr. D. Smail, S.O.A.E.F.D. Marine Laboratory, Aberdeen, U.K. and had been derived from primary cultures of heart cells prepared by Lannan *et al.* (1984), using the technique described by Wolf and Quimby (1976). The cells were

maintained in Eagles minimum essential medium (EMEM; with Earles salts) containing 1% tryptose phosphate broth, 4 mM Tris-HCl buffer (Trizma pH 7.4, Sigma Chemical Co. Ltd., Poole, U.K.), 1% non-essential amino acids, 0.275% sodium bicarbonate, 2 mM HCl, antibiotics (50 I.U. ml^{-1} penicillin and 50 $\mu g.ml^{-1}$ streptomycin) and either 10% or 1 % fetal calf serum (FCS).

2:17 Preparation of PUFA supplements

PUFA supplements were added to the CHH-1 cultures as BSA complexes which were prepared according to the method of Spector and Hoak (1969). To minimise oxidation all procedures were carried out at room temperature under a stream of nitrogen. Twenty five mg of each PUFA were dissolved in 7.5 ml of hexane in a 10 ml test-tube (some PUFA required the addition of a small amount of chloroform/methanol (2:1 v/v) to allow full solubilisation). The dissolved PUFA were added to 800 mg of 'Celite' in a 50 ml conical flask (covered in aluminium foil) and the slurry mixed on a magnetic stirrer. The slurry was dried under a stream of nitrogen until all solvent was evaporated.The top of the flask was then covered with fine wire mesh and the dry slurry vacuum desiccated for 30 min. Twenty five ml of BSA solution (0.25 mM BSA in 16 mM phosphate buffer, 116 mM NaCl, 4.9 mM KCl and 1.2 mM $MgSO_4$ pH 7.4) was added and the slurry stirred for 30 min with constant nitrogen gassing. The mixture was transferred to a 30 ml centrifuge tube and centrifuged (2000 x g, 5 min) to sediment the 'Celite'. The PUFA:BSA complexes in the supernatant were then filter-sterilized through 0.2 μm filters (Flowpore, Flow Laboratories) prior to use. The fatty acid concentrations were assayed after addition of an internal standard (17:0) to an aliquot of the complex. The lipids were then extracted, transmethylated and quantified by gas-liquid chromatography. PUFA concentrations were in the range 1.5-2.0 mM (BSA 0.25 mM) giving a PUFA:BSA ratio of 6-8. The PUFA:BSA mixtures were stored under nitrogen at -20^{o}C in darkened vials.

2:18 Culture of CHH-1 cells

CHH-1 cells were routinely cultured in sealed 75 cm^2 flasks with 15 ml medium containing 10% FCS as described previously. Cells were harvested for analysis or subculture within 24 h of achieving confluence (usually after 5-7 days). Cells incubated with PUFA were subcultured into 225 cm2 flasks with 50 ml medium containing 1% FCS and an appropriate volume of PUFA:BSA complex to give a final fatty acid concentration of 25 μM. FCS contains around 0.37% (w/v) lipid of which around 50% is esterified fatty acid. Previous studies with cultured fish cells indicated that a fatty acid concentration of 25 μM resulted in considerable incorporation and metabolism into phospholipids without causing increased deposition of neutral lipid (Tocher *et al.* 1988, 1989). Control flasks received the same volume of fatty acid free-BSA solution. All incubations were performed at 22^{o}C.

2:19 Cell harvesting and lipid extraction

Cells were harvested within 24 h of reaching confluence. The media were removed by aspiration and the cells were washed with 20 ml phosphate-buffered saline before addition of 3 ml trypsin-EDTA (0.05% and 0.02% (w/v) respectively). Once the cells became dissociated 6 ml of basal EMEM were added to inhibit trypsin activity. Cells from two 225 cm^2 flasks were pooled and centrifuged at 600 x g for 10 min at 4^{o}C, the supernatant poured off and the cell pellet washed twice with 25 ml of phosphate-buffered saline to ensure removal of serum. Total lipid was extracted from the cells by homogenisation in 5 ml of ice-cold chloroform:methanol (2:1, v/v) followed by addition of 1 ml 0.88% (w/v) KCl and mixing on a vortex mixer. The phases were separated by centrifugation, the chloroform layer removed and dried under nitrogen and the residue dissolved in chloroform/methanol (2:1, v/v)

containing 0.05% BHT at a final concentration of 10 mg/ml.

2:20 Protein estimation

Protein in tissue homogenates and intracellular fractions was quantified by the method of Lowry *et al.* (1951). Protein in intact cell preparations was measured using an adaptation of the above method. Standards containing 0-100 μg of protein, prepared from a standard 1 mg/ml solution of BSA, were placed in 5 ml polypropylene tubes and the volume adjusted to 0.1 ml with distilled water. Samples of cell suspensions, containing up to 0.1 ml, were placed in similar tubes. 0.25 ml of a solution containing 1 M NaOH and 0.25% (w/v) sodium dodecyl sulphate (SDS) was added to standard and sample tubes which were incubated for 45 min at 60^{o}C in a water bath. After cooling 2.5 ml of a solution containing 1 ml 1% (w/v) $CuSO_4.5H_2O$, 1 ml 2% (w/v) sodium potassium tartrate and 100 ml 2% (w/v) NaCO3 was added, the contents mixed, and incubated for 15 min. at room temperature. 0.25 ml of Folin-Ciocalteu phenol reagent, diluted 1:1 (v/v) with distilled water, was added to all tubes. After mixing and incubating for a further 30-60 min the absorbance of samples and standards were recorded at 660 nm in a spectrophotometer.

2:21 Enzyme immunoassay of prostaglandins

These assays were performed using commerially available enzyme immunoassay kits. The assay principle is based on the competition between free prostaglandin (e.g. PGE_2) and a PGE_2 tracer (PGE_2 linked to an acetylcholinesterase molecule) for a limited number of PGE_2-specific rabbit antiserum binding sites. The concentration of the PGE_2 tracer remains constant while the concentration of free PGE_2, either standard or sample, is varied. Therefore, the amount of PGE_2 tracer that is able to bind to the rabbit antiserum will be inversely proportional to the concentration of free PGE_2 in the well. The rabbit antiserum-PGE_2 (either free or tracer) binds to the mouse monoclonal antibody which has been previously

attached to the well. The plate is then washed to remove any unbound reagents and then Ellmans reagent, which contains the substrate for the acetylcholinesterase, is added to the well. The chromophore produced by this enzyme reaction is yellow in colour and absorbs strongly at 412 nm. The absorption is determined spectrophotometrically and is proportional to the amount of PGE_2 tracer bound to the well, which is in turn inversely proportional to the amount of free PGE_2 present in the well during the incubation. A standard graph of $\%B/B^0$ (% bound/maximum bound) versus log PGE_2 concentration is shown in Figure 2:21:1. While the kits purport to measure PGE_2, which will be the major PGE analogue in mammals, the antibody also cross-reacts with PGE analogues derived from both EPA and 20:3n-6. The cross-reactivity of the PGE_2 antibody with PGE_3 and PGE_1 was 100% and 6.2% respectively.

2:22 Assay of phospholipase A activity

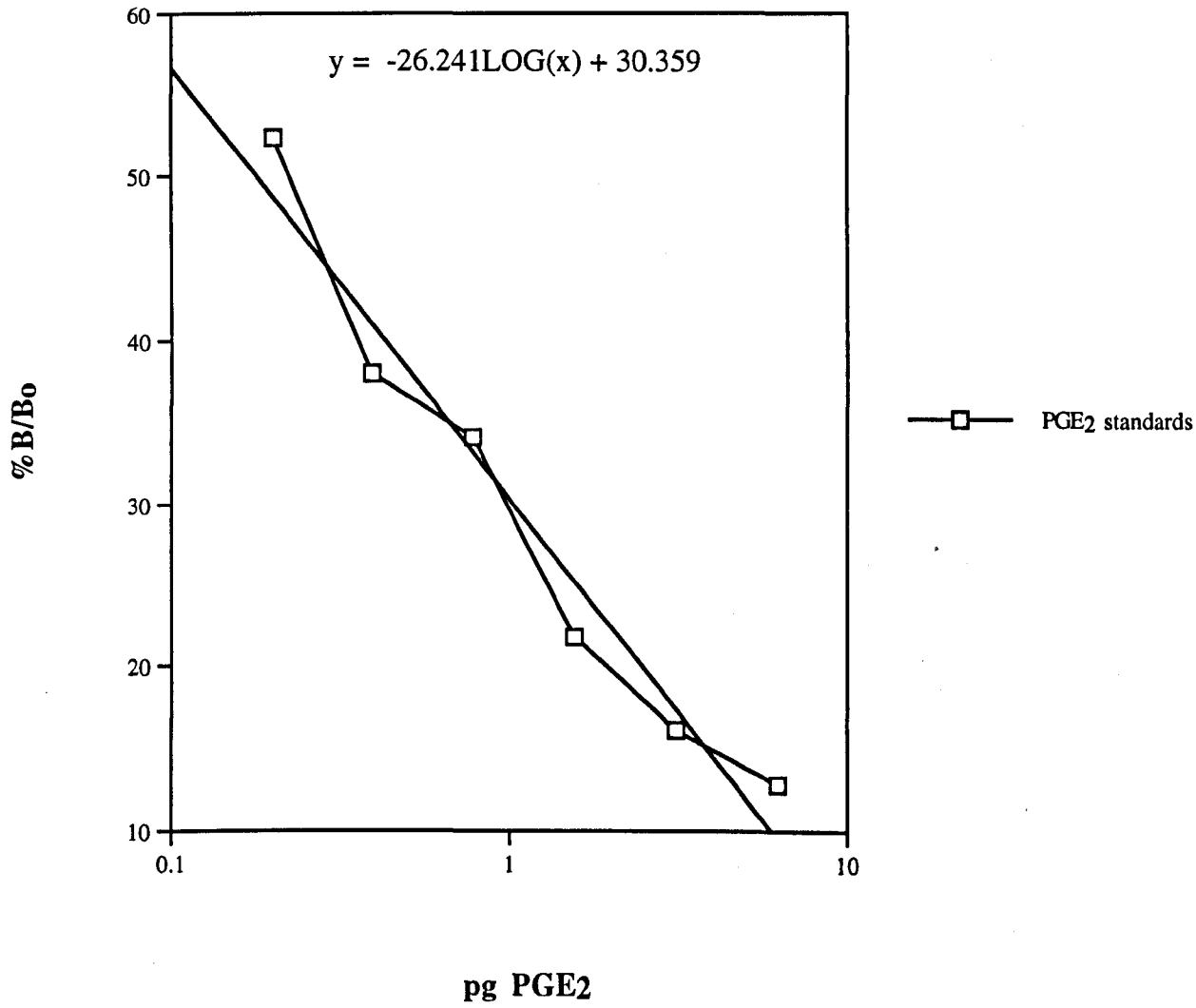
Cardiac phospholipase A activity was assayed using endogenous substrate largely as described by Szymanska *et al.* (1991). One heart was homogenised in 2 ml of 50 mM Tris-HCl (pH 8.5) containing 200 mM KCl and 5 mM $CaCl_2$. One ml of homogenate was immediately extracted by the method of Folch *et al.* (1957), 100 µl was removed for protein determination and the remaining 1 ml was placed in a shaking water bath at 18°C. After 2 h the reaction was terminated by addition of 5 ml of chloroform:methanol (2:1 v/v) containing 0.05% BHT (w/v) and the lipids extracted as above. Free fatty acids, PC and PE were separated in 0 and 2 h samples by HPTLC and quantified as described in section 2:4:2. Phospholipase A activities are expressed either as nmol fatty acid liberated or nmol PC and PE hydrolysed/h per mg protein. The composition of released free fatty acid was determined after separating the free fatty acids using TLC. Lipid extracts were separated on 20 x 20 cm TLC plates using hexane:diethyl ether:acetic acid (70:30:1

Figure 2.21.1

Standard graph of log PGE_2 concentration vs. $\%B/B_0$ (% bound/maximum bound) for PGE_2 enzyme immunoassay (EIA).

The assay is based on competition between free PGE_2 and PGE_2 tracer (PGE_2 linked to acetylcholinesterase) for a limited number of PGE_2-specific antiserum binding sites. The concentration of the tracer remains constant while the concentration of free PGE_2, either standard or sample, is varied. Therefore, the amount of PGE_2 tracer that is able to bind to the antiserum is inversely proportional to the concentration of free PGE_2 in the well.

Figure 2:21:1


y = -26.241LOG(x) + 30.359
60
50
40
30
20
10
0.1
1
10
% B/Bo
pg PGE2
PGE2 standards

v/v/v), the free fatty acid band scraped off and transmethylated, and the fatty acids identified and quantified by gas-liquid chromatography. The average molecular weight of released fatty acid was calculated as 291. The composition of cardiac PC and PE was analysed similarly and average molecular weights for PC and PE of 852 and 804 respectively, were used in calculating phospholipase A activity.

2:23 Histology

Samples of heart, liver, kidney, pancreas, spleen and gills were fixed in 20% buffered formol saline (Na_2HPO_4, 32.1 g; $NaH_2PO_4.2H_20$, 22.8g; NaCl, 22.5g; formalin, 500ml; distilled water, 2L) at the time of sampling. Gills were decalcified in a solution containing 40 ml of 20% nitric acid, 30 ml of 1% chromic acid and 30 ml of absolute ethanol. All tissues were dehydrated by immersion in increasing concentrations of absolute ethanol, from 70-100%, followed by a mixture of absolute ethanol and "Histoclear" (B.S. & S. Ltd, Edinburgh, Scotland) (1:1 v/v) before final clearing in "Histoclear". Tissues were then embedded in paraffin wax and 5 µm sections were cut. Samples were then stained with Harris's haematoxylin and 1% (w/v) aqueous eosin before mounting with "Histomount" (B.S. & S. Ltd., Edinburgh, Scotland) and a coverslip. Pathological assessment was carried out on coded, randomised slides to eliminate bias in interpretation. All slides were randomised, existing identification numbers covered by autoclave tape and the slides renumbered. In all histological examinations 12-24 slides per dietary treatment (one slide per fish) were examined. This allowed estimation of inter-population variability rather than intra-organ variation which would necessitate serial sectioning. Pathological examination was carried out on a Wild M20 compound microscope at an objective magnification of x20 or x40. Each heart ventricle section (and auricle if present) was scanned, in its entirety, at low power (x 20) followed by a second scan focussing on specific areas of interest, at high power (x 40). All histopathological assessments were performed by Dr Alasdair McVicar,

S.O.A.E.F.D. Marine Laboratrory, Aberdeen.

2:24 Statistical analysis

Significance of difference ($P < 0.05$) between treatments was determined either by Student's t test or by analysis of variance (ANOVA). Analyses were performed using a Statgraphics (system 3.0) computer package. Data which were identified as non-homogeneous (using Bartlett's test, Bartlett, 1937) were subjected to either arcsine square root or log transformation before analysis. Data which were non-homogeneous after transformation were analysed by the non-parametric Kruskal-Wallis test (Kruskal and Wallis, 1952).

Section 3. Dietary study A; Effects of feeding diets with high and low n-3/n-6 PUFA ratio on tissue phospholipid fatty acid compositions and eicosanoid production in Atlantic salmon post-smolts.

3:1 Introduction

Most mammals, with the exception of strict carnivores, can metabolise linoleic acid (18:2n-6) by Δ 6-desaturation to gamma-linolenic acid (18:3n-6) which after elongation to dihomogamma-linolenic acid (20:3n-6) can undergo further desaturation by a Δ 5-desaturase to produce arachidonic acid (AA; 20:4n-6) (Crawford *et al.* 1976). The same enzymes are also active in converting a-linolenic acid (18:3n-3) to eicosapentaenoic acid (EPA; 20:5n-3). While most freshwater fish possess the same pathway (Henderson and Tocher, 1987), marine fish such as turbot are apparently deficient in Δ 5-desaturase activity and thus require the long-chain PUFA, EPA, docosahexaenoic acid (DHA; 22:6n-3) and probably AA to be supplied by the diet (Owen *et al.* 1975; Bell *et al.* 1985). In an anadromous fish such as Atlantic salmon (*Salmo salar)* the ability to elongate and desaturate C_{18} PUFA may be diminished during smoltification (Ackman and Takeuchi, 1986) but appears to be present in post-smolting juveniles (Bell *et al.* 1989).

In humans consuming 'Western-type' diets with a high n-6/n-3 PUFA ratio AA is the major precursor available for eicosanoid production, and these diets are possibly responsible for the high incidence of atherothrombotic and inflammatory conditions prevalent in Western populations (Nordoy and Goodnight, 1990; Miller *et al.* 1990). Considerable evidence now exists that many of these conditions can be attenuated by reducing the tissue n-6/n-3 PUFA ratio using dietary fish oil supplements which are rich in EPA and DHA (Lands, 1986). As with humans, intensively farmed Atlantic salmon are susceptible to stress-induced disease conditions. Due to the inclusion of some plant-derived lipids in their dietary regime, farmed salmon contain around five times as much n-6 PUFA compared to their wild

counterparts (Bell, J.G. unpublished data) and it is possible that the subsequent increase in n-6/n-3 PUFA ratio is responsible for increased AA-derived eicosanoid production in farmed salmon. In this experiment two groups of Atlantic salmon post-smolts were fed diets with the same total lipid content, but in which the lipid was supplied either as fish oil, (Fosol, white fish body oil; FO, obtained from Seven Seas Ltd., Hull, U.K.) or sunflower oil (SO; Tesco Ltd., Cheshunt, U.K.), with the latter diet containing adequate levels of n-3 PUFA derived from fishmeal (U.S. National Research Council, 1981). The fatty acid compositions of the experimental diets are shown in Table 3:1:1. The objective was to alter the n-3/n-6 PUFA ratio in salmon and specifically to investigate the incorporation of 18:2n-6 and its metabolites into the individual phospholipids of heart, liver, brain and retina. The former two tissues were chosen because they are tissues of high metabolic activity while the two neural tissues are especially rich in 22:6n-3 (Tocher and Harvie, 1988) which is thought to perform specific functional roles in both brain and retina (Connor *et al.* 1992). Reduction of 22:6n-3 in neural tissues results in impaired physiological function in primates (Neuringer *et al.* 1988) and impaired visual function in herring (Bell *et al.* 1995). The fatty acid compositions of individual phospholipids from both gill and blood leucocytes were also measured since these tissues are particularly active in eicosanoid production (German and Kinsella, 1986; Henderson *et al.* 1985; Pettitt *et al.* 1989). Lipoxygenase products derived from endogenous precursors were measured in isolated gill cells and whole blood challenged with the calcium ionophore A23187.

3:2 Experimental details, growth and mortality

Three hundred Atlantic salmon S1 smolts were obtained from the S.O.A.E.F.D. Fish Cultivation Unit, (Aultbea, Wester Ross) and transferred to seawater at the same facility. The smolts were distributed randomly into two tanks of 2 m x 2 m

Table 3:1:1 **Fatty acid composition of diets** (Values are weight % of total fatty acids)

Fatty acid	Fish oil diet	Sunflower oil diet
14:0	5.7	2.0
16:0	13.8	9.7
18:0	2.5	3.4
16:1n-7	5.2	2.2
18:1n-9	12.0	14.7
18:1n-7	2.3	2.3
20:1n-9	8.9	3.2
22:1n-11	13.8	4.0
24:1	1.4	0.4
18:2n-6	1.4	42.1
18:3n-6	0.2	1.1
20:2n-6	0.2	t
20:3n-6	0.2	0.1
20:4n-6	0.6	0.4
18:3n-3	1.3	0.4
18:4n-3	2.9	0.7
20:5n-3	7.2	3.4
22:5n-3	1.2	0.5
22:6n-3	10.4	4.6
Total n-6	2.5	43.6
Total n-3	23.8	9.9
n-3/n-6	9.4	0.2
Unsaturation index	173.5	168.3

t = trace value < 0.05%. Unsaturation index = weight % x number of double bonds.

square containing 2000 L of seawater which were supplied with non-recirculated at 26 L/min. The tanks were subjected to natural photoperiod and the temperature over the experimental period (June-October) was 9-13^{o}C. The fish meal-based diets contained 46% protein and 17% lipid (see section 2; Materials and Methods). The diets were supplied by automatic feeders, which were activated for a few seconds every 15 min during daylight hours and adjusted to provide 20 g/kg biomass each day. Fish were weighed individually at the start and finish of the experiment and were weighed in bulk every 28 days, and the ration adjusted accordingly. Both dietary treatments achieved satisfactory growth during the 16-week experimental period (See Table 3.2.1). Final weights after 16 weeks were not significantly different although fish fed the FO diet were significantly lighter at the outset of the trial. Mortalities with both diets were low (< 10%) over the experimental period. However, when 30 fish from each treatment were simultaneously transported to the aquarium at the University of Stirling, from the experimental station at Aultbea (a 4 h journey), 30% of SO-fed fish died, while no mortalities occurred in the FO-fed group. The fish were transported in sealed, double, black polythene bags. Each bag contained approximately 15 L of seawater and 2 kg biomass and were sealed after inflation with approximately 30 L of oxygen. The initial water temperature was 13^{o}C and the temperature at the end of the journey was 15^{o}C. Although no fish were dead on arrival, several of the SO-fed fish appeared in a stressed condition, i.e. lying upside down and showing rapid gill movement. The tank was immediately bubbled with oxygen, for a period of 30 min, but within 24 h 30% were dead. These symptoms are similar to the shock syndrome described by Castell *et al.* (1972); Castell (1993, personal communication) in essential fatty acid-deficient rainbow trout.

Table 3.2.1. Growth parameters and mortalities during the 16 weeks experimental period.

Parameter	FO diet	SO diet
Number of fish	152	152
Mean initial weight	46.4 ± 14.1*	55.3 ± 14.9
Mean final weight	243.2 ± 40.7	241.4 ± 43.6
Mortalities (number)	10	12
Specific growth rate (%/d)	1.5	1.3
Feed efficiency ratio	48.6	49.9

Values for initial and final weights are mean ± SD. *Values are significantly different ($P < 0.05$). Specific growth rate calculated as %weight gain/day = $(e^{G_W}-1) \times 100$, where G_W (daily growth rate) = $\ln W_1 - \ln W_0/T$. (W_1 = final weight, W_0 = initial weight, T = time in days). Feed efficiency ratio = gain/feed x 100.

3.3 Tissue histopathology

No significant histopathology was observed in the pancreas, kidney, spleen, heart and gills of the salmon fed the FO diet. One fish fed the SO diet showed areas of focal degenerative necrosis in the liver, while another fish had a similar lesion in the spleen and kidney. These organs were normal in all other fish in the SO group, and therefore, little significance can probably be attributed to these observations. However, a high proportion of fish fed the SO diet showed heart ventricle lesions ranging from mild to severe (Table 3.3.1). At low magnifications 45% of all hearts from the SO-fed group showed a pronounced depletion in the amount of muscle present in both the spongy and compact layers, with (in extreme cases) the heart wall becoming extremely thin (Fig. 3.3.1) compared to the normal organ (Fig. 3.3.2). Closer histological examination of affected hearts, as well as some not showing obvious muscle depletion, generally showed active focal myodegeneration, particularly in the spongy tissue (Fig 3.3.3). The foci of degeneration ranged from single small lesions to multiple extensive areas. Similar lesions were also apparent in atrial muscle. The cardiac myodegenerative lesions found in this study are similar to those described by Roberts and Bullock (1989).

Figure 3.3.1 Light micrograph of heart tissue from Atlantic salmon fed sunflower oil-containing diet for 16 weeks. Micrograph indicates depletion in the amount of muscle present in both spongy (◇) and compact layers (▶), resulting in thinning of the heart wall (Scale = 300 μm).

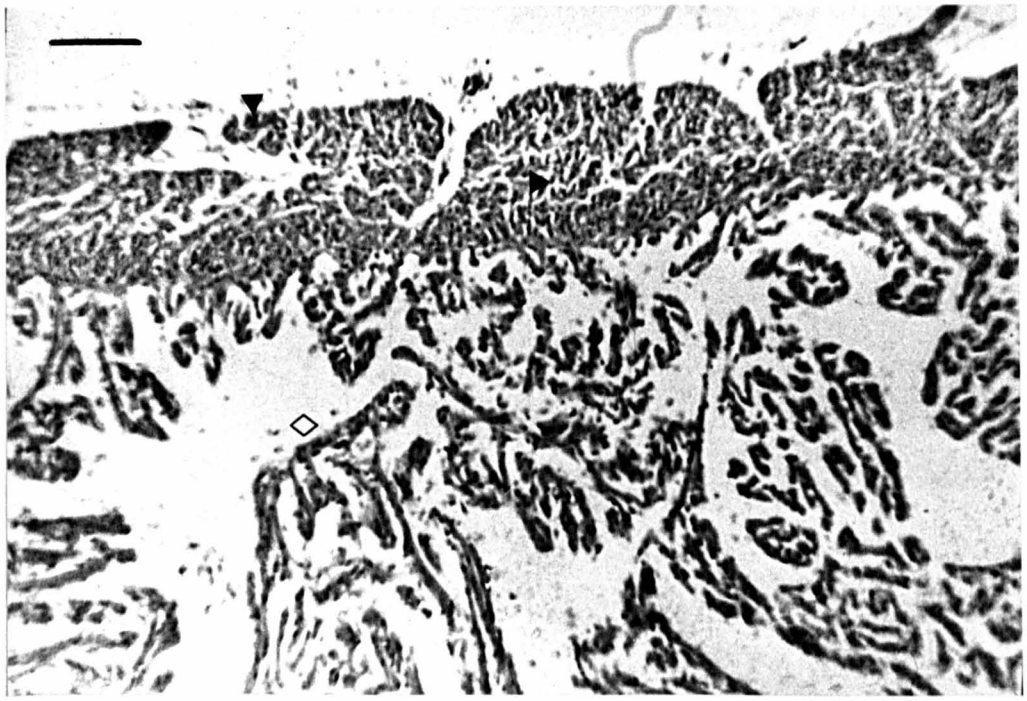


Figure 3.3.2 Light micrograph of heart tissue from Atlantic salmon fed fish oil-containing diet for 16 weeks showing normal appearance of spongy (◇) and compact (▶) muscle layers (Scale = 200 μm)

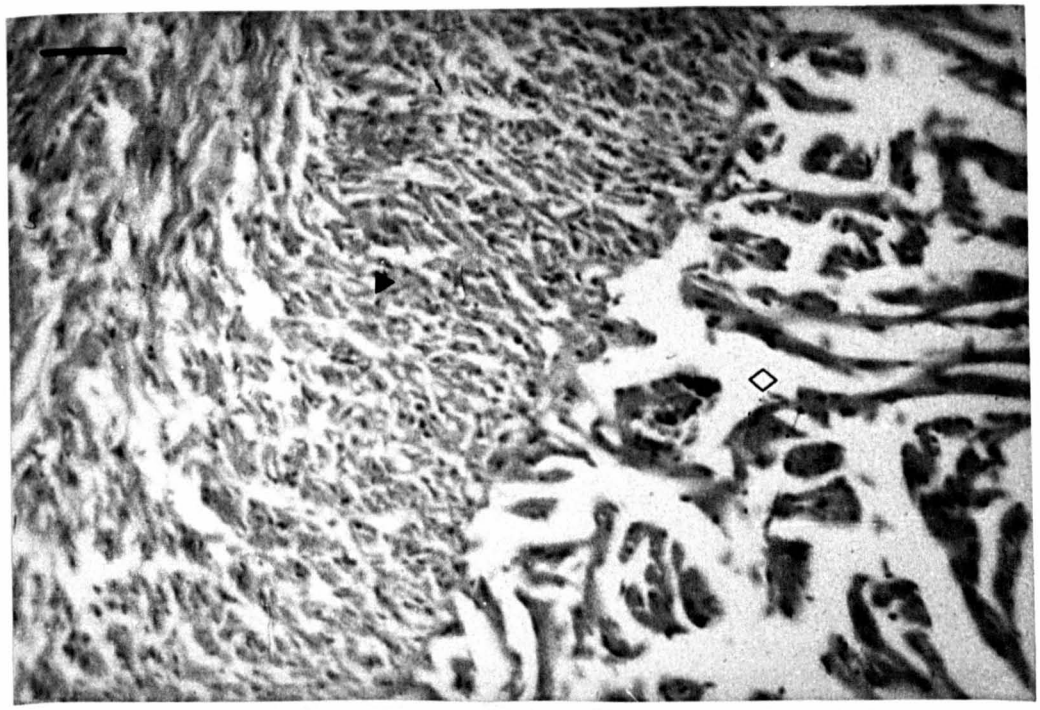


Figure 3.3.3 Light micrograph of heart atrium from Atlantic salmon fed sunflower oil-containing diet for 16 weeks. Micrograph indicates areas of apparent focal myodegeneration and necrosis (arrows) beneath the extensive accumulation and infiltration of leucocytes (L). (Scale = 20 μm).

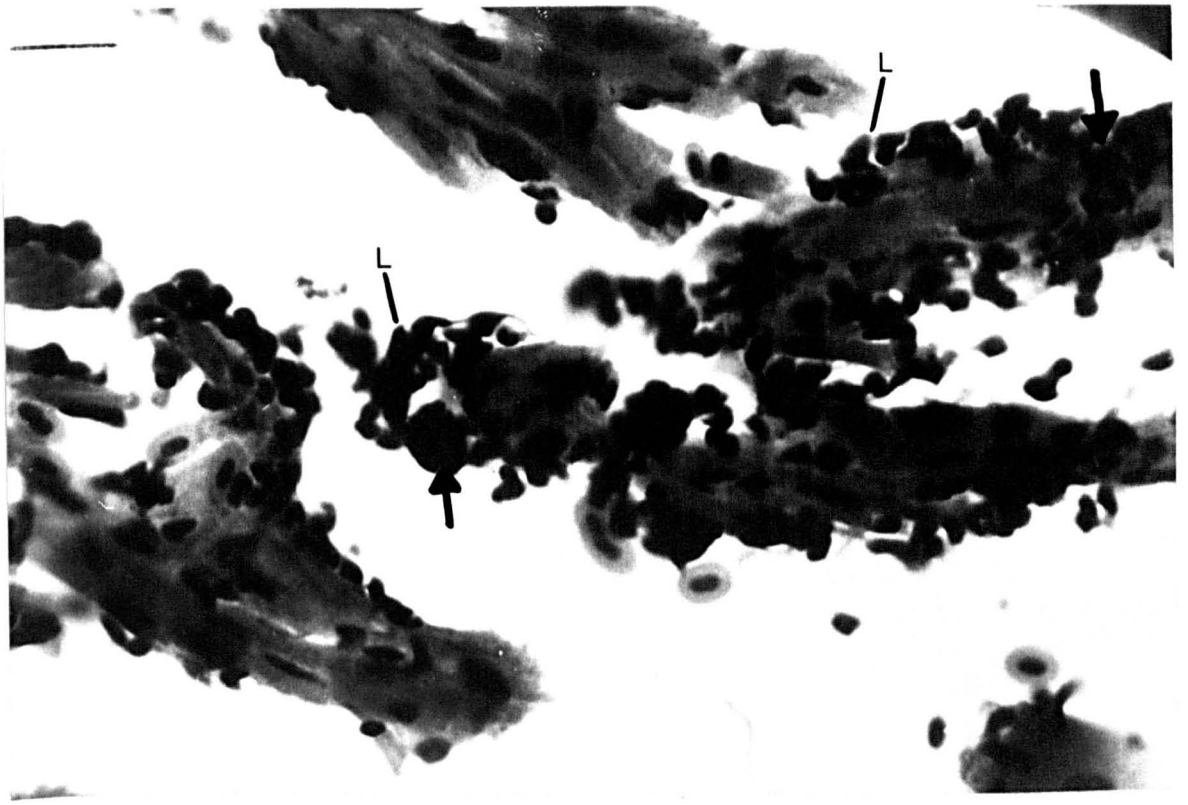


Table 3.3.1 Prevalence of heart muscle necrosis in salmon post-smolts fed diets containing fish oil or sunflower oil.

Sampling time	Fish oil diet			Sunflower oil diet		
(weeks)	Total Sample	Normal Hearts	Necrotic Hearts	Total Sample	Normal Hearts	Necrotic Hearts
16	5	5	0	5	1	4
18	6	6	0	10	1	9
20	10	9	1	9	5	4
Total	21	20	1	24	7	17

3.4 Fatty acid compositions of salmon heart

The fatty acid compositions of heart phospholipid classes are shown in Tables 3.4.1 and 3.4.2. In PC from SO-fed fish there were significant increases in 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6 and 20:4n-6 compared to FO-fed fish. In addition, the SO-fed fish had significantly reduced 20:5n-3 and total monoenes. In PE the same pattern of changes was observed, and in both classes the n-3/n-6 ratio was significantly reduced in SO-fed fish. A significant decrease in total saturates was also seen in PE. In PS of SO-fed fish the n-6 PUFA described above were all significantly increased, with the exception of 20:4n-6, which was unchanged. The levels of 18:3n-3, 20:5n-3 and 22:5n-3 were significantly reduced as were total monoenes. Total saturates were significantly increased in the hearts of SO-fed fish compared with those fed FO. In PI of hearts from SO-fed fish, 18:2n-6, 20:2n-6, 20:3n-6 and 20:4n-6 were significantly increased, while 20:5n-3 and 22:6n-3 were significantly reduced compared to FO-fed fish. The AA/EPA ratio was significantly increased in all phospholipid classes of fish fed SO. The n-3/n-6 PUFA ratio was significantly reduced in both PS and PI of SO-fed fish.

Table 3.4.1 Fatty acid compositions of phosphatidylcholine and phosphatidyl ethanolamine from hearts of salmon fed diets containing fish oil (FO) or sunflower oil (SO)

	Phosphatidylcholine		Phosphatidylethanolamine	
Fatty acid	FO diet	SO diet	FO diet	SO diet
		weight %		
Total saturates	40.8 ± 2.3	40.6 ± 1.3	24.0 ± 0.5*	20.8 ± 0.7
Total monoenes	18.3 ± 1.1*	14.8 ± 1.0*	10.5 ± 0.6*	8.3 ± 0.7
18:2n-6	0.4 ± 0.1*	6.6 ± 0.5	0.7 ± 0.2*	6.7 ± 0.4
18:3n-6	0.1 ± 0.0*	0.3 ± 0.1	0.2 ± 0.0*	0.1 ± 0.0
20:2n-6	0.1 ± 0.0*	0.6 ± 0.1	0.1 ± 0.0*	0.7 ± 0.1
20:3n-6	0.1 ± 0.0*	0.9 ± 0.2	0.1 ± 0.0*	0.8 ± 0.2
20:4n-6	1.6 ± 0.1*	2.6 ± 0.4	1.5 ± 0.2*	2.9 ± 0.5
Total n-6[1]	2.8 ± 0.2*	11.3 ± 0.2	3.6 ± 0.1*	12.1 ± 0.4
18:3n-3	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0
20:5n-3	6.4 ± 0.2*	4.2 ± 0.3	3.9 ± 0.4*	2.5 ± 0.1
22:5n-3	0.9 ± 0.1	0.9 ± 0.0	2.7 ± 0.0	2.5 ± 0.2
22:6n-3	24.2 ± 1.9	22.6 ± 2.1	42.1 ± 1.4	43.5 ± 1.6
Total n-3[2]	32.1 ± 1.6	28.0 ± 2.0	49.4 ± 1.6	48.8 ± 1.6
Total PUFA	34.9 ± 1.8	39.3 ± 1.9	53.0 ± 1.6*	61.0 ± 1.8
n-3/n-6	11.5 ± 0.4*	2.5 ± 0.2	13.5 ± 0.5*	4.0 ± 0.2
20:4/20:5	0.2 ± 0.1*	0.6 ± 0.2	0.4 ± 0.0**	1.1 ± 0.3

Values are means ± SD from three salmon. *Values are significantly different ($P < 0.05$) as determined by Student's *t* test. **Values are significantly different ($P < 0.05$) as determined by Kruskal-Wallis analysis. SD < 0.05 are tabulated as 0.0.

[1]Includes 22:4n-6 and 22:5n-6.

[2]Includes 18:4n-3 and 20:4n-3.

Table 3.4.2 Fatty acid compositions of phosphatidylserine and phosphatidylinositol from hearts of salmon fed diets containing fish oil (FO) or sunflower oil (SO)

	Phosphatidylserine		Phosphatidylinositol	
Fatty acid	FO diet	SO diet	FO diet	SO diet
			Weight %	
Total saturates	30.6 ± 1.4*	34.9 ± 1.7	31.2 ± 0.6	34.6 ± 3.0
Total monoenes	13.3 ± 0.7*	8.0 ± 0.1	9.4 ± 0.1	7.8 ± 1.2
18:2n-6	0.9 ± 0.1*	8.0 ± 0.1	0.6 ± 0.1*	4.2 ± 0.4
18:3n-6	0.7 ± 0.1*	0.4 ± 0.1	0.3 ± 0.0*	0.2 ± 0.0
20:2n-6	0.2 ± 0.0*	0.9 ± 0.2	t*	0.3 ± 0.1
20:3n-6	0.1 ± 0.0*	1.0 ± 0.1	0.1 ± 0.1*	0.8 ± 0.2
20:4n-6	1.8 ± 0.8	1.9 ± 0.3	13.5 ± 0.1**	16.1 ± 2.1
Total n-6[1]	4.4 ± 0.8*	12.8 ± 0.4	15.0 ± 0.3*	22.0 ± 1.9
18:3n-3	0.6 ± 0.1*	0.1 ± 0.1	0.3 ± 0.0*	t
20:5n-3	1.8 ± 0.3*	0.9 ± 0.2	7.6 ± 0.8*	3.6 ± 0.3
22:5n-3	3.2 ± 0.3*	2.1 ± 0.4	1.1 ± 0.2	1.4 ± 0.5
22:6n-3	37.3 ± 0.7	35.4 ± 2.1	28.9 ± 0.8*	22.6 ± 2.5
Total n-3[2]	43.4 ± 0.8*	36.9 ± 1.5	38.3 ± 0.2*	28.0 ± 2.8
Total PUFA	47.8 ± 1.0	49.8 ± 1.9	53.3 ± 0.3*	50.0 ± 4.7
n-3/n-6	10.1 ± 2.0**	2.9 ± 0.1	2.6 ± 0.1*	1.3 ± 0.0
20:4/20:5	1.0 ± 0.4*	2.1 ± 0.3	1.8 ± 0.2*	4.5 ± 0.9

Values are means ± SD from three salmon. *Values are significantly different ($P < 0.05$) as determined by Student's *t* test. **Values are significantly different ($P < 0.05$) as determined by Kruskal-Wallis analysis. SD < 0.05 are tabulated as 0.0.

[1]Includes 22:4n-6 and 22:5n-6.

[2]Includes 18:4n-3 and 20:4n-3.

t = trace value < 0.05%.

3.5 Fatty acid compositions of salmon liver, brain and retina

The fatty acid compositions of the major liver phospholipid classes are shown in Tables 3.5.1 and 3.5.2. Liver PC from SO-fed fish showed significantly increased 18:2n-6, 18:3n-6, 20:2n-6 and 20:3n-6, the latter being some 25 times that in FO-fed fish. Significant decreases in 20:5n-3 and 22:5n-3 were observed in liver PC of fish fed SO. Similar changes also occurred in liver PE, but, in addition, 20:4n-6 and total PUFA were significantly increased while 18:3n-3 and total monoenes were significantly decreased in SO-fed fish. In liver PS, 18:2n-6 and 20:3n-6 were significantly elevated while 18:3n-3 was significantly reduced in SO-fed fish. In liver PI, values for 18:2n-6, 20:2n-6 and 20:3n-6 were significantly increased in fish fed SO. The n-3/n-6 PUFA ratio was significantly reduced in all liver phospholipid classes of SO-fed fish, except in PI. The 20:4n-6/20:5n-3 ratio was generally increased in fish fed SO, although this was only significant in PE.

The fatty acid compositions of the phospholipid classes from brain and retina were much less affected by dietary lipid composition than those of heart and liver. The major changes were generally in PC and PE whose fatty acid compositions in retina and brain are shown in Tables 3.5.3 and 3.5.4. In retinal PC and PE, significant increases were observed in 18:2n-6, 20:2n-6 and 20:3n-6 in SO-fed fish. 20:4n-6 was significantly increased in PC but not PE. In both PC and PE 20:5n-3 was significantly decreased and 22:6n-3 was significantly reduced in PC of SO-fed fish. In retinal PI of SO-fed fish, 22:6n-3 was also significantly decreased (from 23.0% ± 1.6 to 15.2% ± 0.9). A similar pattern was observed in brain PC and PE where 18:2n-6, 20:2n-6 and 20:3n-6 were increased in SO-fed fish but 20:5n-3 was significantly decreased only in PC.

Table 3.5.1 Fatty acid compositions of phosphatidylcholine and phosphatidylethanolamine from liver of salmon fed diets containing fish oil (FO) or sunflower oil (SO)

	Phosphatidylcholine		Phosphatidylethanolamine	
Fatty acid	FO diet	SO diet	FO diet	SO diet
		Weight %		
Total saturates	41.1 ± 4.3	38.2 ± 2.9	25.6 ± 2.7	22.9 ± 2.6
Total monoenes	18.3 ± 2.7	16.5 ± 3.5	23.2 ± 0.7*	15.1 ± 1.9
18:2n-6	0.6 ± 0.1*	11.9 ± 2.9	0.8 ± 0.1**	12.1 ± 2.1
18:3n-6	t*	0.6 ± 0.4	t	t
20:2n-6	0.3 ± 0.2*	2.1 ± 1.1	0.3 ± 0.1*	2.8 ± 0.9
20:3n-6	0.2 ± 0.1*	5.1 ± 1.2	0.2 ± 0.1*	4.3 ± 0.2
20:4n-6	1.3 ± 1.0	2.7 ± 1.6	1.8 ± 0.2*	4.2 ± 0.2
Total n-6[1]	2.9 ± 1.5*	22.8 ± 1.6	4.0 ± 0.5*	24.1 ± 2.4
18:3n-3	0.1 ± 0.0	t	0.2 ± 0.1*	t
20:5n-3	4.8 ± 0.4*	1.9 ± 0.2	5.2 ± 0.4*	2.7 ± 0.4
22:5n-3	1.3 ± 0.1*	0.9 ± 0.1	1.6 ± 0.3	1.0 ± 0.3
22:6n-3	21.2 ± 3.3	15.0 ± 3.1	32.0 ± 1.6	30.1 ± 2.0
Total n-3[2]	27.6 ± 3.6*	17.8 ± 2.9	39.8 ± 1.7*	33.8 ± 1.7
Total PUFA	30.5 ± 4.7	40.6 ± 4.1	43.7 ± 1.5*	57.9 ± 3.4
n-3/n-6	10.7 ± 3.8**	0.8 ± 0.1	10.1 ± 1.5*	1.4 ± 0.1
20:4/20:5	0.3 ± 0.2	1.5 ± 0.9	0.4 ± 0.1*	1.6 ± 0.2

Values are means ± SD from three salmon. *Values are significantly different ($P < 0.05$) as determined by Student's *t* test. **Values are significantly different ($P < 0.05$) as determined by Kruskal-Wallis analysis. SD < 0.05 are tabulated as 0.0.

[1]Includes 22:4n-6 and 22:5n-6.

[2]Includes 18:4n-3 and 20:4n-3.

t = trace value < 0.05%.

Table 3.5.2 Fatty acid compositions of phosphatidylserine and phosphatidylinositol from liver of salmon fed diets containing fish oil (FO) or sunflower oil (SO).

	Phosphatidylserine		Phosphatidylinositol	
Fatty acid	FO diet	SO diet	FO diet	SO diet
	Weight %			
Total saturates	34.9 ± 1.0	34.8 ± 3.8	36.5 ± 3.9	35.1 ± 3.2
Total monoenes	25.9 ± 4.5	24.3 ± 9.2	13.3 ± 1.1	10.4 ± 2.8
18:2n-6	1.2 ± 0.3*	9.2 ± 3.9	0.5 ± 0.0**	3.4 ± 1.2
18:3n-6	0.9 ± 0.3	0.4 ± 0.2	0.6 ± 0.1*	0.2 ± 0.1
20:2n-6	0.3 ± 0.2	1.1 ± 0.5	0.2 ± 0.1*	1.1 ± 0.3
20:3n-6	0.1 ± 0.1*	2.0 ± 0.3	0.7 ± 0.2*	3.9 ± 1.2
20:4n-6	0.4 ± 0.1	1.0 ± 0.5	19.5 ± 1.7	20.6 ± 4.2
Total n-6[1]	2.7 ± 0.2*	13.9 ± 3.9	21.8 ± 1.6*	29.5 ± 2.9
18:3n-3	0.3 ± 0.1*	t	0.2 ± 0.1*	t
20:5n-3	1.4 ± 0.6	0.7 ± 0.1	3.2 ± 0.3	2.6 ± 0.7
22:5n-3	1.1 ± 0.2	1.5 ± 0.5	1.5 ± 0.5	0.9 ± 0.2
22:6n-3	20.5 ± 3.8	13.1 ± 2.9	14.2 ± 1.2	13.1 ± 3.7
Total n-3[2]	24.0 ± 4.6	14.7 ± 3.3	19.4 ± 2.0	16.6 ± 4.2
Total PUFA	26.7 ± 4.7	28.5 ± 5.4	41.2 ± 0.4	46.2 ± 3.0
n-3/n-6	8.7 ± 1.4*	1.1 ± 0.4	0.9 ± 0.2	0.6 ± 0.2
20:4/20:5	0.3 ± 0.1	1.4 ± 0.7	6.2 ± 1.2	8.3 ± 3.0

Values are means ± SD from three salmon. *Values are significantly different ($P < 0.05$) as determined by Student's *t* test. **Values are significantly different ($P < 0.05$) as determined by Kruskal-Wallis analysis. SD < 0.05 are tabulated as 0.0.

[1]Includes 22:4n-6 and 22:5n-6.

[2]Includes 18:4n-3 and 20:4n-3.

t = trace value < 0.05%.

Table 3.4.3 Fatty acid compositions of phosphatidylcholine and phosphatidylethanolamine from retina of salmon fed diets containing fish oil (FO) or sunflower oil (SO)

	Phosphatidylcholine		Phosphatidylethanolamine	
Fatty acid	FO diet	SO diet	FO diet	SO diet
		Weight %		
Total saturates	35.4 ± 1.1	33.3 ± 0.4	14.7 ± 0.9	14.6 ± 1.7
Total monoenes	20.3 ± 0.2	20.8 ± 0.4	18.8 ± 2.9	22.1 ± 3.4
18:2n-6	0.4 ± 0.1*	5.6 ± 0.1	0.5 ± 0.1**	2.8 ± 0.9
18:3n-6	t	0.1 ± 0.0	0.1 ± 0.1	t
20:2n-6	t*	0.8 ± 0.1	t*	0.5 ± 0.1
20:3n-6	t*	0.9 ± 0.1	0.2 ± 0.1*	0.6 ± 0.1
20:4n-6	0.9 ± 0.0*	1.1 ± 0.0	1.7 ± 0.0	2.3 ± 0.6
Total n-6[1]	1.6 ± 0.1*	8.7 ± 0.2	2.7 ± 0.3*	6.5 ± 0.6
18:3n-3	0.1 ± 0.0	t	0.2 ± 0.1	0.2 ± 0.1
20:5n-3	4.4 ± 0.1*	3.9 ± 0.2	3.0 ± 0.3*	2.4 ± 0.1
22:5n-3	0.8 ± 0.1	0.8 ± 0.1	1.9 ± 0.7	1.4 ± 0.1
22:6n-3	31.2 ± 2.6**	26.2 ± 0.6	43.6 ± 0.8	39.9 ± 5.5
Total n-3[2]	36.6 ± 2.2**	31.2 ± 0.5	48.5 ± 0.9	41.8 ± 5.6
Total PUFA	38.4 ± 1.7	39.6 ± 0.3	50.8 ± 0.9	48.3 ± 5.9
n-3/n-6	23.4 ± 1.8*	3.6 ± 0.2	18.1 ± 1.5*	6.5 ± 0.8
20:4/20:5	0.2 ± 0.0*	0.3 ± 0.0	0.6 ± 0.1	1.0 ± 0.3

Values are means ± SD from three salmon. *Values are significantly different ($P < 0.05$) as determined by Student's *t* test. **Values are significantly different ($P < 0.05$) as determined by Kruskal-Wallis analysis. SD < 0.05 are tabulated as 0.0.

[1]Includes 22:4n-6 and 22:5n-6.

[2]Includes 18:4n-3 and 20:4n-3.

t = trace value < 0.05%.

Table 3.5.4 Fatty acid compositions of phosphatidylcholine and phosphatidylethanolamine from brain of salmon fed diets containing fish oil (FO) or sunflower oil (SO)

	Phosphatidylcholine		Phosphatidylethanolamine	
Fatty acid	FO diet	SO diet	FO diet	SO diet
		Weight %		
Total saturates	33.2 ± 2.6	31.3 ± 1.1	16.9 ± 0.2	17.1 ± 0.2
Total monoenes	47.6 ± 2.0	46.5 ± 0.4	30.9 ± 0.3	31.1 ± 0.4
18:2n-6	0.3 ± 0.0*	1.2 ± 0.3	0.3 ± 0.0*	1.1 ± 0.2
18:3n-6	t	t	0.1 ± 0.1	t
20:2n-6	t*	0.3 ± 0.1	t*	0.4 ± 0.1
20:3n-6	t*	0.3 ± 0.0	t*	0.3 ± 0.1
20:4n-6	0.4 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	1.1 ± 0.2
Total n-6[1]	0.9 ± 0.0*	2.7 ± 0.3	1.2 ± 0.2*	3.0 ± 0.1
18:3n-3	t	t	t	t
20:5n-3	2.9 ± 0.1*	2.5 ± 0.1	4.8 ± 0.2	4.4 ± 0.1
22:5n-3	0.9 ± 0.1	1.0 ± 0.4	1.8 ± 0.2	1.6 ± 0.1
22:6n-3	11.5 ± 1.3	12.7 ± 0.7	20.9 ± 1.8	21.5 ± 0.8
Total n-3[2]	15.4 ± 1.5	16.3 ± 0.4	27.8 ± 2.0	27.7 ± 0.8
Total PUFA	16.3 ± 1.5	18.9 ± 0.1	29.0 ± 1.9	30.6 ± 0.8
n-3/n-6	17.2 ± 1.6*	6.2 ± 1.0	22.9 ± 4.3*	9.3 ± 0.1
20:4/20:5	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1

Values are means ± SD from three salmon. *Values are significantly different ($P < 0.05$) as determined by Student's *t* test. **Values are significantly different ($P < 0.05$) as determined by Kruskal-Wallis analysis. SD < 0.05 are tabulated as 0.0.

[1]Includes 22:4n-6 and 22:5n-6.

[2]Includes 18:4n-3 and 20:4n-3.

t = trace value < 0.05%.

3.6 Fatty acid compositions of blood leucocyte and gill phospholipids

The fatty acid compositons of blood leucocyte phospholipid classes are shown in Tables 3.6.1 and 3.6.2. In PC of SO fed fish there were increased levels of 18:2n-6, 18:3n-6, 20:2n-6 and 20:3n-6 compared to fish fed FO. The SO fed fish also had decreased 20:5n-3, 22:6n-3, total saturates and total monoenes. Similar changes were apparent in PE although 20:4n-6 was also increased in fish fed SO. In both lipid classes the n-3/n-6 ratio was reduced in fish fed SO. In PS of fish fed SO the n-6 PUFA were increased and EPA and DHA reduced compared to fish fed FO. In PI 20:3n-6 and 20:4n-6 were increased in SO fed fish, but EPA and DHA were also increased. In general, PI was the least affected by dietary lipid and the n-3/n-6 ratio and 20:4n-6/20:5n-3 ratio was relatively unaffected. In PS, as with PC and PE, the n-3/n-6 ratio was decreased and the AA/EPA ratio was increased in fish fed SO compared to those fed FO.

The fatty acid compositions of gill phospholipid classes are shown in Tables 3.6.3 and 3.6.4. PC from SO fed fish had significantly increased 18:2n-6, 20:2n-6, 20:3n-6 and total PUFA but decreased EPA and total n-3 PUFA compared to FO fed fish. Similar changes were also apparent in gill PE. In gill PS, 18:2n-6, 20:2n-6 and 20:3n-6 were significantly elevated in SO fed fish, while EPA and 22:5n-3 were significantly decreased. Similar changes were observed in gill PI. The n-3/n-6 PUFA ratio was significantly increased in all phospholipid classes of fish fed SO. While the mean value of the AA/EPA ratio was increased in all phospholipid classes this was only significant for PS.

Table 3.6.1 Fatty acid compositions of phosphatidylcholine and phosphatidylethanolamine from blood leucocytes of salmon fed diets containing fish oil (FO) of sunflower oil (SO).

	PC		PE	
Fatty acid	FO diet	SO diet	FO diet	SO diet
		Weight %		
Total saturates	34.8	32.6	22.5	18.2
Total monoenes	25.0	20.6	25.4	14.2
18:2n-6	1.0	8.8	2.4	12.2
18:3n-6	0.2	0.5	0.1	0.6
20:2n-6	0.2	1.6	0.3	2.0
20:3n-6	0.2	1.6	0.2	1.5
20:4n-6	2.8	2.8	2.8	4.2
Total n-6[1]	4.9	15.5	6.5	20.9
18:3n-3	0.2	0.2	0.3	0.2
20:5n-3	8.8	5.1	4.1	2.8
22:5n-3	1.1	0.9	1.0	1.8
22:6n-3	18.6	14.6	29.1	31.1
Total n-3[2]	29.3	20.9	35.5	36.2
Total PUFA	34.2	36.4	42.0	57.0
n-3/n-6	6.0	1.4	5.4	1.7
20:4n-6/20:5n-3	0.3	0.7	0.6	1.5

Results are from pooled leucocyte samples from six salmon.

[1]Includes 22:4n-6 and 22:5n-6.

[2]Includes 18:4n-3 and 20:4n-3.

Table 3.6.2 Fatty acid compositions of phosphatidylserine and phosphatidylinositol of blood leucocytes form salmon fed diets containing fish oil (FO) or sunflower oil (SO).

	PS		PI	
Fatty acid	FO diet	SO diet	FO diet	SO diet
		Weight %		
Total saturates	31.9	34.9	26.7	22.9
Total monoenes	11.4	7.1	12.5	6.6
18:2n-6	1.0	3.8	1.2	1.4
18:3n-6	t	0.2	0.3	0.2
20:2n-6	t	1.0	0.2	0.3
20:3n-6	t	0.9	0.4	0.8
20:4n-6	1.6	2.7	13.0	17.8
Total n-6[1]	2.8	9.1	15.2	21.1
18:3n-3	0.2	0.3	0.3	t
20:5n-3	2.1	1.7	2.4	2.7
22:5n-3	1.7	1.9	1.8	1.8
22:6n-3	41.8	38.4	25.3	30.4
Total n-3[2]	46.0	42.4	31.7	34.9
Total PUFA	48.7	51.4	47.4	56.0
n-3/n-6	16.7	4.7	2.1	1.7
20:4/20:5	0.8	1.6	5.4	6.6

Results are from pooled leucocyte samples from six salmon.

[1]Includes 22:4n-6 and 22:5n-6.

[2]Includes 18:4n-3 and 20:4n-3.

t = trace value < 0.05%.

Table 3.6.3 Fatty acid compositions of phosphatidylcholine and phosphatidylethanolamine from gills of salmon fed diets containing fish oil (FO) or sunflower oil (SO).

	PC		PE	
Fatty acid	FO diet	SO diet	FO diet	SO diet
		Weight %		
Total saturates	40.6 ± 2.3	40.5 ± 1.1	19.1 ± 0.7	21.5 ± 4.1
Total monoenes	24.9 ± 1.5	22.3 ± 0.9	14.8 ± 1.3	16.2 ± 4.1
18:2n-6	0.6 ± 0.1*	7.9 ± 1.0	0.9 ± 0.2*	4.8 ± 0.8
18:3n-6	0.2 ± 0.1	0.2 ± 0.2	0.4 ± 0.2	0.2 ± 0.1
20:2n-6	0.1 ± 0.0*	1.2 ± 0.2	0.2 ± 0.1*	0.9 ± 0.1
20:3n-6	0.1 ± 0.0*	1.6 ± 0.3	0.1 ± 0.0*	0.7 ± 0.3
20:4n-6	1.4 ± 0.2	2.2 ± 0.9	2.8 ± 0.4	4.2 ± 2.3
Total n-6[1]	2.6 ± 0.4*	13.4 ± 0.6	5.0 ± 0.6*	11.4 ± 2.6
18:3n-3	t	t	0.1 ± 0.0	t
20:5n-3	4.9 ± 0.6*	2.6 ± 0.2	4.3 ± 0.5*	2.2 ± 0.2
22:5n-3	0.8 ± 0.3	0.5 ± 0.1	1.2 ± 0.2	0.8 ± 0.3
22:6n-3	15.3 ± 1.3	13.8 ± 0.6	33.7 ± 0.6	26.9 ± 5.2
Total n-3[2]	21.3 ± 2.0*	16.9 ± 0.7	39.4 ± 0.6	30.4 ± 5.2
Total PUFA	23.9 ± 2.4*	30.3 ± 1.3	44.5 ± 0.4	41.8 ± 7.7
n-3/n-6	8.1 ± 0.5*	1.3 ± 0.0	8.0 ± 0.9*	2.7 ± 0.3
20:4n-6/20:5n-3	0.3 ± 0.1	0.9 ± 0.4	0.7 ± 0.2	1.9 ± 0.9

[1]Includes 22:4n-6 and 22:5n-6. [2]Includes 18:4n-3 and 20:4n-3. t = trace value < 0.05%. Results are means ± SD form three fish per dietary treatment. *Values are significantly different ($P < 0.05$). t = trace value < 0.05%. SD less than 0.05 are tabulated as 0.0.

Table 3.6.4. Fatty acid compositions of phosphatidylserine and phosphatidylinositol from gills of salmon fed diets containing fish oil (FO) or sunflower oil (SO).

	PS		PI	
Fatty acid	FO diet	SO diet	FO diet	SO diet
		Weight %		
Total saturates	36.1 ± 0.5*	45.8 ± 1.5	36.0 ± 4.8	40.3 ± 4.5
Total monoenes	15.0 ± 0.7*	10.7 ± 1.2	14.6 ± 0.6	14.6 ± 0.3
18:2n-6	0.8 ± 0.3*	2.8 ± 0.5	1.6 ± 0.1*	3.9 ± 0.4
18:3n-6	0.8 ± 0.1	0.6 ± 0.2	1.0 ± 0.5	0.6 ± 0.2
20:2n-6	0.1 ± 0.1*	0.9 ± 0.1	t*	0.5 ± 0.0
20:3n-6	t*	0.9 ± 0.1	0.4 ± 0.2*	1.2 ± 0.1
20:4n-6	0.8 ± 0.1	1.1 ± 0.2	9.1 ± 0.8	8.7 ± 2.1
Total n-6[1]	3.3 ± 0.4*	6.9 ± 0.3	12.1 ± 0.5	15.4 ± 1.9
18:3n-3	0.1 ± 0.0	t	0.5 ± 0.2	0.2 ± 0.1
20:5n-3	1.3 ± 0.3*	0.4 ± 0.1	2.5 ± 0.3*	1.3 ± 0.1
22:5n-3	1.7 ± 0.1*	1.2 ± 0.2	1.0 ± 0.2	0.6 ± 0.1
22:6n-3	27.6 ± 1.0	25.2 ± 1.4	21.5 ± 2.9	17.6 ± 0.8
Total n-3[2]	30.8 ± 1.2	26.9 ± 1.7	25.4 ± 3.3	19.7 ± 0.7
Total PUFA	34.2 ± 1.0	33.8 ± 1.7	37.5 ± 3.8	35.1 ± 2.5
n-3/n-6	9.3 ± 1.4*	3.9 ± 0.4	2.1 ± 0.3*	1.3 ± 0.2
20:4n-6/20:5n-3	0.7 ± 0.2*	2.5 ± 0.7	3.7 ± 0.3	6.9 ± 1.9

[1]Includes 22:4n-6 and 22:5n-6. [2]Includes 18:4n-3 and 20:4n-3. t = trace value < 0.05%. Results are means ± SD form three fish per dietary treatment. *Values are significantly different ($P < 0.05$). t = trace value < 0.05%. SD less than 0.05 are tabulated as 0.0.

3.7 Lipoxygenase products from gill and blood stimulated with Ca^{2+}ionophore A23187.

The major lipoxygenase products from gill and blood are shown in Tables 3.7.1 and 3.7.2. Peaks were identified by co-elution with commercial standards, and in the case of 12-HEPE, by GC resolution of methyl esters prepared from both commercial standard and from hydroxy fatty acids recovered from the HPLC separation. Gill cells from both dietary treatments produced large amounts of both 12-HEPE (from EPA) and 12-HETE (from AA). The amount of 12-HETE produced was similar for both dietary treatments whereas the amount of 12-HEPE was significantly reduced in fish fed SO. No leukotriene B_4 (LTB_4) or leukotriene B_5 were detected in gill cell extracts. In whole blood stimulated with A23187, LTB_4 production was unaffected by dietary treatment but LTB_5, derived from EPA, was significantly reduced in fish fed SO. Feeding SO significantly reduced formation of both 12-HETE and 12-HEPE by blood leucocytes, compared to fish fed FO.

Table 3.7.1. Lipoxygenase products from isolated salmon gill cells stimulated using the Ca^{2+}-ionophore A23187.

Eicosanoid	FO diet (ng/mg protein)	SO diet (ng/mg protein)
12-HEPE	108.3 ± 7.2*	43.1 ± 1.7
12-HETE	54.8 ± 10.9	52.7 ± 7.4
12-HETE/12-HEPE	0.5 ± 0.1*	1.3 ± 0.2

Values are mean ± SD for five fish per dietary treatment.

*Values are significantly different ($P < 0.05$).

Table 3.7.2. Lipoxgenase products from whole blood of salmon stimulated using the Ca^{2+}-ionophore A23187.

Eicosanoid	FO diet (ng/ml blood)	SO diet (ng/ml blood)
LTB_4	23.2 ± 2.2	18.1 ± 7.4
LTB_5	35.2 ± 10.2*	9.6 ± 1.6
LTB_4/LTB_5	0.7 ± 0.2*	1.9 ± 0.6
12-HEPE	109.0 ± 25.5*	38.1 ± 4.5
12-HETE	185.0 ± 17.3*	97.2 ± 14.8
12-HETE/12-HEPE	2.1 ± 0.3	2.7 ± 0.2

Values are mean ± SD for five fish per dietary treatment.

*Values are significantly different ($P < 0.05$).

3.8 Summary

1. Practical-type diets containing either fish oil (FO) or sunflower oil (SO) were fed to Atlantic salmon post-smolts for a period of 16 weeks. Both diets contained adequate levels of n-3 PUFA. The effect of diet on growth (weight gain) could not be ascertained as the initial weights of the fish were significantly different. However, the specific growth rate was slightly higher in fish fed FO.

2. A number of fish fed SO developed severe heart lesions causing thinning of the ventricular wall and muscle necrosis. In addition the fish fed SO were susceptible to a transportation-induced shock syndrome that caused 30% mortality.

3. The phospholipid classes of heart, liver, gill and leucocytes in fish fed SO had increased levels of 18:2n-6 (2-15 fold), 20:2n-6 (4.5 -12 fold) and 20:3n-6 (2-25 fold). In some tissues, 20:4n-6 was also increased (up to 2 fold), particularly in PC and PE. There was a general decrease in phospholipid 20:5n-3 (up to 3 fold) which was reflected in an increased 20:4n-6/20:5n-3 ratio of up to 6 fold. The fatty acid compositions of brain and retina while showing similar changes to the above tissues were generally much less affected by altered dietary n-3/n-6 PUFA ratio.

4. When isolated gill cells were stimulated with the Ca^{2+}-ionophore A23187, 12-hydroxy-8,10,14,17-eicosapentaenoic acid (12-HEPE) was the major lipoxygenase product in fish fed FO. 12-HEPE was significantly reduced in fish fed SO. The lipoxygenase products derived from whole blood stimulated with A23187 showed decreased levels of LTB_5, 12-HEPE and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) in fish fed SO compared to those fed FO.

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Section 4

Section 4. Dietary study B: Effects of increasing levels of dietary linoleic acid on tissue phospholipid fatty acid composition and eicosanoid production, with particular emphasis on cardiac tissue, in Atlantic salmon.

4.1 Introduction

A number of experiments with mammals have demonstrated that the PUFA composition of heart membrane lipids can be influenced by dietary fat intake (Swanson and Kinsella, 1986; Leonardi *et al.* 1987). The availability of PUFA for eicosanoid production is dictated by phospholipid fatty acid composition and therefore by dietary PUFA intake (Lands, 1989). Prostaglandins are important mediators of cardiac physiology and function (Karmazyn, 1985) and are also involved in muscle protein synthesis and degradation (Palmer, 1990).

Alterations in cell membrane phospholipid fatty acid composition can also have direct effects on the activities of membrane bound enzymes and the control of ion permeability (Stubbs and Smith, 1984). In particular, the activity of the cardiac sarcoplasmic reticulum (SR) Ca^{2+}-Mg^{2+}ATPase (EC 3.6.1.4) can be influenced by the membrane n-3 and n-6 PUFA composition (Swanson *et al.* 1989). In mice fed diets containing either menhaden or corn oil the Ca^{2+}-Mg^{2+} ATPase activity was increased 6.5 fold by the latter dietary treatment. The SR regulates intracellular calcium concentrations during the contraction-relaxation cycle of the myocardium and the calcium-dependent ATPase in the SR membrane is fundamental in this regulatory process. Thus, dietary lipid modifications which influence this enzyme and thereby alter intracellular calcium concentrations can affect the severity of cardiac necrosis resulting from ischemia and reperfusion (Dhalla *et al.* 1978).

In the previous dietary experiment it was established that the fatty acid composition of heart phospholipids could be markedly altered by feeding either fish oil or sunflower oil and that the fish fed the latter developed a severe

cardiomyopathy. In this experiment three diets were prepared containing the same protein and total lipid level (46 and 16% respectively) but with linoleic acid (LA) provided at 10, 25 and 45% of total dietary fatty acids. These percentages of LA were achieved using blends of fish oil, sunflower oil, safflower oil and grapeseed oil. The diet formulations are shown in Table 4.1.1. The fish oil and sunflower oil were as described in trial A and the blend of sunflower, safflower and grapeseed oil ("Vitelma") was supplied by Vandermoortele (UK) Ltd., Hounslow, Middlesex. The fatty acid compositions of the diets are shown in Table 4.1.2. The main objectives were to investigate the effects of increasing levels of LA on the fatty acid compositions of the individual phospholipids of salmon hearts, to measure the activity of Ca^{2+}-Mg^{2+} ATPase in cardiac SR and production of eicosanoids by isolated cardiac myocytes. In addition, the phospholipid fatty acid compositions of the active eicosanoid synthesising cells in blood and gills were measured. Products of cyclooxygenase (6-keto-$PGF_{1\alpha}$ and TXB_2) and lipoxygenases (LTB_4, LTB_5, 12-HETE and 12-HEPE) were measured after stimulating the above cells with A23187, and in plasma.

4.2 Experimental details, growth, mortality and histopathology

Three hundred and thirty Atlantic salmon S1 smolts were obtained from Seafarm Kerry Ltd., Gairloch, Scotland and transferred to tanks at the S.O.A.E.F.D. Fish Cultivation Unit, Aultbea, Wester Ross, Scotland. Fish were distributed randomly into three circular tanks of 1 m diameter containing 500 L of seawater which was supplied at a rate of 10 L/min. The tanks were subjected to natural photoperiod and the water temperature over the experimental period (May-October) varied from 9-14^{o}C. Diets were supplied by automatic feeders, as described in trial A, and were adjusted initially to provide 20 g/kg biomass per day. After 8 weeks the ration was

increased to provide 25 g/kg biomass per day. Fish were weighed individually at the start and finish of the experiment and were weighed in bulk every 28 days as described in trial A. All three groups of fish increased in weight more than 3 fold over the 16 week experimental period (Table 4.2.1). The fish fed the intermediate level of linoleic acid (25%) had significantly increased final weights compared to fish fed the two other dietary treatments and the fish fed the highest level of LA (50%) had the lowest final weights of all the dietary treatments. Mortalities were greater in fish fed the two highest levels of LA (16 and 17% respectively) than those fed 10% LA (10% mortality). The heart histopathological lesion involving thinning of the ventricular wall and active muscle necrosis was apparent in a small number (around 10%) of fish from each dietary treatment. The lack of myodegeneration in the highest LA group might be explained by the slower growth rate achieved by these fish compared to the fish used in the first experiment. The reason for the lower growth rate may have been that this experiment was performed in smaller tanks (1m diameter instead of 2m) so that overcrowding may have retarded growth. However, it may be also be due to the fish being of different genetic stock compared to those used in trial A.

Table 4.1.1. Composition of diets (g/kg)

Ingredient	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
Fishmeal[1]	650	650	650
Starch[2]	150	150	150
Fish oil[3]	78	40	-
Sunflower oil[4]	22	60	-
'Vitelma oil'[5]	-	-	100
Vitamin mix[6]	10	10	10
Mineral mix[7]	24	24	24
α-cellulose	61.6	61.6	61.6
Antioxidant mix[8]	0.4	0.4	0.4
Choline chloride	4	4	4

[1]LT-94 Norwegian low temperature herring meal, Ewos Ltd., Bathgate, Scotland.

[2]Passelli WA4 pre-cooked potato starch, Avebe (U.K.) Ltd., Ulceby, England.

[3]Fosol, Seven Seas Ltd., Hull, U.K. [4]Tesco Ltd., Cheshunt, U.K.

[5]Vandermoortele (UK) Ltd., Hounslow, U.K.

[6]Supplied (mg/kg diet): all-rac -α-tocopheryl acetate, 40; menadione, 10; ascorbic acid, 1000; thiamin hydrochloride, 10; riboflavin, 20; pyridoxine hydrochloride, 12; calcium pantothenate, 44; nicotinic acid, 150; biotin, 1; folic acid, 5; cyanocobalamin, 0.02; myo-inositol, 400; retinyl acetate 7.3; cholecalciferol, 0.06.

[7]Supplied (per kg diet): KH_2PO_4, 22g; $FeSO_4.7H_2O$, 1.0g; $ZnSO_4.7H_2O$, 0.13g; $MnSO_4.4H_2O$, 52.8mg; $CuSO_4.5H_2O$, 12mg; $CoSO_4.7H_2O$, 2mg; KI, 2mg.

[8]Dissolved in propylene glycol and contained (g/L): butylated hydroxyanisole, 60; propyl gallate, 60; citric acid, 40.

Table 4.1.2 Fatty acid composition of experimental diets

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		(Weight %)	
14:0	4.9	3.7	2.1
16:0	12.6	11.9	9.8
18:0	2.6	3.3	3.5
16:1n-7	4.0	3.3	2.4
18:1n-9	12.1	15.0	14.1
18:1n-7	2.1	1.8	1.5
20:1n-9	7.2	5.5	2.8
22:1n-11	9.9	6.7	3.0
24:1	0.9	0.7	0.4
18:2n-6	10.4	25.3	46.3
20:2n-6	0.2	0.2	t
20:3n-6	t	t	t
20:4n-6	0.7	0.6	0.5
18:3n-3	1.0	0.8	0.5
18:4n-3	2.8	2.1	1.1
20:5n-3	6.4	5.1	3.5
22:5n-3	1.3	0.8	0.5
22:6n-3	9.4	6.5	4.7
Total n-6	11.9	26.4	46.8
Total n-3	21.8	15.8	10.6
n-3/n-6	1.8	0.6	0.2

t = trace value < 0.05%.

Table 4.2.1. Growth parameters and mortalities during the 16 weeks experimental period.

Parameter	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
Number of fish	110	110	110
Mean initial weight	36.2 ± 8.1	36.4 ± 7.4	37.1 ± 8.2
Mean final weight	127.3 ± 20.5[a]	137.0 ± 22.7[b]	120.9 ± 19.2[c]
Mortalities (number)	11	18	19
Specific growth rate (%/d)	1.1	1.2	1.1
Feed efficiency ratio	46.5	50.0	42.4

Values for initial and final weights are mean ± SD. Values in the same row having different superscript letters are significantly different ($P < 0.05$). Specific growth rate calculated as %weight gain/day = $(e^{G_w}-1) \times 100$, where G_w (daily growth rate) = $\ln W_1 - \ln W_0/T$. (W_1 = final weight, W_0 = initial weight, T = time in days). Feed efficiency ratio = gain/feed x 100.

4.3 Cardiac sarcoplasmic reticulum Ca^{2+}-Mg^{2+} ATPase activity

The activity of the SR Ca^{2+}-Mg^{2+} ATPase was not significantly affected by dietary treatment. There was no significant difference in total heart lipid content between different dietary treatments. Both these measurements are shown in Table 4.3.1.

Table 4.3.1 Heart total lipid and sarcoplasmic reticulum Ca^{2+}-Mg^{2+} ATPase activity in salmon fed diets containing increasing levels of linoleic acid.

	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
Heart total lipid[1]	1.97 ± 0.28	2.07 ± 0.11	2.16 ± 0.08
Ca^{2+}-Mg^{2+} ATPase[2]	650.7 ± 161.3	366.8 ± 100.7	488.9 ± 173.1

[1]Values are mean % Weight ± SD for four fish per dietary treatment.

[2]nmol P_i released/min per mg protein. Values are mean ± SD for four sarcoplasmic reticula samples per dietary treatment.

4.4 Fatty acid compositions of individual phospholipids from heart.

The fatty acid compositions of heart phospholipids are shown in Tables 4.4.1-4.4.4. In PC (Table 4.4.1), 18:2n-6, 20:2n-6, 20:3n-6 and total n-6 PUFA all increased significantly with increasing dietary intake of LA. Arachidonic acid was significantly increased in fish fed the highest dietary LA (45%), compared to those fed the lowest LA (10%). The fish fed the highest LA also had significantly reduced EPA, 22:5n-3 and total n-3 PUFA compared to those fed the lowest and intermediate

(25%) dietary LA. Total PUFA were significantly increased in fish fed the highest and intermediate level of LA compared to those fed the lowest LA. The n-3/n-6 PUFA ratio decreased significantly with increasing dietary LA and the 20:4/20:5 ratio was significantly increased in fish fed the highest dietary LA.

The fatty acid compositions of heart PE are shown in Table 4.4.2. Total monoenes were significantly reduced in fish fed the highest dietary LA compared to those fed the lowest LA. The changes in n-6 PUFA were similar to those observed in PC but EPA was significantly reduced with each increase in dietary LA. 22:5n-3 was significantly reduced in fish fed the highest LA compared to those fed the lowest dietary LA. The n-3/n-6 ratio decreased significantly with increasing dietary LA, but the 20:4/20:5 ratio was significantly increased only in fish given the highest dietary LA.

The fatty acid compositions of heart PS are shown in Table 4.4.3. Total saturates and 18:2n-6 were significantly increased in fish fed the intermediate and highest dietary LA compared with those fed the lowest LA. 20:2n-6, 20:3n-6 and total n-6 PUFA were all significantly increased, while EPA was significantly decreased with increasing dietary LA. 22:5n-3 was significantly decreased in fish fed the intermediate and highest dietary LA and 22:6n-3 was significantly decreased in fish fed the the highest LA compared to those fed the lowest LA.The n-3/n-6 PUFA ratio was significantly decreased in fish fed the intermediate and highest dietary LA, but the 20:4/20:5 ratio was only increased in those fed the highest dietary LA.

The fatty acid compositions of heart PI are shown in Table 4.4.4. LA, 20:3n-6 and total n-6 PUFA were all significantly increased and EPA and n-3/n-6 ratio were both significantly decreased with increasing dietary LA. 20:2n-6 was significantly increased in fish fed the intermediate and highest dietary LA compared to those fed the lowest LA. Total n-3 PUFA were significantly decreased and both AA and the 20:4/20:5 ratio significantly increased in fish given the highest dietary LA.

Table 4.4.1. Fatty acid compositions of heart phosphatidylcholine from salmon fed increasing levels of dietary linoleic acid.

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		(Weight %)	
Total saturates	45.9 ± 4.8	43.2 ± 2.9	47.3 ± 2.3
Total monoenes	17.2 ± 2.0	16.2 ± 0.9	14.7 ± 1.3
18:2n-6	1.7 ± 0.2^{a}	4.0 ± 0.3^{b}	7.2 ± 0.6^{c}
20:2n-6	0.2 ± 0.1^{a}	0.4 ± 0.1^{b}	0.6 ± 0.1^{c}
20:3n-6	0.2 ± 0.0^{a}	0.5 ± 0.1^{b}	1.1 ± 0.1^{c}
20:4n-6	1.3 ± 0.2^{a}	1.6 ± 0.1^{ab}	2.0 ± 0.4^{b}
Total n-6[1]	3.8 ± 0.6^{a}	7.2 ± 0.2^{b}	11.6 ± 0.4^{c}
20:5n-3	4.4 ± 1.1^{a}	3.9 ± 0.4^{a}	2.2 ± 0.3^{b}
22:5n-3	0.6 ± 0.1^{a}	0.5 ± 0.1^{ab}	0.4 ± 0.1^{b}
22:6n-3	20.9 ± 1.1	22.6 ± 3.1	17.8 ± 0.7
Total n-3[2]	25.5 ± 1.8^{a}	27.2 ± 3.5^{a}	20.8 ± 0.6^{b}
Total PUFA	29.1 ± 2.0^{a}	34.5 ± 3.4^{b}	33.1 ± 1.0^{b}
n-3/n-6	7.5 ± 1.2^{a}	4.0 ± 0.7^{b}	1.7 ± 0.2^{c}
20:4/20:5	0.3 ± 0.1^{a}	0.4 ± 0.0^{a}	0.9 ± 0.3^{b}

Results are mean ± SD for 4 fish per treatment.

[1]Includes 18:3n-6, 22:4n-6 and 22:5n-6.

[2]Includes 18:3n-3, 18:4n-3 and 20:4n-3.

Values in the same row with different superscript letters are significantly different ($P < 0.05$).

Table 4.4.2 Fatty acid compositions of heart phosphatidylethanolamine from salmon fed increasing levels of linoleic acid

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		(Weight %)	
Total saturates	30.3 ± 1.9	31.7 ± 3.0	30.5 ± 1.1
Total monoenes	10.2 ± 0.4^{a}	9.1 ± 1.7^{ab}	7.8 ± 0.2^{b}
18:2n-6	2.5 ± 0.1^{a}	5.3 ± 0.9^{b}	7.9 ± 0.9^{c}
20:2n-6	0.2 ± 0.1^{a}	0.4 ± 0.0^{b}	0.7 ± 0.1^{c}
20:3n-6	0.2 ± 0.1^{a}	0.5 ± 0.1^{b}	1.1 ± 0.3^{c}
20:4n-6	1.6 ± 0.2^{a}	1.7 ± 0.2^{a}	2.5 ± 0.3^{b}
Total n-6[1]	5.1 ± 0.4^{a}	8.7 ± 1.0^{b}	13.5 ± 1.4^{c}
20:5n-3	3.4 ± 0.1^{a}	2.9 ± 0.2^{b}	2.0 ± 0.3^{c}
22:5n-3	2.2 ± 0.1^{a}	2.0 ± 0.4^{ab}	1.6 ± 0.3^{b}
22:6n-3	41.8 ± 1.1	36.9 ± 3.9	37.4 ± 1.3
Total n-3[2]	47.8 ± 1.2	41.9 ± 4.3	42.2 ± 1.6
Total PUFA	52.9 ± 1.0	51.0 ± 3.8	55.6 ± 1.3
n-3/n-6	9.5 ± 0.8^{a}	4.6 ± 0.7^{b}	3.2 ± 0.4^{c}
20:4/20:5	0.5 ± 0.1^{a}	0.6 ± 0.1^{a}	1.3 ± 0.3^{b}

Results are mean ± SD for 4 fish per treatment. [1]Includes 18:3n-6, 22:4n-6 and 22:5n-6. [2]Includes 18:3n-3, 18:4n-3 and 20:4n-3.

Values in the same row with different superscript letters are significantly different ($P < 0.05$).

Table 4.4.3. Fatty acid compositions of heart phosphatidylserine from salmon fed increasing levels of linoleic acid.

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		(Weight %)	
Total saturates	38.1 ± 4.5[a]	46.5 ± 3.5[b]	46.6 ± 1.8[b]
Total monoenes	10.2 ± 0.7[a]	8.5 ± 0.9[b]	5.7 ± 0.5[c]
18:2n-6	3.0 ± 0.6[a]	5.0 ± 0.7[b]	6.4 ± 1.1[c]
20:2n-6	0.3 ± 0.1[a]	0.5 ± 0.1[b]	0.8 ± 0.1[c]
20:3n-6	0.3 ± 0.1[a]	0.6 ± 0.2[b]	1.2 ± 0.2[c]
20:4n-6	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
Total n-6[1]	5.3 ± 0.8[a]	7.6 ± 0.9[b]	9.8 ± 1.2[c]
20:4n-3	0.5 ± 0.1[a]	t[b]	t[b]
20:5n-3	1.2 ± 0.1[a]	0.9 ± 0.1[b]	0.5 ± 0.1[c]
22:5n-3	2.2 ± 0.3[a]	1.6 ± 0.2[b]	1.4 ± 0.2[b]
22:6n-3	34.8 ± 3.9	31.7 ± 2.4	29.9 ± 1.5
Total n-3[2]	38.9 ± 4.1[a]	34.3 ± 2.2[ab]	31.8 ± 1.7[b]
Total PUFA	44.2 ± 3.5	41.9 ± 2.6	41.6 ± 1.7
n-3/n-6	7.6 ± 1.8[a]	4.6 ± 0.6[b]	3.3 ± 0.5[c]
20:4/20:5	0.4 ± 0.1[a]	0.4 ± 0.1[a]	1.2 ± 0.5[b]

Results are mean ± SD for 4 fish per treatment. [1]Includes 18:3n-6, 22:4n-6 and 22:5n-6. [2]Includes 18:3n-3, 18:4n-3 and 20:4n-3.

Values in the same row with different superscript letters are significantly different ($P < 0.05$). t = trace value < 0.05%.

Table 4.4.4. Fatty acid compositions of heart phosphatidylinositol from salmon fed increasing levels of dietary linoleic acid.

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		Weight %	
Total saturates	35.9 ± 4.5	37.2 ± 1.2	40.3 ± 1.6
Total monoenes	9.6 ± 0.6[a]	7.9 ± 0.4[b]	5.9 ± 0.3[c]
18:2n-6	2.6 ± 0.2[a]	5.3 ± 0.5[b]	6.1 ± 0.6[c]
20:2n-6	t[a]	0.3 ± 0.1[b]	0.3 ± 0.1[b]
20:3n-6	0.5 ± 0.1[a]	0.9 ± 0.1[b]	1.3 ± 0.1[c]
20:4n-6	12.2 ± 0.9[a]	13.3 ± 0.6[a]	14.9 ± 0.8[b]
Total n-6[1]	16.5 ± 1.9[a]	20.9 ± 1.2[b]	23.4 ± 0.5[c]
20:4n-3	0.5 ± 0.1[a]	0.4 ± 0.1[a]	t[b]
20:5n-3	6.6 ± 0.7[a]	5.3 ± 0.3[b]	3.0 ± 0.6[c]
22:5n-3	1.0 ± 0.4	0.7 ± 0.1	0.6 ± 0.2
22:6n-3	25.9 ± 5.0	25.7 ± 1.7	21.8 ± 0.6
Total n-3[2]	34.2 ± 5.8[a]	32.2 ± 2.0[a]	25.4 ± 0.5[b]
Total PUFA	50.7 ± 5.6	53.0 ± 2.9	48.8 ± 0.3
n-3/n-6	2.1 ± 0.4[a]	1.6 ± 0.1[b]	1.1 ± 0.1[c]
20:4/20:5	1.9 ± 0.3[a]	2.6 ± 0.1[a]	5.1 ± 1.2[b]

Results are mean ± SD for 4 fish per treatment. [1]Includes 18:3n-6, 22:4n-6 and 22:5n-6. [2]Includes 18:3n-3, 18:4n-3 and 20:4n-3.

Values in the same row with different superscript letters are significantly different ($P < 0.05$).

4.5 Production of eicosanoids by isolated cardiac myocytes

The amounts of 6-ketoPGF$_{1\alpha}$ and TXB$_2$ produced by isolated cardiac myocytes stimulated with A23187 are given in Table 4:5:1. 6-KetoPGF$_{1\alpha}$ concentration was not significantly affected in isolated cardiac myocytes as a result of increasing dietary LA. TXB$_2$ production was increased with increasing dietary LA, but was only significant at the highest level of dietary LA.

Table 4:5:1. Production of eicosanoids by isolated cardiac myocytes from salmon fed increasing levels of linoleic acid.

Eicosanoid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		(pg/mg protein)	
6-ketoPGF$_{1\alpha}$	363.3 ± 76.5	531.7 ± 71.5	533.9 ± 290.1
TXB$_2$	194.8 ± 35.6^a	238.0 ± 42.0^a	439.4 ± 71.9^b

Values are mean ± SD for three cardiac myocyte preparations per treatment. Values in the same row with different superscript letters are significantly different ($P < 0.05$).

4:6 Fatty acid compositions of blood leucocyte phospholipids

The fatty acid compositions of leucocyte phospholipids are summarised in Tables 4:6:1 - 4:6:4. In PC (Table 4:6:1) 18:2n-6, 20:2n-6, 20:3n-6, 20:4n-6 and total n-6 PUFA all increased with increasing dietary intake of LA. Both EPA and total n-3 PUFA decreased with increasing LA intake. Consequently, the n-3/n-6 PUFA ratio decreased and the 20:4/20:5 ratio increased with increasing dietary LA.

Table 4:6:1 Fatty acid compositions of leucocyte phosphatidylcholine from salmon given increasing levels of dietary linoleic acid

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		Weight %	
Total saturates	38.9	36.6	38.3
Total monoenes	26.6	23.4	23.6
18:2n-6	3.1	5.5	8.4
20:2n-6	0.4	1.1	1.3
20:3n-6	0.4	1.1	1.8
20:4n-6	2.7	4.2	4.9
Total n-6[1]	7.4	12.8	17.4
20:5n-3	8.1	5.3	4.7
22:5n-3	0.9	0.7	0.7
22:6n-3	11.7	10.6	11.5
Total n-3[2]	21.6	17.3	17.3
Total PUFA	29.0	30.1	34.7
n-3/n-6	2.9	1.4	1.0
20:4/20:5	0.3	0.8	1.0

Results were obtained from pooled leucocyte samples from 6 fish per dietary treatment (see Materials and Methods for details).

[1]Includes 18:3n-6, 22:4n-6 and 22:5n-6. [2]Includes 18:3n-3, 18:4n-3 and 20:4n-3.

Table 4:6:2. Fatty acid compositions of leucocyte phosphatidylethanolamine from salmon given increasing levels of dietary linoleic acid.

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		(Weight %)	
Total saturates	21.2	20.3	20.6
Total monoenes	22.9	17.6	14.0
18:2n-6	5.6	9.5	12.0
20:2n-6	0.5	1.0	1.2
20:3n-6	0.3	0.9	1.2
20:4n-6	3.4	5.6	6.5
Total n-6[1]	10.2	17.6	21.6
20:5n-3	3.3	2.6	2.0
22:5n-3	0.9	1.0	0.8
22:6n-3	29.2	29.1	29.0
Total n-3[2]	34.3	33.4	32.0
Total PUFA	44.5	51.0	53.6
n-3/n-6	3.4	1.9	1.5
20:4/20:5	1.0	2.2	3.3

Results were obtained from pooled leucocyte samples from 6 fish per dietary treatment (see Materials and Methods for details).

[1]Includes 18:3n-6, 22:4n-6 and 22:5n-6. [2]Includes 18:3n-3, 18:4n-3 and 20:4n-3.

Broadly similar changes occurred in the fatty acid compositions of leucocyte PE and PS (Tables 4:6:2 and 4:6:3). The fatty acid compositon of leucocyte PI was less affected by dietary LA intake but AA content was increased with increasing LA (Table 4:6:4). In general, the saturated and monoenoic fatty acids in leucocyte phospholipids reflected dietary intake.

Table 4:6:3. Fatty acid compositions of leucocyte phosphatidylserine from salmon given increasing levels of dietary linoleic acid.

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		Weight %	
Total saturates	44.7	50.9	41.8
Total monoenes	19.3	13.0	17.6
18:2n-6	1.5	1.7	4.7
20:2n-6	0.3	0.4	0.7
20:3n-6	0.2	0.5	0.6
20:4n-6	0.8	1.7	4.7
Total n-6[1]	3.5	5.2	9.4
20:5n-3	1.0	0.7	0.4
22:5n-3	1.3	1.8	1.0
22:6n-3	21.8	23.7	21.9
Total n-3[2]	24.4	26.3	23.3
Total PUFA	27.9	31.5	32.7
n-3/n-6	7.0	6.1	2.5
20:4/20:5	0.8	2.4	6.8

Results were obtained from pooled leucocyte samples from 6 fish per dietary treatment (see Materials and Methods for details).

[1]Includes 18:3n-6, 22:4n-6 and 22:5n-6. [2]Includes 18:3n-3, 18:4n-3 and 20:4n-3.

Table 4:6:4. Fatty acid composition of leucocyte phosphatidylinositol from salmon given increasing levels of dietary linoleic acid

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		Weight %	
Total saturates	40.3	46.3	47.1
Total monoenes	22.4	10.7	6.8
18:2n-6	2.0	1.6	2.7
20:2n-6	0.2	0.5	0.5
20:3n-6	0.2	0.4	0.6
20:4n-6	22.8	32.0	36.9
Total n-6[1]	25.5	34.5	40.7
20:5n-3	0.5	0.5	0.4
22:5n-3	0.3	t	t
22:6n-3	2.1	3.3	2.9
Total n-3[2]	3.5	3.8	3.3
Total PUFA	29.0	38.3	44.0
n-3/n-6	0.14	0.11	0.08
20:4/20:5	45.6	64.0	92.3

Results were obtained from pooled leucocyte samples from 6 fish per dietary treatment (see Materials and Methods for details).

[1]Includes 18:3n-6, 22:4n-6 and 22:5n-6. [2]Includes 18:3n-3, 18:4n-3 and 20:4n-3

t = trace value < 0.05%.

4:7 Fatty acid compositions of gill phospholipids

The fatty acid compositions of gill phospholipids are shown in Table 4:7:1 - 4:7:4. In PC (Table 4:7:1) 18:2n-6, 20:2n-6, 20:3n-6 and total n-6 PUFA were all increased significantly with increasing dietary LA, but AA and the 20:4/20:5 ratio were only increased significantly in the fish given the highest dietary LA. EPA, total n-3 PUFA and n-3/n-6 PUFA ratio were significantly reduced with increasing dietary LA while DHA was significantly reduced in fish given 25 and 45% LA. A very similar pattern of changes was observed in gill PE (Table 4:7:2) but EPA and total n-3 PUFA were significantly reduced only in fish given the highest dietary LA, while DHA was unaffected. Gill PS fatty acid composition (Table 4:7:3) showed changes similar to those in PE except that 18:2n-6, 20:2n-6 and 20:3n-6 were only significantly increased in fish given the highest dietary level of LA. The n-3/n-6 PUFA ratio was significantly decreased only in fish given the highest dietary LA. In gill PI (Table 4:7:4) 18:2n-6, 20:2n-6, 20:3n-6, total n-6 PUFA and the 20:4/20:5 ratio were all increased significantly with increasing dietary LA, while total PUFA were significantly increased only in fish fed the highest dietary LA. EPA, total n-3 PUFA and n-3/n-6 PUFA ratio were all significantly reduced with increasing dietary LA, while 22:5n-3 and DHA were significantly reduced in fish given 25 and 45% LA.

Table 4:7:1 Fatty acid compositions of gill phosphatidylcholine from salmon fed increasing levels of dietary linoleic acid.

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		(Weight %)	
Total saturates	41.8 ± 2.6	43.1 ± 0.9	44.1 ± 2.2
Total monoenes	26.1 ± 1.7[a]	26.6 ± 1.5[a]	23.7 ± 1.0[b]
18:2n-6	2.4 ± 0.2[a]	5.0 ± 0.3[b]	8.9 ± 0.8[b]
20:2n-6	0.3 ± 0.1[a]	0.7 ± 0.1[b]	1.2 ± 0.2[c]
20:3n-6	0.2 ± 0.1[a]	0.6 ± 0.1[b]	1.5 ± 0.3[c]
20:4n-6	1.3 ± 0.2[a]	1.5 ± 0.3[a]	2.5 ± 0.7[b]
Total n-6[1]	4.8 ± 0.6[a]	8.3 ± 0.6[b]	14.4 ± 1.5[c]
20:5n-3	3.7 ± 0.3[a]	2.9 ± 0.7[a]	1.7 ± 0.2[b]
22:5n-3	0.5 ± 0.0	0.4 ± 0.1	0.4 ± 0.1
22:6n-3	15.7 ± 1.7[a]	13.6 ± 0.6[b]	12.1 ± 0.5[b]
Total n-3[2]	20.1 ± 1.7[a]	17.1 ± 1.2[a]	14.2 ± 0.6[b]
Total PUFA	24.9 ± 1.8	25.4 ± 1.8	28.6 ± 2.2
n-3/n-6	4.3 ± 0.6[a]	2.1 ± 0.0[b]	1.0 ± 0.1[c]
20:4/20:5	0.4 ± 0.1[a]	0.5 ± 0.1[a]	1.5 ± 0.5[b]

Values are means ± SD for 4 fish per dietary treatment.

[1]Includes 18:3n-6, 22:4n-6 and 22:5n-6. [2]Includes 18:3n-3, 18:4n-3 and 20:4n-3.

Values in the same row having different superscript letters are significantly different.

SD of < 0.05 is tabulated as 0.0.

Table 4:7:2. Fatty acid compositions of gill phosphatidylethanolamine from salmon given increasing levels of dietary linoleic acid.

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
Total saturates	25.7 ± 0.8	25.4 ± 1.9	27.3 ± 2.0
Total monoenes	14.2 ± 0.9[a]	13.2 ± 0.8[a]	10.8 ± 0.6[b]
18:2n-6	2.8 ± 0.3[a]	5.9 ± 0.6[b]	8.4 ± 0.6[c]
20:2n-6	0.4 ± 0.1[a]	0.8 ± 0.2[b]	1.2 ± 0.2[c]
20:3n-6	0.2 ± 0.1[a]	0.6 ± 0.1[b]	1.2 ± 0.2[c]
20:4n-6	2.6 ± 0.5[a]	2.9 ± 0.4[a]	5.1 ± 1.3[b]
Total n-6[1]	6.6 ± 1.0[a]	10.9 ± 1.1[b]	16.7 ± 1.7[c]
20:5n-3	3.2 ± 0.6[a]	2.9 ± 0.4[a]	2.0 ± 0.3[b]
22:5n-3	0.8 ± 0.1	0.8 ± 0.1	0.6 ± 0.1
22:6n-3	36.2 ± 1.5	32.6 ± 2.9	31.6 ± 2.7
Total n-3[2]	40.4 ± 1.2[a]	36.3 ± 2.7[ab]	34.2 ± 3.1[b]
Total PUFA	47.0 ± 1.3	47.2 ± 2.3	51.5 ± 3.3
n-3/n-6	6.2 ± 1.1[a]	3.4 ± 0.5[b]	2.0 ± 0.2[c]
20:4/20:5	0.8 ± 0.0[a]	1.0 ± 0.2[a]	2.7 ± 1.0[b]

Values are means ± SD for 4 fish per dietary treatment.

[1]Includes 18:3n-6, 22:4n-6 and 22:5n-6. [2]Includes 18:3n-3, 18:4n-3 and 20:4n-3.

Values in the same row having different superscript letters are significantly different.

SD of < 0.05 is tabulated as 0.0.

Table 2:7:3. Fatty acid compositions of gill phosphatidylserine from salmon given increasing levels of dietary linoleic acid.

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
Total saturates	32.8 ± 1.5^{a}	33.6 ± 2.8^{ab}	36.2 ± 0.8^{b}
Total monoenes	14.2 ± 1.5^{a}	11.7 ± 1.0^{b}	9.0 ± 0.4^{c}
18:2n-6	1.9 ± 0.2^{a}	2.3 ± 0.3^{ab}	2.9 ± 0.4^{b}
20:2n-6	0.4 ± 0.1^{a}	0.7 ± 0.2^{a}	1.1 ± 0.1^{b}
20:3n-6	0.5 ± 0.1^{a}	0.7 ± 0.2^{ab}	1.1 ± 0.2^{b}
20:4n-6	0.9 ± 0.1^{a}	1.5 ± 0.6^{ab}	1.6 ± 0.2^{b}
Total n-6[1]	5.4 ± 0.2^{a}	6.3 ± 0.7^{a}	8.1 ± 0.6^{b}
20:5n-3	0.9 ± 0.1^{a}	0.9 ± 0.1^{a}	0.6 ± 0.1^{b}
22:5n-3	1.3 ± 0.1	1.3 ± 0.2	1.1 ± 0.1
22:6n-3	33.5 ± 1.1	35.0 ± 3.3	32.1 ± 0.3
Total n-3[2]	36.6 ± 1.3	37.6 ± 3.5	33.8 ± 0.1
Total PUFA	42.0 ± 1.5	43.9 ± 3.8	41.9 ± 0.6
n-3/n-6	6.7 ± 0.2^{a}	6.0 ± 0.6^{a}	4.2 ± 0.4
20:4/20:5	0.7 ± 0.2^{a}	1.6 ± 0.5^{a}	2.8 ± 0.4^{b}

Values are means ± SD for 4 fish per dietary treatment.

[1]Includes 18:3n-6, 22:4n-6 and 22:5n-6. [2]Includes 18:3n-3, 18:4n-3 and 20:4n-3.

Values in the same row having different superscript letters are significantly different.

SD of < 0.05 is tabulated as 0.0.

Table 4.7.4. Fatty acid compositions of gill phosphatidylinositol from salmon given increasing levels of dietary linoleic acid.

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		Weight %	
Total saturates	35.6 ± 1.0	38.5 ± 2.4	36.4 ± 0.7
Total monoenes	16.0 ± 0.7^{a}	19.7 ± 1.2^{b}	18.8 ± 0.2
18:2n-6	2.2 ± 0.2^{a}	4.7 ± 0.5^{b}	6.8 ± 0.3^{c}
20:2n-6	0.3 ± 0.1^{a}	0.5 ± 0.1^{a}	1.0 ± 0.1^{b}
20:3n-6	0.5 ± 0.1^{a}	1.1 ± 0.1^{b}	1.5 ± 0.1^{c}
20:4n-6	8.2 ± 0.3	8.0 ± 0.8	9.0 ± 0.3
Total n-6[1]	12.5 ± 0.3^{a}	17.5 ± 0.8^{b}	20.3 ± 0.7^{c}
20:5n-3	2.7 ± 0.2^{a}	2.0 ± 0.2^{b}	1.2 ± 0.2^{c}
22:5n-3	1.2 ± 0.2^{a}	0.7 ± 0.1^{b}	0.5 ± 0.1^{b}
22:6n-3	17.2 ± 0.4^{a}	14.5 ± 0.6^{b}	14.6 ± 0.3^{b}
Total n-3[2]	22.2 ± 0.1^{a}	18.2 ± 0.4^{b}	17.2 ± 0.4^{c}
Total PUFA	34.7 ± 0.2^{a}	35.7 ± 0.4^{a}	37.5 ± 0.9^{b}
n-3/n-6	1.8 ± 0.1^{a}	1.0 ± 0.1^{b}	0.8 ± 0.1^{b}
20:4/20:5	3.0 ± 0.2^{a}	4.1 ± 0.3^{b}	7.9 ± 1.1^{c}

Values are means ± SD for 4 fish per dietary treatment.

[1]Includes 18:3n-6, 22:4n-6 and 22:5n-6. [2]Includes 18:3n-3, 18:4n-3 and 20:4n-3.

Values in the same row having different superscript letters are significantly different.

SD of < 0.05 is tabulated as 0.0.

4.8 Plasma eicosanoid concentrations and production of eicosanoids following stimulation with A23187 in whole blood and gill cells.

The concentrations of plasma 6-keto-$PGF_{1\alpha}$ and TXB_2 are shown in Table 4.8.1. Both cyclooxygenase products were significantly increased in plasma of salmon fed the highest dietary level of LA. However, while 6-keto-$PGF_{1\alpha}$ was increased around 1.5-fold, TXB_2 was increased over 3.5-fold. The production of eicosanoids by whole blood stimulated with the calcium ionophore A23187 is shown in Table 4.8.2. LTB_4, 12-HETE, TXB_2 and the 12-HETE/12-HEPE ratio were all significantly increased, whereas 12-HEPE was significantly reduced in fish fed the highest dietary LA. While LTB_5 was not significantly affected, the ratio of LTB_4/LTB_5 was significantly increased in fish given the highest dietary LA.

The production of eicosanoids by isolated gill cells stimulated with A23187 is shown in Table 4.8.3. The production of both 12-HEPE and 12-HETE was significantly reduced with increasing dietary linoleic acid although the ratio 12-HETE/12-HEPE indicated that the EPA-derived species was more affected. The production of a DHA-derived 12-lipoxygenase product, eluting immediately before 12-HETE on HPLC, was confirmed by labeling gill cells with [1-^{14}C]-DHA. The peak detected by absorbance at 235 nm co-eluted with the maximum radioactive peak when eicosanoids derived from gill cells labeled with [1-^{14}C]-DHA were analysed by HPLC. This was presumed to be the 12-lipoxygenase product of DHA, 14-hydroxy-4, 7, 10, 13, 16, 19-docosahexaenoic acid (14-HDHE). Production of the putative 14-HDHE was also decreased with increasing dietary LA. Production of TXB_2 by isolated gill cells was not affected by increasing dietary LA but the amount of 6-keto-$PGF_{1\alpha}$ was significantly reduced.

Table 4.8.1. Plasma 6-keto-$PGF_{1\alpha}$ and TXB_2 concentrations in salmon given increasing dietary levels of linoleic acid.

Eicosanoid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		pg/ml plasma	
6-keto-$PGF_{1\alpha}$	1103 ± 84^a	1005 ± 177^a	1664 ± 323^b
TXB_2	402 ± 130^a	293 ± 54^a	1418 ± 1083^b

Values are mean ± SD for 4 fish per treatment.
Values in the same row with different superscript letters are significantly different ($P < 0.05$).

Table 4.8.2. Eicosanoid production by whole blood of salmon fed increasing dietary linoleic acid, after stimulation with calcium ionophore A23187.

Eicosanoid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		(ng/ml blood)	
LTB_4	54.8 ± 21.8^a	75.3 ± 15.0^{ab}	101.4 ± 27.3^b
LTB_5	38.3 ± 10.2	28.7 ± 8.3	29.2 ± 2.2
LTB_4/LTB_5	1.44 ± 0.60^a	2.85 ± 0.99^{ab}	3.53 ± 1.17^b
12-HETE	55.6 ± 7.1^a	53.3 ± 14.1^a	92.7 ± 8.8^b
12-HEPE	40.5 ± 6.8^a	24.7 ± 3.9^b	20.8 ± 3.4^b
12-HETE/12-HEPE	1.69 ± 0.39^a	2.20 ± 0.60^{ab}	3.81 ± 1.51^b
TXB_2	36.4 ± 8.7^a	53.1 ± 19.9^a	136.5 ± 41.1^b

Footnotes as described in Table 4.8.1.

Table 4.8.3. Eicosanoid production by isolated gill cells from salmon given diets with increasing linoleic acid, after stimulation with calcium ionophore A23187.

Eicosanoid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		ng/mg protein	
12-HEPE	48.8 ± 11.3[a]	22.1 ± 5.9[b]	5.9 ± 2.1[c]
12-HETE	17.7 ± 4.9[a]	13.1 ± 2.5[ab]	6.9 ± 3.8[b]
12-HETE/12-HEPE	0.33 ± 0.04[a]	0.64 ± 0.16[b]	0.98 ± 0.15[c]
14-HDHE	43.1 ± 15.4[a]	19.7 ± 4.4[b]	10.8 ± 4.4[c]
6-keto-$PGF_{1\alpha}$	0.23 ± 0.06[a]	ND	0.14 ± 0.06[b]
TXB_2	0.30 ± 0.14	ND	0.30 ± 0.12

Values are mean ± SD for 4 fish per treatment.
Values in the same row with different superscript letters are significantly different ($P < 0.05$). ND = not determined.

4.9. Summary.

1. Diets containing linoleic acid at 10, 25 and 45% of total dietary fatty acids were fed to three groups of post-smolt Atlantic salmon (*Salmo salar)* for 16 weeks. Cardiac lesions, similar to those described in the previous experiment, were recorded in a small number of individuals from all dietary treatments although number of fish and lesion severity was lower than in the first study. While the reason for this is not clear it may be due to the fish being of different genetic stock compared to those used in trial A. The fish fed the intermediate level of linoleic acid (25%) had significantly increased final weights compared to fish fed the two other dietary treatments and the fish fed the highest level of LA (50%) had the lowest final weights of all the dietary treatments.

2. Incorporation of linoleic acid into membrane phospholipids of heart, gill and blood leucocytes increased in response to dietary intake. In addition to linoleic acid individual phospholipids from the above tissues showed increased 20:2n-6 (2 to 4-fold), 20:3n-6 (2 to7-fold) and 20:4n-6 (1 to3-fold) and decreased 20:5n-3 (up to 2.3 fold) in response to increasing dietary linoleic acid. The ratio of n-3/n-6 PUFA decreased (up to 4.3-fold) in heart, gill and blood leucocyte phospholipids and the 20:4n-6/20:5n-3 ratio increased (up to 8.5-fold) in response to increasing dietary linoleic acid.

3. These changes in eicosanoid precursors were reflected in significantly increased plasma concentrations of 6-keto-$PGF_{1\alpha}$ and TXB_2 in salmon fed the highest dietary level of linoleic acid. In isolated cardiac myocytes, stimulated with A23187, TXB_2 production was increased in salmon fed the highest dietary level of linoleic acid. In whole blood stimulated with A23187, LTB_4, 12-HETE and TXB_2 were significantly increased and 12-HEPE significantly decreased in response to increasing dietary linoleic acid. In isolated gill cells stimulated with A23187, 12-HEPE, 12-HETE, 14-HDHE and TXB_2 were all **decreased** in response to increasing dietary linoleic

acid, although the ratio of 12-HETE/12-HEPE was increased. The activity of heart sarcoplasmic reticulum Ca^{2+}-Mg^{2+} ATPase was not affected by dietary linoleic acid.

Section 5. Dietary study C: Effects of diets rich in linoleic acid or α-linolenic acid on phospholipid fatty acid compositions, development of cardiac lesions, phospholipase activity and eicosanoid production in Atlantic salmon (*Salmo salar)*.

5.1 Introduction

The essential fatty acids, linoleic acid and linolenic acid (18:3n-3), are the precursors of the long-chain n-6 and n-3 species which are important components of the phospholipid bilayer of cell membranes. It is generally accepted that both the n-6 and n-3 fatty acids are metabolised by the same enzyme systems of sequential desaturation and elongation to yield their long-chain products (Sprecher, 1981). In these pathways the desaturation steps are generally rate-limiting while elongation is rapid (Horrobin, 1991), and thus competition between substrate fatty acids at desaturase binding sites will determine the nature of the resulting PUFA and ultimately the composition of cellular membranes. While an excess of n-6 PUFA may inhibit metabolism of 18:3n-3, in competitive terms, the n-3 PUFA are much more potent inhibitors of n-6 PUFA metabolism than *vice versa* although the relative concentrations of the two substrates will also determine the resulting products (Garcia and Holman, 1965).

Evidence suggests that AA is the preferred eicosanoid substrate in fish (Tocher and Sargent, 1987; Anderson *et al.* 1981) although the presence of EPA can reduce production of AA-derived eicosanoids in fish cells in culture (Bell *et al.* 1994b). EPA, DHA and 18:3n-3 can act directly to inhibit cyclooxygenase activity in mammals, thereby reducing production of AA-derived prostaglandins and thromboxanes (Lands *et al.* 1973; Garg *et al.* 1990). A number of recent studies have suggested that feeding oils rich in 18:3n-3 can reduce production of AA-derived eicosanoids by increasing levels of EPA in membrane phospholipids (Hwang *et al.* 1988; Olomu and Baracos, 1991).

In the first study (trial A), salmon fed diets rich in 18:2n-6 developed a severe cardiomyopathy which caused extensive thinning of the ventricular muscle and active necrosis in both the atrium and ventricle. Salmon fed high dietary levels of 18:2n-6 also showed increased levels of AA-derived eicosanoids in plasma and from stimulated cell preparations from heart, gill and whole blood. In the present study the objective was to investigate the metabolism of 18:2n-6 and 18:3n-3 in salmon fed diets containing either sunflower oil (SO), linseed oil (LO) or fish oil (FO) by measuring phospholipid fatty acid compositions in heart, liver, gill and blood leucocytes. The fatty acid compositions of diets are shown in Table 5.1.1. Eicosanoids, derived from both cyclooxygenase and lipoxygenase, would be measured in plasma and stimulated gill cells, cardiac myocytes and blood leucocytes. The occurrence of cardiac pathology is also be recorded as well as the measurement of cardiac phospholipase A activity.

5.2 Experimental details, growth, mortality and cardiac pathology.

Three hundred and thirty Atlantic salmon S1 smolts were obtained from theS.O.A.E.F.D. Fish Cultivation Unit, Aultbea, Wester Ross. Fish were distributed randomly into three circular tanks of 1 m diameter containing 500 L of seawater supplied at a rate of 10 L/min. After 5 weeks the fish were transferred to 2 m x 2 m square tanks containing 2000 L of seawater supplied at a rate of 26 L/min. The tanks were subjected to natural photoperiod and the water temperature over the experimental period (August-November) varied from 10-15^{o}C. The diets were supplied by automatic feeders as described in the previous experiments and were adjusted to supply 28 g/kg biomass initially and were adjusted to provide 20 g/kg biomass after 4 weeks. Fish were weighed individually at the start and finish of the experiment and were bulk weighed every 28 days as described in the previous trials. The diets contained 47% protein and 16% lipid with the latter being supplied

Table 5.1.1. Fatty acid composition of experimental diets. Values are weight %

Fatty acid	FO diet	SO diet	LO diet
14:0	6.6	1.8	1.8
16:0	14.8	9.5	9.1
18:0	2.4	4.3	3.5
Total saturates[1]	24.6	15.9	14.9
16:1n-7	5.1	2.0	2.0
18:1n-9	9.7	17.8	17.4
18:1n-7	2.0	1.0	1.0
20:1n-9	10.5	3.1	3.1
22:1n-11	16.6	4.8	4.4
24:1	1.0	0.6	0.5
Total monoenes[2]	45.2	29.5	28.7
18:2n-6	1.4	40.3	12.2
18:3n-6	0.2	0.1	t
20:2n-6	0.3	0.1	0.1
20:3n-6	0.1	t	t
20:4n-6	0.6	0.5	0.5
Total n-6[3]	2.8	41.1	12.9
18:3n-3	1.3	0.5	31.8
18:4n-3	3.1	1.0	0.9
20:4n-3	0.7	0.2	0.2
20:5n-3	6.5	3.6	3.4
22:5n-3	0.9	0.5	0.5
22:6n-3	8.8	4.9	4.5
Total n-3	21.3	10.7	41.3
Total PUFA	25.0	51.8	54.2
n-3/n-6	7.6	0.3	3.4

[1]Includes 15:0, 17:0, 20:0 and 22:0. [2]Includes 20:1n-11, 20:1n-7 and 22:1n-9.

[3]Includes 22:4n-6 and 22:5n-6. t = trace value < 0.05%.

as FO, SO (suppliers as described previously) or LO (ICN Biomedicals Ltd., High Wycombe, U.K.). Cardiosomatic index was calculated as heart weight in mg/ body weight in g. Satisfactory growth was observed in all dietary treatments over the 10 week experimental period and no differences were apparent between treatments (Table 5.2.1). Mortalities were low in all dietary treatments (< 5%). There was no significant effect of dietary treatment on cardiosomatic index (1.35 ± 0.21, 1.30 ± 0.18 and 1.32 ± 0.19 for FO, SO and LO diets respectively) or heart total lipid content and no gross pathologies were evident. The only cardiac histopathological lesion evident was focal degeneration of the myocardium of the spongy layer of the ventricle with associated leucocyte accumulation (see Fig. 5.2.1). For assessment purposes the ventricle muscle tissue of each fish was categorised into one of five groups ranging from no detectable degeneration through to severe lesion (Table 5.2.2). Similar lesions were also evident in the muscle of the auricle. The greatest incidence and severity of lesion was in the SO-fed fish although some evidence of tissue damage was also apparent in FO-fed fish. Feeding LO appeared to suppress lesion occurrence to a greater extent than FO.

Table 5.2.1. Growth parameters and mortalities during the 10 week experimental period.

Parameter	FO diet	SO diet	LO diet
Number of fish	110	110	110
Mean initial weight	82.9 ± 15.7	84.9 ± 15.6	85.6 ± 14.5
Mean final weight	190.5 ± 43.2	191.0 ± 45.6	200.0 ± 53.7
Mortalities (number)	3	10	5
Specific growth rate (%/d)	1.2	1.2	1.2
Feed efficiency ratio	54.5	54.8	58.1

Values for initial and final weights are mean ± SD. Specific growth rate calculated as %weight gain/day = $(e^{G_W}-1) \times 100$, where G_W (daily growth rate) = $\ln W_1 - \ln W_0/T$. (W_1 = final weight, W_0 = initial weight, T = time in days). Feed efficiency ratio = gain/feed x 100.

Figure 5.2.1

Areas of focal myotomal degeneration (arrowed) in the heart ventricle of a salmon fed diet containing sunflower oil. The leucocytic accumulation overlies each of these areas. Scale bar = 20 μm.

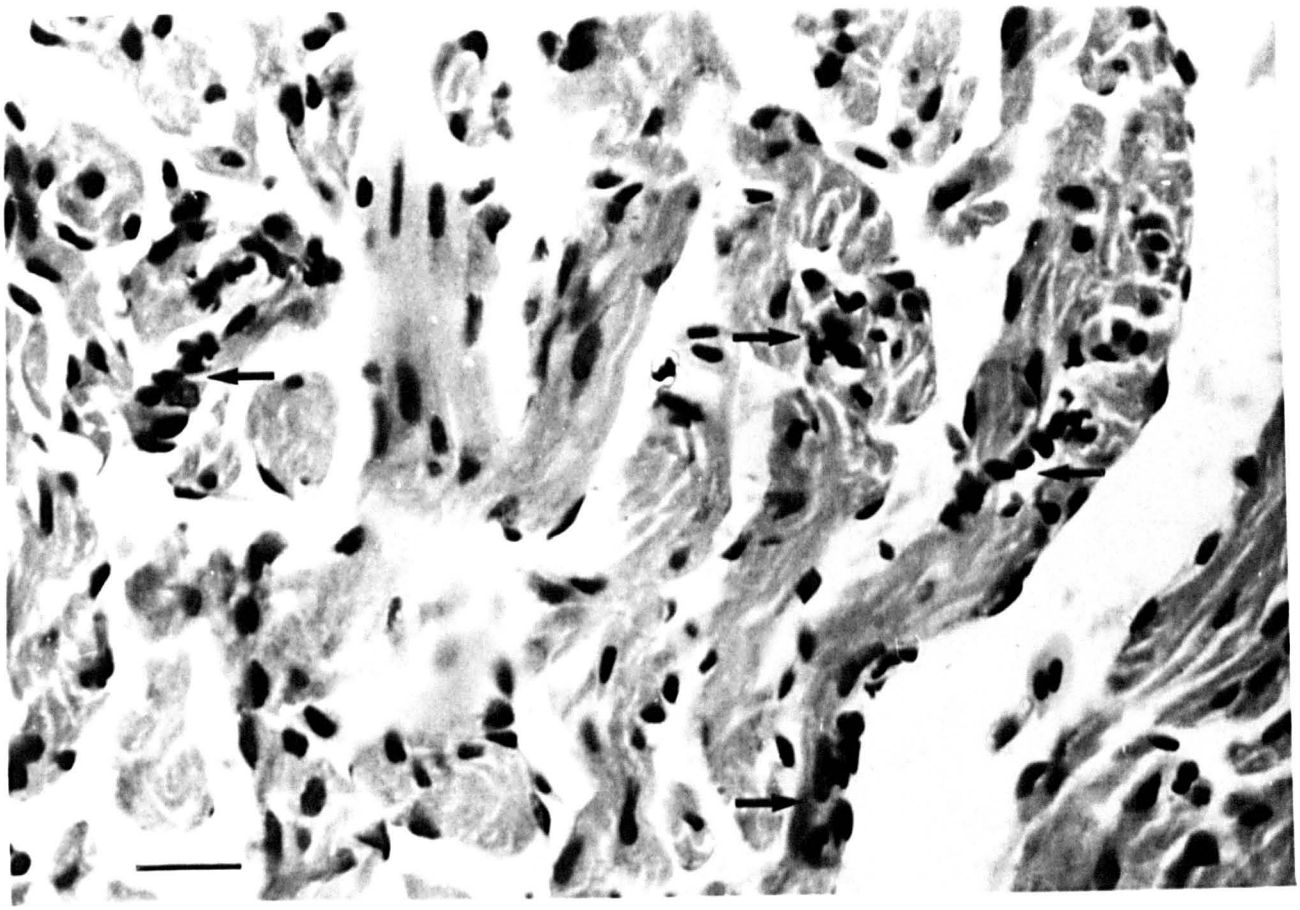


Table 5.2.2. Severity of heart histopathology in salmon fed diets containing fish (FO), sunflower (SO), or linseed oils (LO).

Lesion severity[a]	FO diet	SO diet	LO diet
		Number of fish	
0	7	6	11
1	2	0	0
2	2	3	1
3	0	2	0
4	0	0	0
Total sample	11	11	12

[a]Lesion severity categories; 0 = no changes evident. 1 = focal increase of endocardial cellularity without evidence of myocardial damage. 2 = focal lymphocyte accumulation with myocardial degeneration, 1-3 lesions per ventrical section. 3 = as 2 but with 4-10 lesions. 4 = as 2 with 10 + lesions.

5.3 Activity of cardiac phospholipase A

The activities of cardiac phospholipase A, measured with endogenous substrate, are shown in Table 5.3.1. Results are calculated as both accumulation of free fatty acid or hydrolysis of PC and PE/h per mg protein. In both cases the phospholipase activity in fish given dietary SO was significantly increased compared to those fed either FO or LO.

Table 5.3.1. Phospholipase A activities in hearts of salmon fed diets containing fish, sunflower or linseed oils

Phospholipase A activity	FO diet	SO diet	LO diet
nmol fatty acid liberated/ h per mg protein	53.1 ± 4.9^a	96.4 ± 6.9^b	59.9 ± 13.4^a
nmol PC and PE hydrolysed/h per mg protein	11.2 ± 1.4^a	26.3 ± 7.0^b	13.9 ± 3.4^a

Values are means ± SD for 4 fish per treatment.

Values in the same row with different superscript letters are significantly different ($P < 0.05$).

5.4 Cardiac lipid class compositions

The lipid class composition of heart is shown in Table 5.4.1. The amount of PI was significantly reduced in fish fed SO compared to those fed FO and LO and PC was significantly reduced in fish fed LO compared to both other dietary treatments. The latter observation significantly reduced the ratio PC:PE in LO-fed fish compared to both other dietary treatments so that in LO-fed fish PE was the major polar lipid class.

Table 5.4.1. Lipid class compositions of hearts from salmon fed diets containing either fish, sunflower or linseed oils

Lipid class	FO diet	SO diet	LO diet
		weight %	
Total phospholipids	60.1 ± 8.6	54.5 ± 8.3	53.5 ± 3.5
Total neutral lipids	39.8 ± 8.6	45.3 ± 8.3	46.3 ± 3.7
SM	3.0 ± 0.5	2.4 ± 0.4	2.6 ± 0.2
PC*	22.1 ± 3.1[a]	20.4 ± 3.1[a]	16.1 ± 0.9[b]
PS	3.3 ± 0.6	2.7 ± 0.4	3.1 ± 0.2
PI	3.5 ± 0.6[a]	3.0 ± 0.5[b]	3.7 ± 0.3[a]
PA/CL	8.7 ± 1.4	7.9 ± 1.5	8.8 ± 0.8
PE	19.4 ± 2.7	18.1 ± 2.7	19.3 ± 2.6
PC:PE ratio+	1.14 ± 0.03[a]	1.13 ± 0.08[a]	0.84 ± 0.04[b]
Triacylglycerol	27.8 ± 9.8	32.8 ± 8.8	33.6 ± 5.1
Free fatty acids	0.4 ± 0.1	0.5 ± 0.1	0.8 ± 0.6
Cholesterol	11.0 ± 1.8	12.3 ± 1.7	12.0 ± 1.6

Values are means ± SD for 12 fish per dietary treatment.

Values in the same row with different superscript letters are significantly different ($P < 0.05$.

*Values subjected to arc sine square root transformation before ANOVA.

+Values subjected to log transformation before ANOVA.

5.5. Fatty acid compositions of phospholipids from heart and blood leucocytes and eicosanoid production in blood and cardiac myocytes

The fatty acid compositions of heart PC are shown in Table 5.5.1. Fish fed SO had significantly reduced total saturates compared to FO-fed fish . SO-fed fish had significantly increased 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, AA and total n-6 PUFA compared to both other dietary treatments. Fish fed LO had significantly increased 18:2n-6, 20:2n-6, 20:3n-6 and total n-6 PUFA compared to fish fed FO but had significantly reduced AA compared to both other treatments. The 20:4/20:5 ratio was also significantly increased in fish fed SO compared to both other treatments while total PUFA were significantly increased compared to FO-fed fish. Fish fed LO had significantly increased 18:3n-3 and 20:4n-3 but decreased EPA, DHA and n-3/n-6 PUFA ratio compared to FO-fed fish.

The fatty acid compositions of heart PE are shown in Table 5.5.2. Total monoenes were significantly different for each dietary treatment with the highest levels in FO-fed fish and the lowest in SO-fed fish. SO-fed fish had significantly increased 18:2n-6, 20:2n-6, 20:3n-6, AA and total n-6 PUFA compared to the other two dietary treatments. Fish fed LO had significantly greater 18:2n-6, 20:2n-6, 20:3n-6 and total n-6 PUFA compared to FO-fed fish. 18:3n-3, 20:3n-3, 20:4n-3 and total n-3 PUFA were significantly increased in LO-fed fish and significantly reduced in SO-fed fish compared to fish fed FO. EPA was significantly reduced in fish fed SO compared to both other treatments. 22:5n-3 and the n-3/n-6 PUFA ratio were significantly different in all dietary treatments with the highest level in FO-fed fish and the lowest in SO-fed fish. Total n-3 PUFA were also significantly different in each dietary treatment with the highest level in LO-fed fish and the lowest in SO-fed fish. The 20:4/20:5 eicosanoid precursor ratio was significantly increased in SO-fed fish. Total PUFA were significantly increased in SO and LO-fed fish compared to those given fish oil. Total dimethyl acetals derived from plasmalogens were significantly reduced in SO-

fed fish compared to FO-fed fish.

Table 5.5.1 Fatty acid compositions of phosphatidylcholine from hearts of salmon fed diets containing either fish, sunflower or linseed oils

Fatty acid	FO diet	SO diet	LO diet
		Weight %	
Total saturates	32.9 ± 0.8^{a}	31.3 ± 0.1^{b}	32.4 ± 0.3^{ab}
Total monoenes	14.2 ± 0.8	12.9 ± 0.9	13.7 ± 0.5
18:2n-6	0.5 ± 0.1^{a}	8.0 ± 0.1^{b}	2.5 ± 0.1^{c}
18:3n-6	t^{a}	0.2 ± 0.1^{b}	t^{a}
20:2n-6	0.1 ± 0.0^{a}	0.8 ± 0.0^{b}	0.2 ± 0.0^{c}
20:3n-6	0.1 ± 0.0^{a}	1.5 ± 0.1^{a}	0.4 ± 0.1^{c}
20:4n-6	2.1 ± 0.2^{a}	3.0 ± 0.2^{b}	1.6 ± 0.1^{c}
Total n-6[1]	3.3 ± 0.3^{a}	13.9 ± 0.4^{b}	4.9 ± 0.1^{c}
18:3n-3	0.5 ± 0.2^{a}	0.2 ± 0.0^{a}	5.7 ± 0.3^{b}
18:4n-3	0.3 ± 0.1^{a}	0.1 ± 0.0^{b}	0.4 ± 0.1^{a}
20:4n-3	0.5 ± 0.1^{a}	0.3 ± 0.1^{a}	1.0 ± 0.0^{b}
20:5n-3	12.2 ± 0.1^{a}	7.7 ± 0.1^{b}	10.3 ± 0.6^{c}
22:5n-3	1.4 ± 0.1	1.2 ± 0.1	1.4 ± 0.2
22:6n-3	32.6 ± 1.6^{a}	30.4 ± 0.3^{ab}	28.9 ± 1.2^{b}
Total n-3	47.5 ± 1.4^{a}	39.9 ± 0.2^{b}	48.8 ± 0.9^{a}
Total PUFA	50.8 ± 1.2^{a}	53.8 ± 0.5^{b}	52.9 ± 0.9^{ab}
n-3/n-6*	14.5 ± 1.6^{a}	2.8 ± 0.1^{b}	9.8 ± 0.3^{c}
20:4/20:5	0.2 ± 0.0^{a}	0.4 ± 0.0^{b}	0.2 ± 0.0^{a}

Values are mean ± SD for 3 fish per treatment. Values in the same row with different superscript letters are significantly different. [1]Includes 22:4n-6 and 22:5n-6. *Values were subjected to log transformation before ANOVA.

Table 5.5.2. Fatty acid compositions of heart phosphatidylethanolamine from salmon fed diets containing either fish, sunflower or linseed oils.

Fatty acid	FO diet	SO diet	LO diet
		Weight %	
Total saturates	15.7 ± 0.2	15.4 ± 0.4	15.4 ± 0.6
Total monoenes	12.4 ± 0.4^{a}	7.6 ± 0.2^{b}	9.1 ± 0.2^{c}
18:2n-6	1.1 ± 0.1^{a}	8.2 ± 0.3^{b}	3.1 ± 0.1^{c}
20:2n-6	0.2 ± 0.0^{a}	1.0 ± 0.0^{b}	0.3 ± 0.0^{c}
20:3n-6	0.1 ± 0.0^{a}	1.1 ± 0.1^{b}	0.3 ± 0.1^{c}
20:4n-6	2.2 ± 0.3^{a}	3.0 ± 0.2^{b}	1.7 ± 0.1^{a}
Total n-6[1]	4.5 ± 0.2^{a}	14.4 ± 0.4^{b}	6.3 ± 0.2^{c}
18:3n-3	1.0 ± 0.1^{a}	0.4 ± 0.1^{b}	5.2 ± 0.1^{c}
20:3n-3	0.1 ± 0.1^{a}	t^{b}	0.7 ± 0.1^{c}
20:4n-3	0.7 ± 0.1^{a}	0.4 ± 0.0^{b}	1.2 ± 0.0^{c}
20:5n-3	6.1 ± 0.1^{a}	4.7 ± 0.2^{b}	6.5 ± 0.4^{a}
22:5n-3	4.0 ± 0.1^{a}	3.3 ± 0.1^{b}	3.6 ± 0.1^{c}
22:6n-3	47.9 ± 0.6	46.5 ± 0.4	45.7 ± 1.6
Total n-3	59.8 ± 0.5^{a}	55.3 ± 0.4^{b}	63.0 ± 1.3^{c}
Total PUFA	64.3 ± 0.3^{a}	69.7 ± 0.3^{b}	69.3 ± 1.4^{b}
n-3/n-6	13.4 ± 0.7^{a}	3.9 ± 0.1^{b}	10.0 ± 0.2^{c}
20:4/20:5	0.4 ± 0.1^{a}	0.7 ± 0.1^{b}	0.3 ± 0.0^{a}
Total dimethyl acetals	5.4 ± 0.5^{a}	4.6 ± 0.1^{b}	4.7 ± 0.2^{ab}

Values are mean ± SD for 3 fish per treatment. Values in the same row with different superscript letters are significantly different. [1]Includes 22:4n-6 and 22:5n-6.

Heart phosphatidylserine (PS) fatty acid composition was less affected by dietary lipid than PC and PE. Linoleic acid and 18:3n-3 levels reflected dietary intake and 20:2n-6, 20:3n-6 and total n-6 PUFA levels were significantly increased in SO-fed fish (data not shown). The fatty acid compositions of heart PI are shown in Table 5.5.3. Total monoenes were significantly reduced in fish fed SO and LO compared to those fed FO. Linoleic acid was significantly different in each dietary treatment with highest levels in SO and lowest levels in FO-fed fish. 20:2n-6, 20:3n-6, AA and total n-6 PUFA were significantly increased in SO-fed fish compared to the other two treatments. Linolenic acid was significantly increased in fish fed the LO containing diet. EPA, 22:5n-3, total n-3 PUFA and n-3/n-6 PUFA ratio were all significantly reduced in SO-fed fish compared to fish fed the other two dietary treatments. The 20:4/20:5 ratio was significantly increased in fish fed SO.

The fatty acid compositions of leucocyte PC and PE are shown in Table 5.5.4. The dietary induced changes in fatty acid compositions are largely similar for both phospholipid classes. In general, feeding SO increased 18:2n-6 and all C_{20} n-6 PUFA while reducing all n-3 PUFA compared to FO-fed fish. This resulted in a decreased n-3/n-6 PUFA ratio and an increased 20:4/20:5 eicosanoid precursor ratio compared to FO-fed fish. Feeding LO also increased 18:2n-6, 20:2n-6 and 20:3n-6 but decreased AA compared to FO-fed fish. EPA was increased in LO-fed fish compared to FO-fed fish and the resulting n-3/n-6 PUFA ratio and 20:4/20:5 ratio were similar. The fatty acid compositions of leucocyte PS and PI are shown in Table 5.5.5. Dietary induced changes in PS were minimal although 18:2n-6 and C_{20} n-6 PUFA were increased in SO-fed fish compared to the other two treatments. In LO-fed fish all n-3 PUFA were increased compared to the other two treatments. In PI, feeding LO did not reduce AA but did increase EPA such that the resulting 20:4/20:5 ratio was similar to that in FO-fed fish but was considerably less than SO-fed fish.

The levels of plasma PGE_2 and TXB_2, and production of the same prostanoids by

whole blood and cardiac myocytes stimulated with A23187, are shown in Table 5.5.6. There were no significant dietary-induced changes in concentration of either eicosanoid in plasma. In stimulated blood cells TXB_2 was significantly increased in fish fed SO compared to those fed LO, whereas PGE_2 was significantly decreased in fish fed LO compared to those fed either SO or FO. In stimulated cardiac myocytes TXB_2 production was not affected by dietary treatment whereas PGE_2 was significantly increased in fish given both SO and LO compared to those given FO.

Table 5.5.3. Fatty acid compositions of heart phosphatidylinositol from salmon fed diets containing either fish, sunflower or linseed oils.

Fatty acid	FO diet	SO diet	LO diet
		Weight %	
Total saturates	32.9 ± 2.0	35.8 ± 0.9	35.1 ± 2.7
Total monoenes	11.0 ± 0.9^{a}	8.8 ± 0.2^{b}	8.6 ± 0.2^{b}
18:2n-6	0.4 ± 0.0^{a}	3.6 ± 0.2^{b}	1.1 ± 0.1^{c}
18:3n-6	0.4 ± 0.2	0.2 ± 0.1	0.4 ± 0.3
20:2n-6	0.2 ± 0.1^{a}	0.4 ± 0.0^{b}	0.1 ± 0.1^{a}
20:3n-6	0.2 ± 0.0^{a}	1.2 ± 0.1^{b}	1.0 ± 0.2^{b}
20:4n-6	19.6 ± 2.1^{a}	23.1 ± 0.6^{b}	18.7 ± 0.9^{a}
Total n-6[1]	20.8 ± 1.8^{a}	28.5 ± 0.7^{b}	21.2 ± 0.7^{a}
18:3n-3	0.4 ± 0.2^{a}	0.2 ± 0.0^{a}	3.0 ± 0.2^{b}
20:3n-3	t^{a}	t^{a}	0.2 ± 0.0^{b}
20:4n-3	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.1
20:5n-3	10.3 ± 0.5^{a}	6.6 ± 0.5^{b}	10.3 ± 0.8^{a}
22:5n-3	1.2 ± 0.1^{a}	0.9 ± 0.1^{b}	1.0 ± 0.1^{ab}
22:6n-3	16.9 ± 2.8	13.4 ± 0.7	13.2 ± 0.5
Total n-3	29.3 ± 2.7^{a}	21.5 ± 0.4^{b}	28.2 ± 1.6^{a}
Total PUFA	50.1 ± 1.3	50.0 ± 0.3	49.4 ± 2.0
n-3/n-6	1.4 ± 0.2^{a}	0.8 ± 0.1^{b}	1.3 ± 0.1^{a}
20:4/20:5	1.9 ± 0.2^{a}	3.5 ± 0.2^{b}	1.8 ± 0.1^{a}

Values are mean ± SD for 3 fish per treatment. Values in the same row with different superscript letters are significantly different. [1]Includes 22:4n-6 and 22:5n-6.

Table 5.5.4. Fatty acid compositions of blood leucocyte phosphatidylcholine and phosphatidylethanolamine from salmon fed diets containing either fish, sunflower or linseed oil.

	PC			PE		
Fatty acid	FO diet	SO diet	LO diet	FO diet	SO diet	LO diet
Total saturates	33.0	36.3	35.2	22.4	16.6	15.1
Total monoenes	30.1	25.4	27.7	16.0	10.3	15.1
18:2n-6	1.7	9.6	2.6	2.0	11.3	4.4
18:3n-6	0.4	0.2	0.2	0.2	0.2	0.3
20:2n-6	0.2	1.7	0.4	0.3	1.5	0.5
20:3n-6	0.3	1.7	0.5	0.2	1.0	0.3
20:4n-6	2.1	3.0	1.7	2.9	4.7	2.6
Total n-6[1]	4.7	16.2	5.6	5.6	18.9	8.1
18:3n-3	0.5	0.2	3.0	0.3	0.3	3.2
20:3n-3	t	-	0.7	-	-	0.5
20:4n-3	0.5	0.2	0.7	t	t	0.4
20:5n-3	8.9	6.1	9.3	4.0	3.2	4.9
22:5n-3	1.5	0.7	0.9	1.1	1.0	1.2
22:6n-3	16.4	13.4	15.2	36.2	36.2	39.0
Total n-3	28.0	20.6	29.8	41.6	40.7	49.2
Total PUFA	32.7	36.8	35.4	47.2	59.6	57.3
n-3/n-6	6.0	1.3	5.3	7.4	2.2	6.1
20:4/20:5	0.2	0.5	0.2	0.7	1.5	0.5
Total dimethyl acetals	-	-	-	10.1	8.8	10.4

Results were obtained from pooled leucocyte samples from 6 fish per dietary treatment. t = trace value < 0.05%. - = not determined. [1]Includes 22:4n-6 and 22:5n-6.

Table 5.5.5. Fatty acid compositions of blood leucocyte phosphatidylserine and phosphatidylinositol from salmon fed diets containing either fish oil, sunflower oil or linseed oil.

	PS			PI		
Fatty acid	FO diet	SO diet	LO diet	FO diet	SO diet	LO diet
Total saturates	39.2	45.3	43.0	40.4	43.7	43.9
Total monoenes	19.6	11.0	9.5	24.3	7.9	6.0
18:2n-6	1.7	2.6	1.0	1.2	1.5	0.6
18:3n-6	0.3	0.4	0.2	2.0	0.5	0.2
20:2n-6	t	0.8	0.3	t	0.4	0.1
20:3n-6	t	0.6	0.2	t	0.9	0.7
20:4n-6	0.6	1.1	0.7	19.4	32.5	35.6
Total n-6[1]	2.6	5.7	2.4	22.6	35.8	37.2
18:3n-3	0.5	0.2	1.0	0.6	t	0.5
20:5n-3	1.5	1.3	2.0	1.2	0.9	1.8
22:5n-3	1.7	1.6	2.1	0.6	1.1	1.3
22:6n-3	30.9	31.5	37.9	8.2	6.7	6.1
Total n-3[2]	35.3	34.6	43.6	10.6	9.2	10.1
Total PUFA	39.5	40.3	46.0	33.2	45.0	47.3
n-3/n-6	13.6	6.1	18.2	0.5	0.3	0.3
20:4/20:5	0.4	0.9	0.4	16.2	36.1	19.8

Results were obtained from pooled leucocyte samples from 6 fish per dietary treatment. t = trace value < 0.05%. - = not determined. [1]Includes 22:4n-6 and 22:5n-6. [2]Includes 18:4n-3, 20:3n-3 and 20:4n-3.

Table 5.5.6. PGE_2 and TXB_2 concentrations in plasma and extracts from blood and cardiac myocytes stimulated with calcium-ionophore A23187 in salmon fed diets containing FO, SO or LO.

Eicosanoid	FO diet	SO diet	LO diet
Plasma TXB_2 (ng/ml)	1.24 ± 0.35	0.77 ± 0.55	1.02 ± 0.67
Plasma PGE_2 (pg/ml)	109.0 ± 15.5	125.3 ± 46.1	165.7 ± 75.3
Stimulated blood TXB_2 (ng/ml)	2.54 ± 2.00^{ab}	12.27 ± 6.20^{a}	2.20 ± 2.17^{b}
Stimulated blood PGE_2 (ng/ml)*	1.39 ± 0.33^{a}	2.93 ± 0.88^{a}	0.58 ± 0.21^{b}
Stimulated cardiac myocytes TXB_2 (pg/mg protein)	112.2 ± 48.4	108.7 ± 27.1	98.0 ± 56.8
Stimulated cardiac myocytes PGE_2 (pg/mg protein)	268.0 ± 141.9^{b}	857.0 ± 141.9^{a}	706.3 ± 157.9^{a}

Values are mean ± SD for 4 fish per treatment.

Values in the same row with different superscript letters are significantly different ($P < 0.05$).

*Values were subjected to log transformation before ANOVA.

5.6 Fatty acid compositions of salmon liver and gills and eicosanoid production by isolated gill cells

The fatty acid compositions of liver PC are shown in Table 5.6.1. Total monoenoic fatty acids were significantly reduced in SO-fed fish compared to fish fed FO. In SO-fed fish 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6 were all significantly increased compared to both other dietary treatments. Fish fed LO had significantly increased 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6 and total n-6 PUFA but reduced 22:5n-6 compared to fish fed FO. LO-fed fish had significantly increased 18:3n-3, 18:4n-3, 20:3n-3 and 20:4n-3 compared to both other dietary treatments but had significantly reduced DHA compared to FO-fed fish. In fish fed SO 18:3n-3, 20:4n-3, EPA, 22:5n-3 and total n-3 PUFA were significantly reduced compared to both other dietary treatments while DHA was significantly reduced compared to FO-fed fish. The n-3/n-6 PUFA ratio was significantly different in the three dietary treatments with the highest value in FO-fed fish and the lowest in SO-fed fish.

The fatty acid compositions of liver PE are shown in Table 5.6.2. Total monoenoic fatty acids were significantly different for each dietary treatment with the highest levels in FO-fed fish and the lowest levels in SO-fed fish. Also, 18:2n-6, 20:2n-6, 20:3n-6 and total n-6 PUFA were significantly affected by dietary treatment with the highest levels in SO-fed fish and the lowest levels in FO-fed fish. Levels of 22:5n-6 were also significantly different in each dietary group with highest values in SO-fed fish and lowest in LO-fed fish. AA and 22:4n-6 were significantly increased in fish fed SO compared to both other dietary treatments. Also, 18:3n-3, 20:4n-3 and EPA were significantly different in each dietary treatment with highest levels in LO-fed fish and lowest in SO-fed fish. 22:5n-3 was significantly increased in LO-fed fish compared to SO-fed fish whereas DHA was significantly greater in FO-fed fish compared to both other treatments. Total n-3 PUFA were significantly lower in SO-fed fish while total PUFA were significantly different in each dietary treatment with

Table 5.6.1. Fatty acid compositions of liver phosphatidylcholine from salmon fed diets containing fish oil, sunflower oil or linseed oil.

Fatty acids	FO diet	SO diet	LO diet
		Weight %	
Total saturated	29.3 ± 1.6	29.9 ± 3.5	31.7 ± 3.7
Total monoenoic	16.0 ± 1.6^{b}	13.2 ± 0.8^{c}	14.6 ± 1.1^{bc}
18:2n-6	1.1 ± 0.3^{d}	10.6 ± 1.7^{b}	4.0 ± 0.5^{c}
18:3n-6	t^{d}	0.5 ± 0.3^{b}	0.2 ± 0.0^{c}
20:2n-6	0.3 ± 0.1^{d}	1.7 ± 0.5^{b}	0.6 ± 0.1^{c}
20:3n-6	0.3 ± 0.1^{d}	6.4 ± 1.0^{b}	2.0 ± 0.5^{c}
20:4n-6	0.7 ± 0.1^{c}	5.5 ± 2.2^{b}	0.6 ± 0.1^{c}
22:4n-6	0.1 ± 0.0^{c}	0.7 ± 0.4^{b}	0.1 ± 0.0^{c}
22:5n-6	0.3 ± 0.1^{c}	0.7 ± 0.3^{b}	0.1 ± 0.0^{c}
Total n-6	2.8 ± 0.3^{d}	26.0 ± 0.4^{b}	7.4 ± 0.4^{c}
18:3n-3	0.3 ± 0.1^{c}	0.1 ± 0.0^{d}	3.6 ± 1.2^{b}
18:4n-3	0.1 ± 0.0^{c}	0.1 ± 0.0^{c}	0.7 ± 0.2^{b}
20:3n-3	t^{c}	t^{c}	0.4 ± 0.1^{b}
20:4n-3	0.8 ± 0.2^{c}	0.2 ± 0.1^{d}	2.9 ± 0.8^{b}
20:5n-3	9.8 ± 1.0^{b}	3.8 ± 0.4^{c}	9.5 ± 1.2^{b}
22:5n-3	3.0 ± 0.6^{b}	2.0 ± 0.5^{c}	3.3 ± 0.4^{b}
22:6n-3	35.9 ± 2.6^{b}	23.8 ± 3.2^{c}	25.5 ± 1.7^{c}
Total n-3	49.6 ± 1.8^{b}	29.7 ± 3.1^{c}	45.8 ± 2.2^{b}
Total PUFA	52.4 ± 1.8	55.7 ± 3.2	53.1 ± 2.1
n-3/n-6	18.0 ± 1.6^{b}	1.2 ± 0.1^{d}	6.2 ± 0.5^{c}
20:4/20:5	0.1 ± 0.0^{c}	1.5 ± 0.6^{b}	0.1 ± 0.0^{c}

Results are means ± standard deviation from 4 fish per treatment. t = trace value < 0.05%. Standard deviation < 0.05 are recorded as 0.0.
Values in the same row with different superscript letters are significantly different ($P < 0.05$).

Table 5.6.2. Fatty acid compositions of liver phosphatidylethanolamine from salmon fed diets containing fish oil, sunflower oil or linseed oil.

Fatty acids	FO diet	SO diet	LO diet
		Weight %	
Total saturated	12.6 ± 0.7	12.7 ± 0.6	13.6 ± 1.4
Total monoenoic	25.2 ± 1.8^{b}	15.5 ± 2.5^{d}	21.1 ± 0.4^{c}
18:2n-6	1.7 ± 0.4^{d}	12.0 ± 2.1^{b}	4.7 ± 1.1^{c}
18:3n-6	0.1 ± 0.0	0.2 ± 0.1	t
20:2n-6	0.4 ± 0.1^{d}	2.3 ± 0.5^{b}	0.7 ± 0.1^{c}
20:3n-6	0.3 ± 0.1^{d}	5.1 ± 0.6^{b}	1.5 ± 0.2^{c}
20:4n-6	1.3 ±0.4^{c}	9.6 ± 2.7^{b}	1.1 ± 0.1^{c}
22:4n-6	t^{c}	0.8 ± 0.4^{b}	t^{c}
22:5n-6	0.5 ± 0.0^{c}	1.0 ± 0.3^{b}	0.3 ± 0.1^{d}
Total n-6	4.2 ± 0.4^{d}	30.9 ± 2.5^{b}	8.1 ± 1.1^{c}
18:3n-3	0.8 ± 0.2^{c}	0.3 ± 0.1^{d}	3.0 ± 0.6^{b}
20:3n-3	t^{c}	t^{c}	0.7 ± 0.1^{b}
20:4n-3	0.5 ± 0.1^{c}	t^{d}	1.7 ± 0.4^{b}
20:5n-3	8.8 ± 1.2^{c}	3.6 ± 1.0^{d}	11.1 ± 1.1^{b}
22:5n-3	2.1 ± 0.4^{bc}	1.8 ± 0.4^{c}	2.8 ± 0.4^{b}
22:6n-3	41.9 ± 1.9^{b}	32.5 ± 2.7^{c}	36.0 ± 0.9^{c}
Total n-3	54.1 ± 0.8^{b}	38.2 ± 2.2^{c}	55.4 ± 0.3^{b}
Total PUFA	58.3 ± 1.2^{d}	69.2 ± 2.7^{b}	63.5 ± 1.4^{c}
n-3/n-6	12.9 ± 1.2^{b}	1.3 ± 0.1^{d}	6.9 ± 0.9^{c}
20:4/20:5	0.2 ± 0.1^{c}	2.9 ± 1.4^{b}	0.1 ± 0.0^{c}

Results are means ± standard deviation from 4 fish per treatment. t = trace value < 0.05%. Standard deviation < 0.05 are recorded as 0.0.
Values in the same row with different superscript letters are significantly different ($P < 0.05$).

the highest levels in SO-fed fish and the lowest in FO-fed fish. The ratio of n-3/n-6 PUFA was significantly different in each dietary treatment with the highest value in FO-fed fish and the lowest in SO-fed fish. The AA/EPA ratio of eicosanoid precursors was significantly increased in SO-fed fish compared to both other treatments.

The fatty acid compositions of liver PS are shown in Table 5.6.3. Total saturated fatty acids were significantly increased whereas total monoenoic fatty acids were significantly decreased in fish fed SO and LO compared to those fed FO. The level of 20:3n-6 was significantly different in each dietary treatment with the highest levels in SO-fed fish and the lowest in FO-fed fish. AA and 22:5n-6 were also significantly different in each treatment with the highest levels in SO-fed fish and the lowest in LO-fed fish. 18:2n-6, 20:2n-6, 22:4n-6 and total n-6 PUFA were all significantly increased in fish fed SO compared to the other dietary treatments. Levels of 18:3n-3 and 20:4n-3 were significantly greater in fish fed LO than in the two other treatments while EPA was significantly reduced in SO-fed fish compared to LO-fed fish. Total n-3 PUFA were significantly reduced in SO-fed fish compared to both other treatments while DHA was reduced in fish fed SO compared to those fed FO. Total PUFA were significantly greater in SO-fed fish compared to LO-fed fish. The n-3/n-6 PUFA ratio was significantly lower and the AA/EPA ratio significantly greater in SO-fed fish compared to both other treatments.

The fatty acid compositions of liver PI are shown in Table 5.6.4. Total saturated fatty acids were significantly elevated in fish fed SO and LO compared with those fed FO. Total monoenoic fatty acids were significantly different in all treatments with the highest levels in fish fed FO and the lowest in those fed SO. Levels of 18:2n-6 and total n-6 PUFA were significantly increased in SO-fed fish compared to the other two treatments whereas 20:3n-6 was significantly greater in LO-fed fish compared to the other treatments. AA was significantly different in all dietary treatments with the highest level in SO-fed fish and the lowest level in LO-fed fish.

Also, 18:3n-3, 20:4n-3 and total n-3 PUFA were significantly reduced in fish fed SO compared to the other two treatments. EPA was significantly different in all treatments with the highest level in LO-fed fish and the lowest in SO-fed fish. Levels of 22:5n-3 and DHA were significantly different in all treatments with the highest levels in FO-fed fish and the lowest in SO-fed fish. Total PUFA were significantly greater in SO-fed fish compared to LO-fed fish. The n-3/n-6 PUFA ratio was significantly lower and the AA/EPA ratio significantly greater in fish fed SO compared to both other treatments.

The major PUFA in gill PC and PE are shown in Figure 5.6.1. In PC 18:2n-6 and 20:3n-6 were significantly different in all dietary treatments with the highest levels in SO-fed fish and the lowest in FO-fed fish. AA was significantly increased and EPA significantly decreased in SO-fed fish compared to the other treatments. Consequently, the AA/EPA ratio was significantly greater in SO-fed fish compared to the other two treatments. DHA and total n-3 PUFA were significantly decreased in SO-fed fish compared to both other treatments. Largely similar effects occurred in gill PE except that 20:3n-6 was significantly greater in SO-fed fish compared to both other treatments and DHA was significantly reduced in SO-fed fish compared to FO-fed fish.

The major PUFA of gill PS and PI are shown in Figure 5.6.2. In gill PS 18:2n-6 and 20:3n-6 were significantly different in all dietary treatments with the highest levels in SO-fed fish and the lowest levels in FO-fed fish. AA was significantly increased and EPA significantly reduced in SO-fed fish resulting in an increased AA/EPA ratio when compared to fish fed either FO or LO. In gill PI the results were largely similar to PS except that DHA was significantly greater in fish fed FO than in both other dietary treatments.

Table 5.6.3. Fatty acid compositions of liver phosphatidylserine from salmon fed diets containing fish oil, sunflower oil or linseed oil.

Fatty acids	FO diet	SO diet	LO diet
		Weight %	
Total saturated	31.7 ± 1.5^{c}	35.5 ± 1.4^{b}	35.8 ± 1.8^{b}
Total monoenes	10.3 ± 0.8^{b}	6.2 ± 0.5^{c}	6.7 ± 1.0^{c}
18:2n-6	0.5 ± 0.1^{c}	1.5 ± 0.2^{b}	0.6 ± 0.1^{c}
18:3n-6	0.2 ± 0.2	0.2 ± 0.1	t
20:2n-6	0.2 ± 0.0^{c}	1.0 ± 0.3^{b}	0.3 ± 0.1^{c}
20:3n-6	0.2 ± 0.1^{d}	1.6 ± 0.5^{b}	0.6 ± 0.1^{c}
20:4n-6	0.5 ± 0.1^{c}	1.7 ± 0.3^{b}	0.2 ± 0.1^{d}
22:4n-6	t^{c}	0.8 ± 0.5^{b}	t^{c}
22:5n-6	0.6 ± 0.1^{c}	1.1 ± 0.4^{b}	0.3 ± 0.0^{d}
Total n-6	2.0 ± 0.2^{c}	8.0 ± 0.7^{b}	2.0 ± 0.1^{c}
18:3n-3	0.4 ± 0.1^{bc}	0.2 ± 0.1^{c}	0.6 ± 0.2^{b}
18:4n-3	0.4 ± 0.1^{b}	t^{c}	t^{c}
20:4n-3	t^{c}	t^{c}	0.5 ± 0.1^{b}
20:5n-3	1.4 ± 0.5^{bc}	0.7 ± 0.3^{c}	1.4 ± 0.3^{b}
22:5n-3	2.7 ± 0.4	3.2 ± 0.8	3.8 ± 0.6
22:6n-3	47.3 ± 1.6^{b}	43.2 ± 2.3^{c}	44.6 ± 0.7^{bc}
total n-3	52.3 ± 0.3^{b}	47.2 ± 1.7^{c}	50.9 ± 0.7^{b}
total PUFA	54.3 ± 1.0^{bc}	55.2 ± 1.3^{b}	52.9 ± 0.7^{c}
n-3/n-6	25.9 ± 2.1^{b}	5.9 ± 0.6^{c}	25.8 ± 1.3^{b}
20:4/20:5	0.4 ± 0.2^{c}	3.1 ± 1.6^{b}	0.2 ± 0.1^{c}

Results are means ± standard deviation from 4 fish per treatment. t = trace value < 0.05%. Standard deviation < 0.05 are recorded as 0.0.
Values in the same row with different superscript letters are significantly different ($P < 0.05$).

Table 5.6.4. Fatty acid compositions of liver phosphatidylinositol from salmon fed diets containing either fish oil, sunflower oil or linseed oil.

Fatty acids	FO diet	SO diet	LO diet
		Weight %	
Total saturated	33.3 ± 1.6^{c}	38.1 ± 0.5^{b}	38.4 ± 1.1^{b}
Total monoenoic	12.5 ± 1.4^{b}	6.7 ± 0.7^{d}	9.2 ± 0.6^{c}
18:2n-6	0.5 ± 0.1^{c}	1.4 ± 0.3^{b}	0.6 ± 0.1^{c}
20:2n-6	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.1
20:3n-6	2.0 ± 0.3^{c}	1.8 ± 0.7^{c}	5.8 ± 1.2^{b}
20:4n-6	28.4 ± 0.3^{c}	41.8 ± 0.4^{b}	21.0 ± 1.2^{d}
Total n-6	31.5 ± 0.2^{c}	46.1 ± 0.5^{b}	28.3 ± 1.7^{c}
18:3n-3	0.4 ± 0.1^{b}	t^{c}	0.5 ± 0.1^{b}
20:4n-3	0.3 ± 0.1^{b}	t^{c}	0.5 ± 0.1^{b}
20:5n-3	4.9 ± 0.9^{c}	0.7 ± 0.3^{d}	10.1 ± 2.5^{b}
22:5n-3	2.2 ± 0.3^{b}	0.6 ± 0.1^{d}	1.7 ± 0.2^{c}
22:6n-3	12.4 ± 1.2^{b}	4.7 ± 0.5^{d}	8.1 ± 0.4^{c}
Total n-3	19.7 ± 0.4^{b}	6.2 ± 0.6^{c}	21.5 ± 2.9^{b}
Total PUFA	51.4 ± 0.7^{bc}	52.2 ± 1.1^{b}	49.7 ± 1.1^{c}
n-3/n-6	0.6 ± 0.0^{b}	0.1 ± 0.0^{c}	0.7 ± 0.2^{b}
20:4/20:5	7.0 ± 2.2^{c}	76.1 ± 28.2^{b}	3.2 ± 2.2^{c}

Results are means ± standard deviation from 4 fish per treatment. t = trace value < 0.05%. Standard deviation < 0.05 are recorded as 0.0.
Values in the same row with different superscript letters are significantly different ($P < 0.05$).

The production of eicosanoids by isolated gill cells stimulated with A23187 is shown in Table 5.6.5. The production of 12-HETE, 12-HEPE and 14-HDHE were all significantly reduced in fish fed both LO and SO compared to those fed FO. However, the ratio of 12-HETE/12-HEPE was significantly increased in fish fed SO compared to both other treatments. While mean values of both TXB_2 and PGE_2 were greatest in fish fed SO they were not significantly different from either of the other dietary treatments.

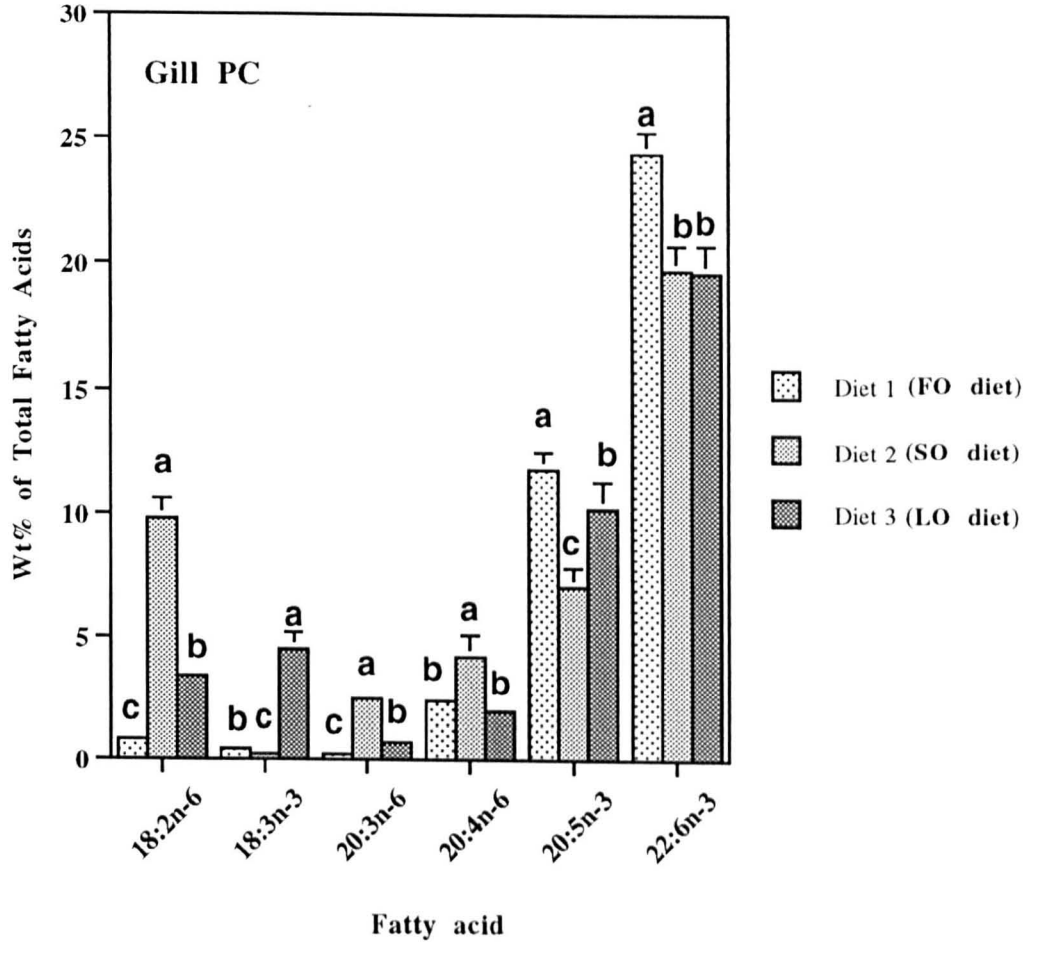
Table 5.6.5. Eicosanoids produced by isolated salmon gill cells stimulated with the Ca^{2+}-ionophore A23187.

Eicosanoid	FO diet	SO diet	LO diet
12-HETE	17.7 ± 9.6^b	5.7 ± 2.9^c	5.8 ± 0.5^c
12-HEPE	45.2 ± 12.4^b	7.8 ± 3.2^c	21.2 ± 8.2^c
12-HETE/12-HEPE	0.354 ± 0.094^c	0.791 ± 0.189^b	0.327 ± 0.138^c
14-HDHE	34.1 ± 11.8^b	6.8 ± 3.8^c	10.4 ± 1.9^c
TXB_2	142.8 ± 80.4	229.0 ± 120.9	172.0 ± 59.6
PGE_2	192.3 ± 54.0	807.3 ± 562.3	510.3 ± 177.9

Values are mean ± standard deviation from four fish per treatment. Values for 12-HETE, 12-HEPE and 14-HDHE are ng/mg protein whereas those for TXB_2 and PGE_2 are pg/mg protein. Values in the same row with different superscript letters are significantly different ($P < 0.05$).

Fig. 5.6.1. Levels of the major polyunsaturated fatty acids of gill phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from salmon fed diets containing either fish oil (Diet 1;FO), sunflower oil (Diet 2; SO) or linseed oil (Diet 3; LO). Values are means ± SD for four fish per treatment. Values for each fatty acid having a different column letter are significantly different ($P < 0.05$).

Figure 5.6.1


Gill PC
Wt% of Total Fatty Acids
30
25
20
15
10
5
0
18:2n-6
18:3n-3
20:3n-6
20:4n-6
20:5n-3
22:6n-3
Fatty acid
Diet 1 (FO diet)
Diet 2 (SO diet)
Diet 3 (LO diet)

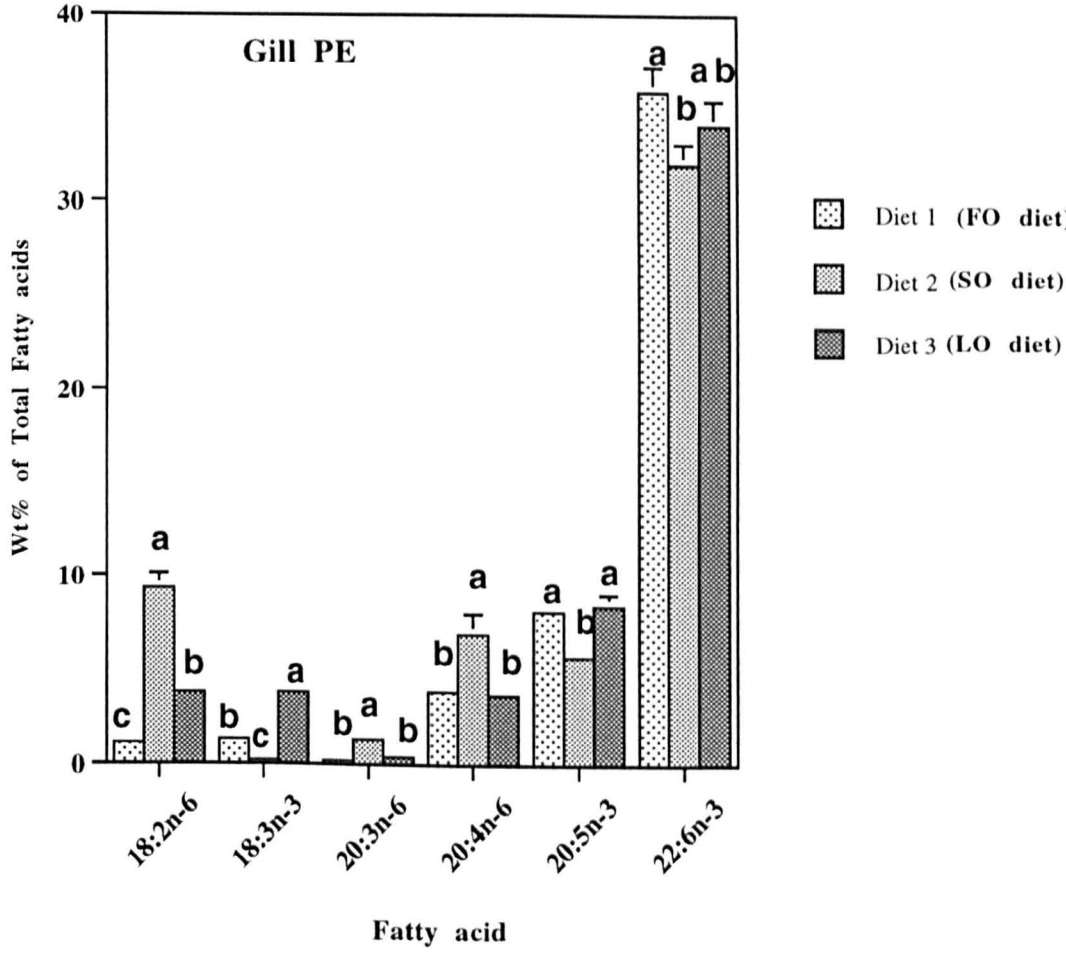
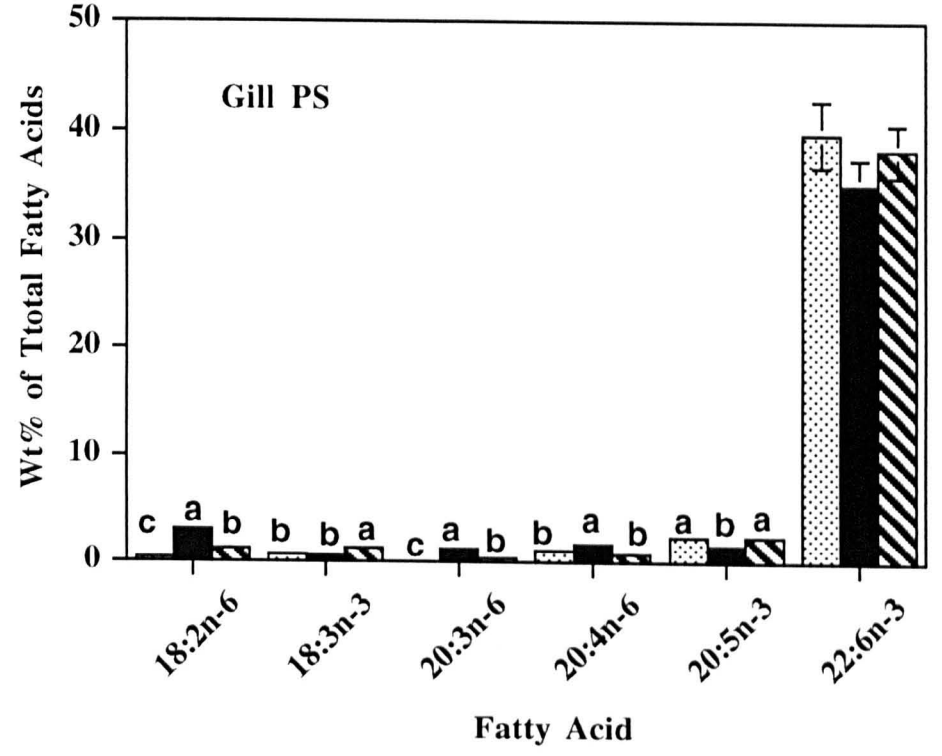
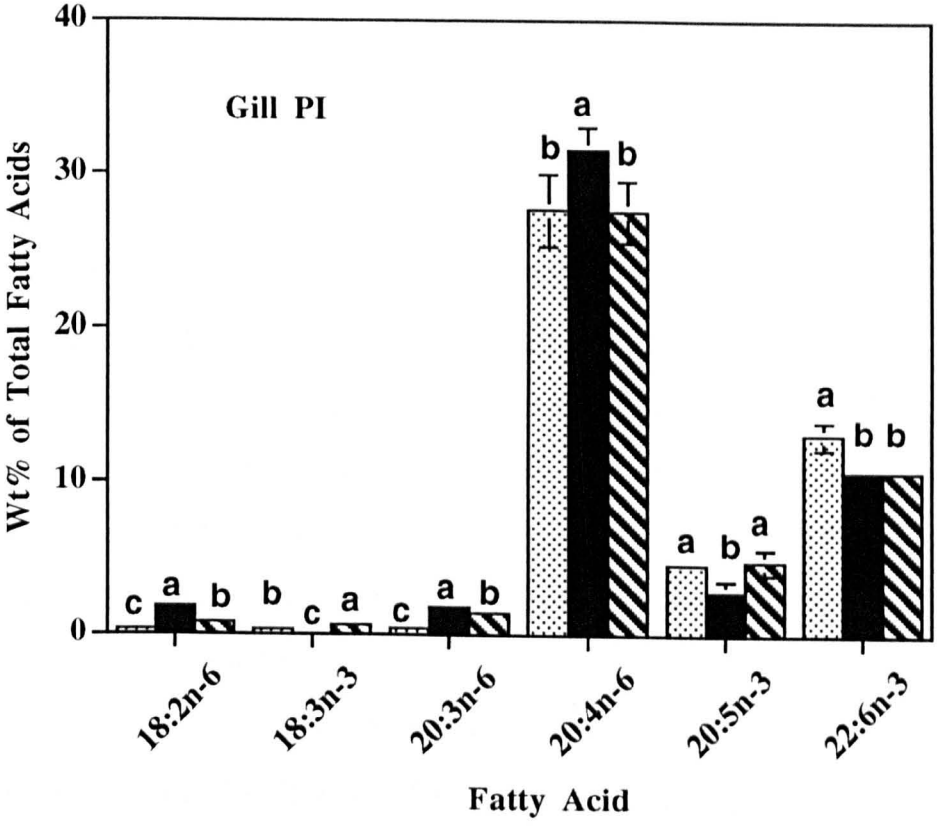

Gill PE
Wt% of Total Fatty acids
40
30
20
10
0
18:2n-6
18:3n-3
20:3n-6
20:4n-6
20:5n-3
22:6n-3
Fatty acid
Diet 1 (FO diet)
Diet 2 (SO diet)
Diet 3 (LO diet)

Fig. 5.6.2. Levels of the major polyunsaturated fatty acids of gill phosphatidyl serine (PS) and phosphatidylinositol (PI) from salmon fed diets containing fish oil (dotted bars), sunflower oil (filled bars) or linseed oil (hatched bars). Values are means ± SD for four fish per treatment. Values for each fatty acid having a different column letter are significantly different ($P < 0.05$).

Figure 5.6.2


Gill PS
Wt% of Ttotal Fatty Acids
50
40
30
20
10
0
c a b b b a c a b b a b a b a a
18:2n-6
18:3n-3
20:3n-6
20:4n-6
20:5n-3
22:6n-3
Fatty Acid


Gill PI
Wt% of Total Fatty Acids
40
30
20
10
0
c a b b c a c a b a b b a b a a b b
18:2n-6
18:3n-3
20:3n-6
20:4n-6
20:5n-3
22:6n-3
Fatty Acid

5.7. Summary

1. Atlantic salmon post-smolts were fed diets in which the lipid component was supplied either as fish oil (FO), sunflower oil (SO) or linseed oil (LO) for 12 weeks. Fish fed SO developed a marked cardiac histopathology which, while present in FO-fed fish, albeit in a less severe form, was virtually absent in fish fed LO. Fish fed SO had increased heart phospholipase A activity compared to those given either FO or LO. There were no significant differences between final weights of fish fed the three experimental diets.

2. The tissue phospholipids from fish fed SO had increased levels of 18:2n-6, 20:2n-6, 20:3n-6 and 20:4n-6 and liver PC, PE and PS also had elevated 22:4n-6 and 22:5n-6 compared to the other dietary treatments. This was reflected in a decreased n-3/n-6 PUFA ratio and an increased 20:4n-6/20:5n-3 eicosanoid precursor ratio in SO-fed fish. While the tissue phospholipids from fish fed LO had increased levels of 18:2n-6, 20:2n-6 and 20:3n-6, the levels of 20:4n-6, 22:4n-6 and 22:5n-6 were similar, or significantly reduced compared to fish fed FO. Tissue phospholipid compositions from fish fed LO also had increased 18:3n-3 and 20:4n-3 compared to both other treatments while in some tissues and phospholipid classes 20:5n-3 was increased compared to fish fed FO.

3. Fish fed LO had reduced PC in heart cell membranes compared to both other treatments, resulting in a PC:PE ratio of less than one.

4. Blood leucocytes stimulated with A23187 produced less TXB_2 in fish fed LO compared to those fed SO and PGE_2 production was reduced in LO-fed fish compared to both other treatments. In stimulated cardiac myocytes PGE_2 production was significantly increased in fish fed both SO and LO compared to those fed FO. Production of 12-lipoxygenase metabolites in isolated gill cells stimulated with A23187 were significantly reduced in fish fed both SO or LO compared to those fed FO. However, the ratio of 12-HETE/12-HEPE was significantly elevated in stimulated

gill cells from SO-fed fish compared to both other treatments.

Section 6. The incorporation and metabolism of polyunsaturated fatty acids in phospholipids of an established cell line derived from chum salmon (*Oncorhynchus keta)* heart.

6.1 Introduction

Recent studies with established fish cell lines have confirmed the metabolic pathways of fatty acid elongation and desaturation which have been observed in intact fish. Thus, cells derived from turbot fin have been demonstrated to be deficient in Δ5-desaturase while those derived from rainbow trout gonad have been capable of synthesising 22:6n-3 from added 18:3n-3 (Tocher, 1990; Tocher and MacKinlay, 1990). In the preceding studies with Atlantic salmon the development of a severe cardiomyopathy in fish fed sunflower oil rich in 18:2n-6 has been observed whereas lesions have been largely absent in fish given fish oil or linseed oil, which are rich in n-3 PUFA. A number of other cardiac abnormalities including epicarditis and haemopericarditis have also been observed in farmed Atlantic salmon (Ferguson *et al.* 1986; Raynard and Houghton, 1992).

While the preceding experiments, and similar studies in mammals (Swanson and Kinsella, 1986) demonstrate that dietary n-3/n-6 PUFA ratio can influence cardiac tissue fatty acid composition and subsequent alteration of membrane-bound enzyme activities (Leonardi *et al.* 1987), much less is known about the cellular mechanisms regulating uptake and intracellular metabolism of PUFA in cardiac cells. One study utilising isolated rat cardiac myocytes suggested that chain elongation and desaturation was not performed by myocytes indicating that the fatty acid composition of these cells was determined by selective uptake of PUFA which had been synthesised by other organs, probably the liver, and carried to the heart in the bloodstream (Hagve and Sprecher, 1989).

In cells which are routinely cultured, the only added lipid tends to be derived from

the serum supplememt, which is usually of mammalian origin. This results in cultured cells having a fatty acid composition which largely reflects the serum supplement (Spector *et al.* 1981). Fetal calf serum (FCS) is normally the serum of choice but fish cells cultured in FCS are generally deficient in n-3 PUFA and usually contain elevated concentrations of n-6 PUFA (Tocher *et al.* 1988). In this study the incorporation and metabolism of n-3 and n-6 PUFA was investigated in an established epithelial cell line (CHH-1) from chum salmon (*Oncorhynchus keta)* (Lannan *et al.* 1984). These cells had been derived from primary cultures of heart cells from juvenile chum salmon prepared as described by Wolf and Quimby (1976). The aim of the present experiments was to determine the pathways of desaturation and elongation present in these cardiac-derived cells.

6.2 Lipid class compositions of CHH-1 cells

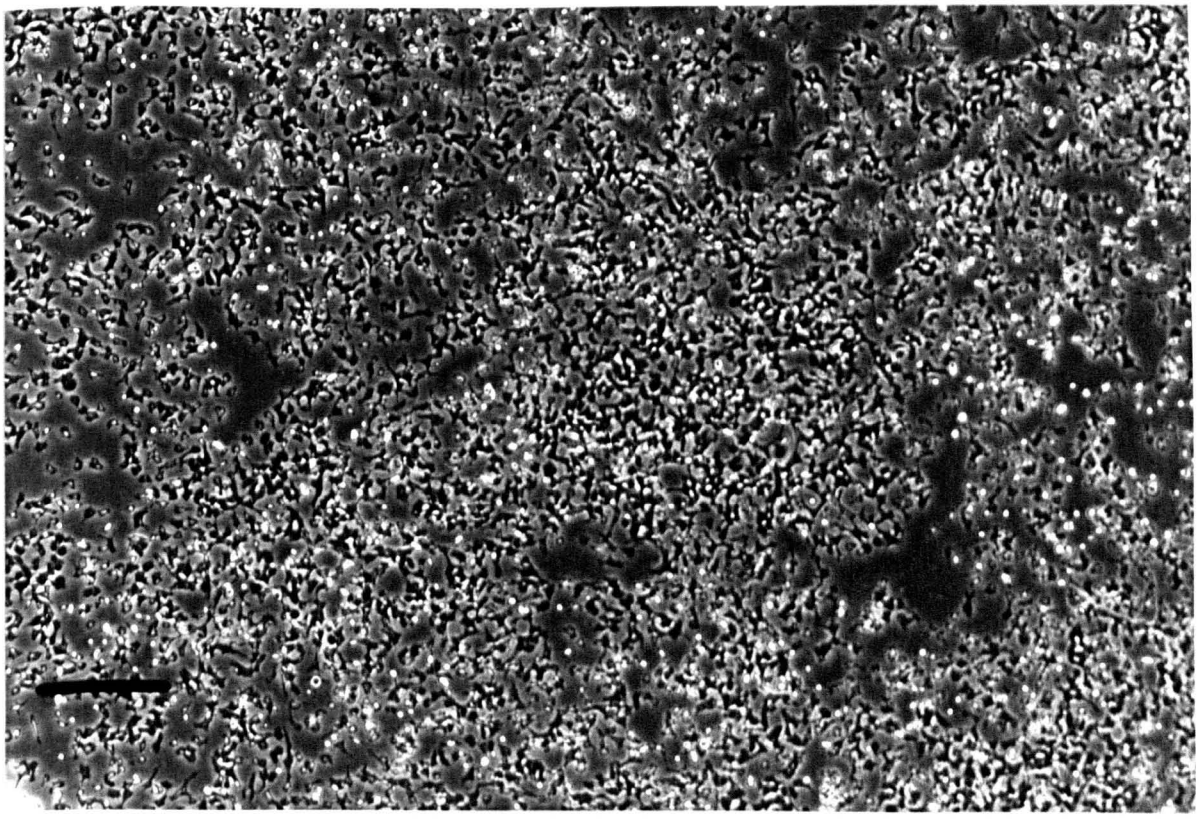
The lipid class compositions of CHH-1 cells grown in 10% FCS or 1% FCS plus various fatty acids at 25 μM are shown in Table 6.2.1. In all treatments, PC was the predominant phospholipid closely followed by PE which together accounted for approximately 40% of the total lipid. PS and PI were present in approximately equal amounts with PI always the more abundant followed by PA/CL and SM. In all cases polar lipid was in excess of neutral lipid. Some accumulation of neutral lipid was observed in cells supplemented with PUFA compared to FCS. Highest levels were in cells supplemented with 18:3n-3, 20:3n-6 and 22:6n-3 but microscopic examination of the cells indicated minimal presence of lipid droplets in all of the experimental treatments. Cells grew well and attained confluence in 5-7 days with all fatty acid supplements except 20:3n-6. Cells supplemented with 20:3n-6 did not attain confluence and after 6-7 days they began to detach from the flask (Figures 6.2.1 and 6.2.2). The supplementation with 20:3n-6 was repeated twice, using the same fatty acid concentration, and with two different sources of 20:3n-6. In all cases

Table 6.2.1. Lipid class compositions of CHH-1 cells cultured in 10% fetal calf serum (FCS) or 1% FCS supplemented with various fatty acids. (Values are weight % of total lipid).

	Lipid supplement							
Lipid Class	10% FCS	18:2n-6	18:3n-3	18:3n-6	20:3n-6	20:4n-6	20:5n-3	22:6n-3
Sphingomyelin	3.1 ± 0.4	3.5 ± 0.1	3.5 ± 0.1	3.6 ± 0.1	3.9 ± 0.5	3.9 ± 0.2	3.7 ± 0.1	3.6 ± 0.1
PC	23.4 ± 1.8^{ab}	22.9 ± 1.9^{ac}	20.1 ± 0.6^{a}	22.1 ± 1.6^{ac}	28.8 ± 0.0^{b}	27.4 ± 1.5^{ab}	23.6 ± 1.3^{ab}	24.0 ± 1.5^{ab}
PE	16.1 ± 1.0^{b}	17.6 ± 0.3^{ab}	16.1 ± 0.6^{b}	16.9 ± 1.1^{b}	18.1 ± 0.4^{ab}	19.9 ± 0.8^{a}	18.6 ± 0.5^{ab}	16.7 ± 1.0^{b}
PS	9.1 ± 0.6^{a}	6.6 ± 0.1^{bc}	6.2 ± 0.2^{bc}	6.5 ± 0.0^{bc}	4.7 ± 0.2^{d}	7.1 ± 0.8^{bc}	7.3 ± 0.1^{b}	5.7 ± 0.1^{cd}
PI	11.7 ± 0.9^{a}	8.9 ± 0.4^{b}	7.4 ± 0.2^{bc}	7.2 ± 0.1^{bc}	6.8 ± 0.9^{bc}	7.8 ± 0.6^{bc}	7.8 ± 0.0^{bc}	6.3 ± 0.3^{c}
PA/CL	4.3 ± 0.2	4.4 ± 0.0	3.9 ± 0.6	3.7 ± 0.1	4.3 ± 0.5	3.6 ± 0.2	4.0 ± 0.1	4.0 ± 0.2
Total polar lipid	67.5 ± 5.0^{ac}	63.8 ± 1.1^{ab}	57.1 ± 0.6^{b}	59.7 ± 2.6^{bc}	66.4 ± 1.5^{ab}	69.5 ± 0.6^{a}	64.8 ± 0.7^{ab}	60.2 ± 3.0^{ab}
Triacylglycerol	5.5 ± 0.1^{a}	8.8 ± 0.3^{b}	12.3 ± 0.1^{c}	11.7 ± 1.9^{bc}	12.6 ± 1.3^{c}	9.0 ± 0.1^{b}	6.4 ± 0.4^{ab}	16.3 ± 0.1^{d}
Free fatty acid	0.7 ± 0.5^{a}	1.0 ± 0.6^{ab}	3.7 ± 0.2^{c}	3.3 ± 0.6^{bc}	5.0 ± 0.1^{cd}	5.4 ± 0.2^{cd}	6.3 ± 0.2^{de}	7.6 ± 0.9^{e}
Diacylglycerol	0.3 ± 0.0^{a}	1.0 ± 0.6^{ab}	3.2 ± 0.4^{bc}	2.7 ± 0.1^{bc}	1.9 ± 0.5^{b}	3.3 ± 0.1^{bc}	3.7 ± 0.2^{d}	3.0 ± 0.5^{bcd}
Cholesterol	21.3 ± 0.9^{a}	23.6 ± 1.8^{a}	21.3 ± 1.3^{a}	21.0 ± 0.9^{a}	14.1 ± 1.8^{bc}	12.8 ± 1.0^{c}	18.7 ± 0.9^{ab}	13.1 ± 1.9^{c}
Total neutral lipid	27.6 ± 1.3^{a}	34.6 ± 0.1^{bcd}	40.4 ± 0.6^{d}	38.6 ± 1.8^{cd}	33.6 ± 1.2^{bc}	30.5 ± 0.8^{ab}	34.9 ± 1.3^{bcd}	39.8 ± 3.2^{d}

Values are mean ± SD for 3 experiments. Values in the same row having different superscript letters are significantly different ($P < 0.05$). PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA/CL, phosphatidic acid/cardiolipin.

Figure 6.2.1 Low magnification light micrograph of CHH-1 cells grown with 25μM 20:3n-6 (top) and 20:5n-3 supplements (bottom) after 6 days in culture. Note that cells grown with 20:5n-3 have nearly attained confluence whereas those with 20:3n-6 are not confluent. The brighter spots on the 20:3n-6-supplemented flask probably indicate dying cells about to detach from the flask. Scale bar = 100 μm.



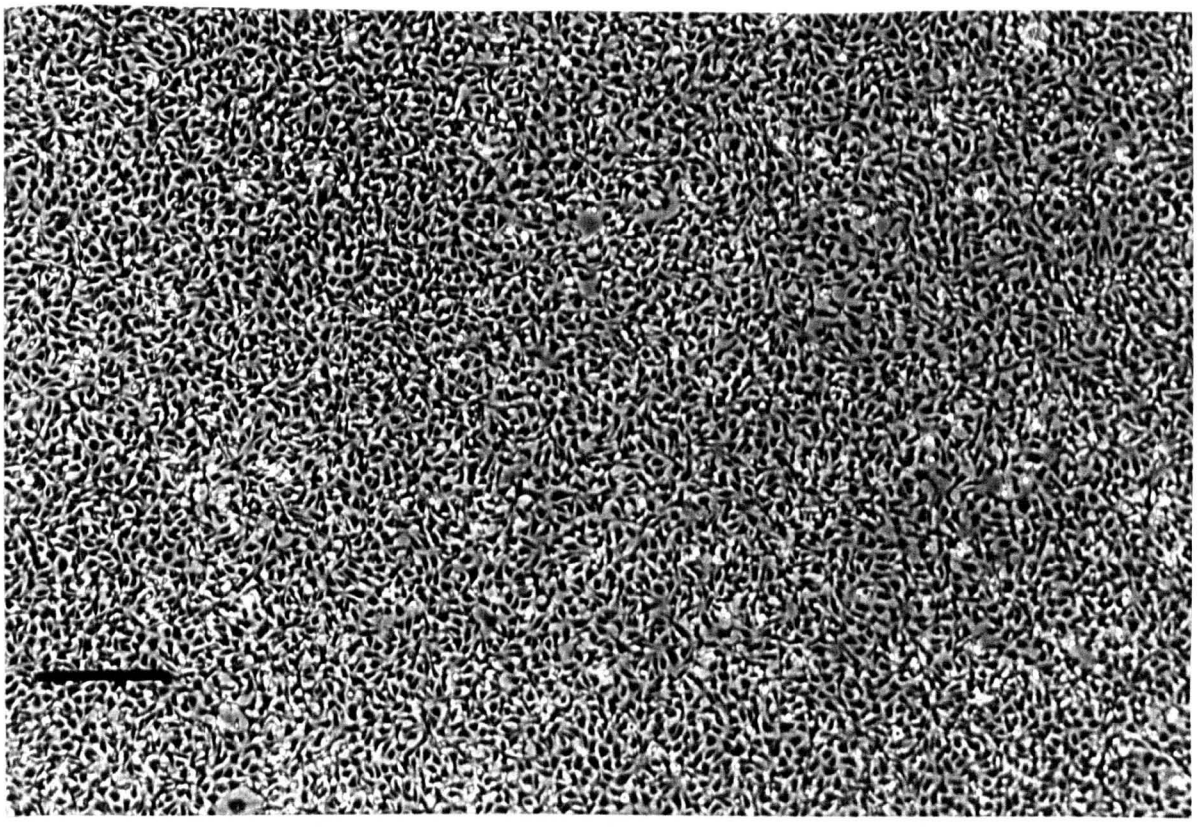
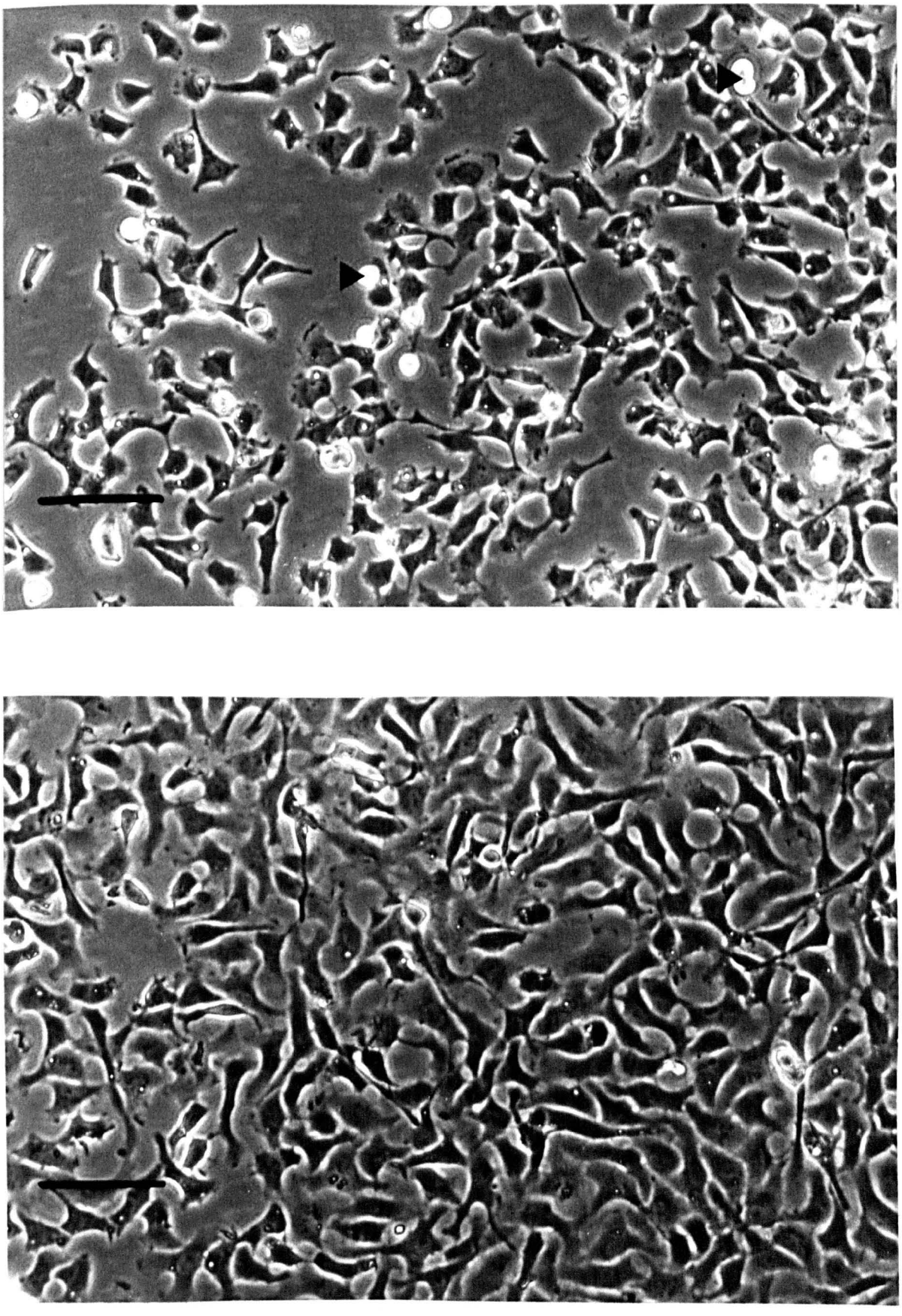


Figure 6.2.2. High magnification light micrograph of CHH-1 cells grown with 20:3n-6 (top) and 20:5n-3 supplements (bottom) after 6 days in culture. Note characteristic phase-bright cells (arrowed) perhaps indicative of dead cells detaching from the flask and lower cell density in cultures supplemented with 20:3n-6. Scale bar = 50 μm.



the same effect on cell growth was recorded.

6.3 Fatty acid compositions of phospholipid classes of CHH-1 cells cultured in medium containing 10% FCS or 1% FCS plus 25 μM PUFA

The fatty acid compositions of phospholipid classes of cells cultured in 10% FCS are shown in Table 6.3.1. The n-9 series PUFA are dominant in all phospholipid classes, except PI, where n-6 series PUFA predominate. 18:2n-9 is the predominant n-9 PUFA in all phospholipid classes except PI where 20:2n-9 predominates. 20:3n-9, which could not be separated from 20:2n-6 on this chromatographic system, did not accumulate in CHH-1 cells. The n-6 PUFA were generally more abundant than n-3 PUFA, except in PS, and the major n-6 fatty acids were 20:4, 18:2 and 20:2. The major n-3 PUFA were 22:5 and 22:6. The highest PUFA levels were in PI with the lowest in PC, but in all classes the monoenes, especially 18:1n-9, and the saturates, especially 16:0 and 18:0, formed the main bulk of phospholipid fatty acids.

In the following tables (6.3.2-6.3.8) the fatty acid compositions are compared to those in Table 6.3.1. The n-6 PUFA composition of phospholipid classes from CHH-1 cells supplemented with 18:2n-6 is shown in Table 6.3.2, where the total n-6 PUFA content of all phospholipid classes is significantly increased (compared to those grown in 10% FCS, Table 6.3.1). Apart from 18:2n-6, there were significant increases in the Δ6-desaturation product 18:3n-6 and the elongation product 22:3n-6, in PC and PE. 20:2n-6 and 20:3n-6 were significantly increased in all phospholipid classes. 20:3n-6 showed the greatest increase, particularly in PI. The highest percentage of 18:2n-6 was observed in PE.

The n-3 PUFA composition of phospholipid classes from CHH-1 cells supplemented with 18:3n-3 is shown in Table 6.3.3. Supplementing the media with 25 μM 18:3n-3 significantly increased the total n-3 PUFA content of all phospholipid classes with the highest levels being found in PE. The Δ6-desaturase product,

Table 6.3.1. Fatty acid compositions of phospholipid classes of CHH-1 cells cultured in medium containing 10% fetal calf serum.

Fatty acid	PC	PE	PS	PI
		(Weight %)		
14:0	1.5 ± 0.1	0.3 ± 0.0	0.8 ± 0.1	0.4 ± 0.0
16:0	18.8 ± 0.2	5.0 ± 0.2	9.8 ± 0.1	4.4 ± 0.1
18:0	6.5 ± 0.1	5.1 ± 0.3	26.4 ± 1.4	19.2 ± 1.7
Total saturates[1]	27.6 ± 0.3	10.8 ± 0.4	37.7 ± 1.4	25.0 ± 1.5
16:1n-9	5.2 ± 0.2	3.3 ± 0.0	3.1 ± 0.1	0.8 ± 0.1
16:1n-7	4.9 ± 0.0	3.2 ± 0.2	2.8 ± 0.1	1.1 ± 0.1
18:1n-9	44.2 ± 1.0	36.2 ± 0.4	25.1 ± 0.3	25.8 ± 0.9
18:1n-7	3.1 ± 0.1	2.9 ± 0.1	1.7 ± 0.1	2.2 ± 0.5
20:1n-9	0.8 ± 0.4	0.5 ± 0.4	1.0 ± 0.1	0.4 ± 0.0
24:1	0.7 ± 0.2	0.5 ± 0.1	0.9 ± 0.4	1.0 ± 0.5
Total monoenes	58.9 ± 0.5	46.6 ± 0.4	34.6 ± 0.6	31.3 ± 0.9
18:2n-9	4.3 ± 0.2	5.3 ± 0.1	5.6 ± 0.0	2.6 ± 0.3
20:2n-9	3.8 ± 0.1	4.3 ± 0.1	4.6 ± 0.1	11.1 ± 0.4
Total n-9	8.1 ± 0.3	9.6 ± 0.0	10.2 ± 0.2	13.7 ± 0.7
18:2n-6	0.6 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.7 ± 0.0
18:3n-6	0.2 ± 0.1	0.1 ± 0.1	0.7 ± 0.4	0.5 ± 0.3
20:2n-6[2]	0.3 ± 0.0	1.5 ± 0.1	0.3 ± 0.0	3.6 ± 0.1
20:3n-6	0.3 ± 0.0	0.5 ± 0.0	0.6 ± 0.2	2.1 ± 0.2
20:4n-6	0.4 ± 0.1	4.5 ± 0.2	0.6 ± 0.1	7.8 ± 1.0
22:2n-6	-	0.3 ± 0.1	0.4 ± 0.0	0.5 ± 0.2
22:4n-6	-	0.4 ± 0.0	0.6 ± 0.1	0.5 ± 0.1
22:5n-6	0.4 ± 0.3	0.4 ± 0.2	0.7 ± 0.1	0.4 ± 0.0
Total n-6	2.2 ± 0.4	8.7 ± 0.2	4.9 ± 0.3	16.1 ± 1.5
18:3n-3	t	t	0.1 ± 0.0	0.1 ± 0.0
18:4n-3	t	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:5n-3	0.1 ± 0.0	1.4 ± 0.0	0.1 ± 0.0	0.8 ± 0.3
22:5n-3	0.3 ± 0.0	2.8 ± 0.0	3.6 ± 0.5	4.2 ± 0.2
22:6n-3	0.2 ± 0.0	4.2 ± 0.2	2.2 ± 0.3	3.7 ± 0.8
Total n-3	0.6 ± 0.0	8.5 ± 0.2	6.2 ± 0.7	9.0 ± 1.2
Total PUFA	10.9 ± 0.6	26.8 ± 0.4	21.3 ± 0.6	38.8 ± 0.5
n-3/n-6	0.3 ± 0.1	1.0 ± 0.1	1.4 ± 0.4	0.6 ± 0.2
Total dimethyl acetals	0.4 ± 0.0	13.3 ± 0.7	-	-

Data are means ± SD of triplicate experiments. [1]includes 15:0, 17:0 and 20:0. [2]Includes 20:3n-9. t = trace value < 0.05%. - = not detected.

18:4n-3 was significantly increased in all phospholipid classes as were the elongation products 20:3n-3 and 20:4n-3, with the latter being the main metabolite of 18:3n-3. The Δ5-desaturation product 20:5n-3 was significantly increased in all phospholipid classes with the biggest increase occurring in PI. The elongation and desaturation products of 20:5n-3 were significantly decreased in PS, PI and PE.

The n-6 PUFA compositions of phospholipid classes from CHH-1 cells supplemented with 18:3n-6 are shown in Table 6.3.4. The increase in total n-6 PUFA resulting from 18:3n-6 supplementation was generally greater than that for 18:2n-6 with the exception of PE. PC showed the highest incorporation of 18:3n-6 and its metabolites including a significant increase in the Δ5-desaturation product, 20:4n-6. The major metabolite was the elongation product, 20:3n-6 which showed greatest incorporation into PI but was also significantly increased in all phospholipids. The incorporation of the elongation product 22:3n-6 was significantly increased in all phospholipid classes, especially PE.

The n-6 PUFA compositions of phospholipid classes from CHH-1 cells supplemented with 20:3n-6 are shown in Table 6.3.5. The increase in total n-6 PUFA was of a similar magnitude to that achieved with 18:3n-6 with the highest incorporation into PC. PC showed a significant increase in the Δ5-desaturation product 20:4n-6 but the same fatty acid was significantly reduced in PE. All classes had significantly increased levels of the elongation product 22:3n-6. All phospholipid classes showed significantly increased 18:3n-6 demonstrating the presence of chain shortening activity.

The n-6 PUFA compositions of phospholipid classes from CHH-1 cells supplemented with 20:4n-6 are shown in Table 6.3.6. Supplemented 20:4n-6 generated total n-6 PUFA levels similar to 20:3n-6 in all phospholipid classes but was preferentially incorporated into PI. Chain elongation to 22:4n-6 was extensive with maximum incorporation into PS. No Δ4-desaturation and elongation to 22:5n-6

was observed.

The n-3 PUFA compositions of phospholipid classes from CHH-1 cells supplemented with 20:5n-3 are shown in Table 6.3.7. Incorporation of 20:5n-3 was greatest into PI although, as a result of extensive elongation to 22:5n-3, the highest levels of total n-3 PUFA were in PE. The incorporation of 22:5n-3 was greatest in PE and PS where it comprised 70 and 91% of the total n-3 PUFA respectively. There was no evidence of Δ4-desaturation and elongation to 22:6n-3.

The n-3 PUFA compositions of phospholipid classes from CHH-1 cells supplemented with 22:6n-3 are shown in Table 6.3.8. Supplementation with 22:6n-3 produced levels of total n-3 PUFA similar to those supplemented with 20:5n-3. Incorporation of 22:6n-3 was particularly high in PE and PS (93 and 92% of total n-3 PUFA respectively) while increased 20:5n-3 in all phospholipid classes suggested that significant retroconversion of 22:6n-3 was occurring.

Table 6.3.2. n-6 PUFA compositions of phospholipid classes from CHH-1 cells cultured in medium supplemented with 18:2n-6.

n-6 PUFA	PC	PS	PI	PE
18:2	23.8 ± 1.5*	19.7 ± 1.5*	17.2 ± 1.1*	34.4 ± 0.2*
18:3	0.8 ± 0.1*	1.0 ± 0.1	0.7 ± 0.1	0.9 ± 0.0*
20:2	0.8 ± 0.1*	1.4 ± 0.3*	5.1 ± 0.2*	1.9 ± 0.9*
20:3	1.2 ± 0.2*	3.7 ± 0.5*	11.8 ± 0.7*	1.8 ± 0.1*
20:4	0.2 ± 0.1	0.5 ± 0.2	5.2 ± 0.8*	1.3 ± 0.2*
22:2	0.2 ± 0.0*	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.2
22:3	0.1 ± 0.0*	0.6 ± 0.2	0.2 ± 0.2	0.3 ± 0.1*
22:4	t	0.2 ± 0.0*	0.1 ± 0.1*	0.2 ± 0.0
22:5	0.2 ± 0.1	0.2 ± 0.1*	0.2 ± 0.1*	t
Total	27.3 ± 1.8*	27.7 ± 1.0*	40.9 ± 2.6*	41.4 ± 0.4*

Data are shown as means ± SD of triplicate experiments, results are expressed as % of total fatty acids. t = trace value < 0.05%. SD of < 0.05 are tabulated as 0.0. *Values are significantly different ($P < 0.05$) compared to cells cultured in 10% FCS.

Table 6.3.3. n-3 PUFA compositions of phospholipid classes from CHH-1 cells cultured in medium supplemented with 18:3n-3.

n-3 PUFA	PC	PS	PI	PE
18:3	24.9 ± 2.3*	21.0 ± 0.8*	12.5 ± 1.0*	30.1 ± 0.4*
18:4	1.4 ± 0.1*	1.4 ± 0.1*	0.7 ± 0.0*	1.3 ± 0.1*
20:3	2.0 ± 0.5*	1.8 ± 0.3*	2.4 ± 0.2*	2.8 ± 0.6*
20:4	3.2 ± 0.5*	4.2 ± 0.2*	9.4 ± 0.1*	5.3 ± 0.8*
20:5	0.3 ± 0.1*	0.5 ± 0.1*	4.5 ± 0.8*	2.6 ± 0.0*
22:5	0.2 ± 0.0	1.8 ± 0.3*	1.2 ± 0.3*	1.4 ± 0.1*
22:6	0.1 ± 0.0	1.3 ± 0.2*	0.7 ± 0.3*	1.2 ± 0.1*
Total	32.1 ± 3.4*	32.0 ± 0.9*	31.0 ± 1.3*	44.7 ± 1.9*

Data are shown as means ± SD of triplicate experiments, results are expressed as % of total fatty acids. t = trace value < 0.05%. SD of < 0.05 are tabulated as 0.0. *Values are significantly different ($P < 0.05$) compared to cells cultured in 10% FCS.

Table 6.3.4. n-6 PUFA composition of phospholipid classes from CHH-1 cells cultured in medium supplemented with 18:3n-6.

n-6 PUFA	PC	PS	PI	PE
18:2	0.4 ± 0.1	0.5 ± 0.0*	0.5 ± 0.1*	0.5 ± 0.1
18:3	27.4 ± 0.3*	22.9 ± 0.1*	12.7 ± 0.9*	18.0 ± 2.4*
20:2	0.7 ± 0.0*	0.3 ± 0.0	3.6 ± 0.4	1.4 ± 0.3
20:3	14.3 ± 0.4*	6.1 ± 0.0*	16.3 ± 0.2*	10.4 ± 1.0*
20:4	1.3 ± 0.0*	0.8 ± 0.2	6.5 ± 0.4	2.9 ± 0.4*
22:2	0.2 ± 0.0*	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1*
22:3	1.9 ± 0.4*	0.9 ± 0.0*	1.8 ± 0.2*	3.1 ± 0.5*
22:4	0.2 ± 0.0	0.5 ± 0.0	0.4 ± 0.1	0.6 ± 0.1*
Total	46.4 ± 0.2*	32.4 ± 0.4*	42.3 ± 1.9*	37.5 ± 4.5*

Footnotes as described in Table 6.3.3.

Table 6.3.5. n-6 PUFA compositions of phospholipid classes from CHH-1 cells cultured in medium supplemented with 20:3n-6.

n-6 PUFA	PC	PS	PI	PE
18:2	0.4 ± 0.0*	0.7 ± 0.1*	0.4 ± 0.0*	0.4 ± 0.1*
18:3	2.7 ± 0.1*	2.0 ± 0.1*	1.4 ± 0.1*	1.2 ± 0.1*
20:2	1.0 ± 0.1*	0.7 ± 0.1*	5.6 ± 0.4*	2.0 ± 0.1*
20:3	30.5 ± 1.9*	19.2 ± 1.1*	26.3 ± 0.4*	22.3 ± 0.5*
20:4	2.0 ± 0.3*	0.8 ± 0.2	7.8 ± 1.3	3.0 ± 0.2*
22:2	0.2 ± 0.0*	0.6 ± 0.1*	0.4 ± 0.1	0.7 ± 0.2*
22:3	4.5 ± 0.3*	4.6 ± 0.3*	3.4 ± 0.2*	9.7 ± 1.1*
22:4	0.3 ± 0.1*	0.7 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
Total	41.6 ± 1.3*	29.3 ± 1.7*	45.7 ± 1.4*	39.8 ± 1.1*

Data are shown as means ± SD of triplicate experiments, results are expressed as % of total fatty acids. t = trace value < 0.05%. SD of < 0.05 are tabulated as 0.0. *Values are significantly different ($P < 0.05$) compared to cells cultured in 10% FCS.

Table 6.3.6. n-6 PUFA compositions of phospholipid classes from CHH-1 cells cultured in medium supplemented with 20:4n-6

n-6 PUFA	PC	PS	PI	PE
18:2	0.7 ± 0.3	1.0 ± 0.4	0.6 ± 0.0	0.4 ± 0.0*
18:3	0.7 ± 0.1*	0.8 ± 0.3	0.8 ± 0.3	0.5 ± 0.1*
20:2	0.4 ± 0.1	t	1.7 ± 0.4*	0.7 ± 0.1*
20:3	1.2 ± 0.2*	0.4 ± 0.0	0.8 ± 0.1*	0.9 ± 0.3*
20:4	12.5 ± 0.3*	2.2 ± 0.5*	23.6 ± 1.6*	13.3 ± 0.1*
22:2	0.2 ± 0.0*	0.2 ± 0.0*	0.2 ± 0.1	0.2 ± 0.0
22:3	0.2 ± 0.1*	0.2 ± 0.0*	0.2 ± 0.0*	0.2 ± 0.0*
22:4	21.5 ± 1.2*	26.8 ± 2.4*	14.7 ± 0.8*	22.7 ± 1.8*
22:5	0.1 ± 0.0	0.1 ± 0.1*	t*	0.3 ± 0.1
Total	37.5 ± 1.4*	35.3 ± 2.8*	42.6 ± 1.8*	39.2 ± 1.9*

Footnotes as descreibed in Table 6.3.5.

Table 6.3.7. n-3 PUFA compositions of phospholipid classes from CHH-1 cells cultured in medium supplemented with 20:5n-3

n-3 PUFA	PC	PS	PI	PE
18:3	t	t	-	-
18:4	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	-
20:3	t	t	0.3 ± 0.1*	t
20:4	0.5 ± 0.1*	0.4 ± 0.1*	0.3 ± 0.1*	0.3 ± 0.0*
20:5	11.0 ± 0.3*	1.6 ± 0.3*	14.8 ± 1.1*	9.7 ± 0.1*
22:5	26.3 ± 0.3*	34.6 ± 0.2*	23.4 ± 1.0*	30.7 ± 0.7*
22:6	0.5 ± 0.0*	1.3 ± 0.1*	1.0 ± 0.1*	2.9 ± 0.1*
Total	38.4 ± 0.6*	38.0 ± 0.2*	39.9 ± 0.3*	43.6 ± 0.7*

Data are shown as means ± SD of triplicate experiments, results are expressed as % of total fatty acids. t = trace value < 0.05%. SD of < 0.05 are tabulated as 0.0. *Values are significantly different ($P < 0.05$) compared to cells cultured in 10% FCS.

Table 6.3.8 n-3 PUFA compositions of phospholipid classes from CHH-1 cells cultured in medium supplemented with 22:6n-3

n-3 PUFA	PC	PS	PI	PE
18:3	-	-	0.1 ± 0.0	-
18:4	-	-	-	-
20:3	-	0.3 ± 0.1*	0.3 ± 0.1*	0.3 ± 0.1*
20:4	0.1 ± 0.0*	t	t	0.1 ± 0.0*
20:5	5.2 ± 1.0*	1.2 ± 0.0*	3.8 ± 0.2*	2.0 ± 0.2*
22:5	1.9 ± 0.2*	1.8 ± 0.0*	1.8 ± 0.0*	1.1 ± 0.1*
22:6	27.2 ± 5.4*	37.2 ± 2.6*	33.3 ± 1.1*	44.0 ± 1.5*
Total	34.4 ± 6.5*	40.5 ± 2.5*	40.2 ± 1.5*	47.5 ± 1.0*

Footnotes as described in Table 6.3.7.

6.4 Summary

1. Supplementing media with 25 μM fatty acid considerably altered the fatty acid compositions of CHH-1 cells but had only minor affects on the lipid class composition and did not result in the appearance of cytoplasmic lipid droplets.

2. CHH-1 cells supplemented with 20:3n-6 showed reduced growth rate, did not achieve confluence and had an increased incidence of cell death, compared to cells supplemented with other PUFA.

3. CHH-1 cells exhibited considerable Δ6-desaturase activity but showed no preference for n-3 PUFA over n-6 PUFA.

4. CHH-1 cells also contain significant Δ5-desaturase activity which showed a preference towards n-3 PUFA. However, Δ4-desaturation activity was totally absent.

5. Elongation of C_{20} PUFA was especially active in CHH-1 cells with C_{22} PUFA being specifically incorporated into PE and PS.

6. The C_{20} PUFA were specifically incorporated into PI.

7. Supplementation with 22:6n-3 generated fatty acid compositions most closely resembling those in salmonid cardiac tissue. Substantial chain shortening of 22:6n-3 to 20:5n-3 was observed.

Section 7; Comparison of lipid compositions of heart and liver from wild and farmed Atlantic salmon parr.

7.1 Introduction

In the previous dietary studies we have shown that the composition of individual membrane phospholipids can be altered profoundly by feeding different dietary lipids. We felt it would be of interest to examine the lipids of wild Atlantic salmon to compare the heart and liver compositions with those found in farmed fish. However, the wild adult salmon caught by coastal netting are not a good comparison with farmed fish since they will not have been actively feeding for some months while undergoing their spawning migration. Therefore, since actively feeding wild post-smolts are extremely difficult to obtain we decided to compare the compositions of wild parr and pre-smolts with their farmed equivalents.

It has been established that, while farmed salmonid parr may appear outwardly healthy, they may be different in terms of their lipid metabolic activities, when compared to wild fish found within the same watershed. A study comparing wild and farmed salmon undergoing smoltification has revealed that the wild fish contain only one quarter of the carcass lipid compared to equivalent farmed fish (Ackman and Takeuchi, 1986). In addition , the wild fish contained higher levels of AA compared to their farmed counterparts despite the latter containing higher levels of 18:2n-6, the precursor of AA formation. The farmed fish also developed dermal lesions resulting in erosion of the dorsal, pectoral and caudal fins.

The seaward migration of anadromous salmonid juveniles is accompanied by profound changes in morphology, behaviour and physiology known as parr-smolt-transformation (Folmar and Dickhoff, 1980). The process involves activation of neuroendocrine and endocrine systems which result in biochemical and molecular reorganisation within the tissues which prepares the fish, while still in freshwater, for life in the marine environment (Hoar, 1976; Wedemeyer *et al.* 1980). This is an

extremely stressful period in the salmonid life cycle, in some ways similar to metamorphosis, which renders the fish susceptible to poor husbandry, disease and nutritional inadequacy (Folmar and Dickhoff, 1980). Prostaglandins are known to mediate fluid and electrolyte fluxes in fish gill and kidney and are important in adaptation to changes in salinity (Brown and Bucknall, 1986; Mustafa and Srivastava, 1989). In addition, a dietary deficiency of essential PUFA can decrease ionic permeability in trout brush border membranes (Di Constanzo *et al.* 1983) and causes destruction of gill epithelia in turbot (Bell *et al.* 1985a and b).

In this experiment the lipid compositions of heart and liver from Atlantic salmon parr collected by electrofishing in October, and from pre-smolts collected from smolt traps in April, both from tributaries of the River Dee, Grampian, Scotland, were compared with farmed salmon collected at the same time points.

7.2 Lipid compositions of hearts and livers from wild and farmed salmon parr

The lipid class compositions of heart from wild and farmed salmon parr are shown in Table 7.2.1. The level of PC, PS, PI, PA/CL, PE, total polar lipid, cholesterol and free fatty acid were all significantly greater in wild fish compared to farmed. The increased level of polar lipids were compensated in farmed salmon parr by a significantly elevated (almost 2-fold) triacylglycerol concentration.

The fatty acid compositions of PC, PS, PI, PE and TAG from hearts of wild and farmed salmon parr are shown in table 7.2.2. In PC there was little difference between wild and farmed fish in terms of saturated and monounsaturated fatty acids but considerable differences in PUFA content. Wild parr heart PC contained considerably more 18:2n-6, AA, 18:3n-3, EPA and 22:5n-3 but much less DHA compared to farmed hearts.

Table 7.2.1. Lipid class compositions of hearts from wild and farmed Atlantic salmon parr

Lipid Class	Wild parr	Farmed parr
	(Weight %)	
Lyso phosphatidylcholine	0.1 ± 0.0	0.1 ± 0.1
Sphingomyelin	1.5 ± 0.1	1.5 ± 0.0
Phosphatidylcholine	14.5 ± 0.6	11.0 ± 0.2*
Phosphatidylserine	2.6 ± 0.2	1.2 ± 0.1*
Phosphatidylinositol	2.3 ± 0.1	1.6 ± 0.1*
Phosphatidic acid/Cardiolipin	5.4 ± 0.9	3.5 ± 0.3*
Phosphatidylethanolamine	13.6 ± 0.9	7.8 ± 0.3*
Total polar lipid	40.1 ± 2.1	26.7 ± 0.8*
Cholesterol	17.5 ± 1.2	12.5 ± 0.3*
Free fatty acid	4.5 ± 2.3	1.7 ± 0.1*
Triacylglycerol	21.2 ± 4.6	40.5 ± 3.1*
Sterol ester	13.3 ± 1.8	16.7 ± 2.5
Total neutral lipid	56.6 ± 2.1	71.5 ± 0.5

Values represent mean ± SD from 3 samples of wild and farmed parr where each sample comprised hearts from 2 fish. *Values are significantly different ($P < 0.05$).

Wild parr heart PS contained more saturated fatty acids (mainly 16:0), AA, 18:3n-3 and EPA but less 22:5n-3 and DHA compared to farmed hearts, whereas wild parr heart PI and PE had broadly similar compositions to those of farmed parr. Triacylglycerol composition of wild parr hearts contained more 18:2n-6, 18:3n-3 and AA but considerably less 20:1n-9, 22:1n-11and DHA compared to farmed salmon hearts.

The lipid class compositions of livers from wild and farmed salmon parr are shown in Table 7.2.3. The liver lipid class compositions were very similar in both wild and farmed parr with the only significant differences being reduced free fatty acids and increased sterol esters in wild compared to farmed parr.

The fatty acid compositions of individual phospholipids and TAG in livers from wild and farmed parr are shown in Table 7.2.4. The differences in fatty acid compositions are broadly similar to those found in hearts. Farmed fish contained higher levels of 20:1n-9 and 22:1n-11but reduced levels of 18:2n-6 and 18:3n-3 compared to wild fish. In the long-chain PUFA, AA and EPA were increased in livers of wild fish whereas DHA was reduced compared to farmed fish. These changes in composition resulted in a considerably higher n-3/n-6 PUFA ratio in farmed parr.

Table 7.2.2. The fatty acid compositions of individual phospholipids and triacylglycerol from hearts of wild and farmed Atlantic salmon parr (Values are weight % of total fatty acids).

Fatty acid	Wild PC	Farmed PC	Wild PS	Farmed PS	Wild PI	Farmed PI	Wild PE	Farmed PE	Wild TAG	Farmed TAG
14:0	0.7	1.6	0.4	0.2	0.1	0.2	0.2	0.3	2.7	4.8
16:0	28.7	29.4	11.6	5.3	5.9	7.3	7.6	6.9	19.6	15.3
18:0	4.7	2.0	15.3	12.6	24.1	25.7	6.8	6.4	7.4	3.9
Total sats.[1]	34.7	33.2	28.3	18.8	30.2	33.4	15.2	13.9	29.9	25.9
16:1n-7	3.9	1.3	3.2	0.6	1.8	2.4	1.9	1.3	11.8	6.4
18:1n-9	9.0	7.5	6.8	4.6	5.2	5.3	5.1	5.1	14.6	17.2
18:1n-7	3.1	1.6	2.5	2.8	3.7	3.3	4.3	3.8	7.1	4.0
20:1n-9	0.5	1.6	1.0	2.7	0.2	2.1	0.2	3.0	t	9.1
22:1n-11	-	0.8	-	0.7	-	0.1	-	t	-	8.8
24:1	0.5	0.9	1.2	0.5	1.4	1.2	0.4	0.5	3.4	0.9
Total monos.[2]	17.0	13.7	14.7	11.9	12.3	14.4	11.9	8.3	36.9	47.8
18:2n-6	2.0	1.1	1.6	1.3	1.4	1.5	2.8	3.1	5.8	4.1
20:2n-6	0.5	0.3	0.2	0.2	0.6	0.2	0.4	0.3	0.2	0.1
20:4n-6	4.5	1.6	7.9	2.6	24.0	23.3	4.7	4.5	1.4	0.5
Total n-6[3]	7.0	3.0	10.2	4.1	26.6	25.2	8.1	8.3	9.0	4.7
18:3n-3	4.5	0.5	1.4	0.3	1.8	1.7	3.0	0.4	5.3	1.0
20:4n-3	0.4	0.6	0.5	0.9	0.4	0.2	0.7	0.6	t	0.9
20:5n-3	12.0	7.4	7.6	4.4	7.4	6.4	6.4	6.4	2.7	2.4
22:5n-3	2.1	1.4	1.9	3.8	1.7	1.6	4.8	4.6	1.5	1.3
22:6n-3	20.8	34.8	27.0	50.6	12.2	10.8	35.7	36.3	3.3	9.9
Total n-3[4]	40.0	44.7	38.4	60.0	23.5	20.7	51.0	48.3	14.5	17.0
Total PUFA	47.0	47.7	48.6	64.1	50.1	45.9	59.1	56.6	23.5	21.7
n-3/n-6	5.7	14.9	3.8	14.6	0.9	0.8	6.3	5.8	1.6	3.6
Total dimethyl acetals	-	-	-	-	-	-	6.3	4.7	-	-

Values were obtained from pooled samples of 6 hearts. t = trace value < 0.05%. - = not detected.

[1] Includes 15:0, 17:0 and 20:0. [2] Includes 16:1n-9, 20:1n-11, 20:1n-7 and 22:1n-9. [3] Includes 18:3n-6, 20:3n-6 and 22:5n- 6. [4] Includes 18:4n-3 and 20:3n-3.

7.2.3. Lipid class compositions of livers from wild and farmed Atlantic salmon parr. (Values are weight % of total lipid)

Lipid class	Wild parr	Farmed parr
Sphingomyelin	3.6 ± 0.6	3.6 ± 0.3
Phosphatidylcholine	28.0 ± 1.9	29.8 ± 1.1
Phosphatidylserine	3.3 ± 0.4	3.8 ± 0.1
Phosphatidylinositol	5.8 ± 0.3	6.1 ± 0.3
Phosphatidic acid/cardiolipin	4.5 ± 0.4	4.1 ± 0.4
Phosphatidylethanolamine	16.2 ± 1.1	18.3 ± 0.9
Total polar lipid	61.3 ± 4.2	65.7 ± 2.3
Cholesterol	13.5 ± 0.8	12.9 ± 0.8
Free fatty acid	t	0.7 ± 0.4*
Triacylglycerol	8.7 ± 0.9	10.8 ± 2.6
Sterol ester	14.5 ± 3.5	7.1 ± 0.7*
Total neutral lipid	36.7 ± 3.5	31.3 ± 2.4

Values are mean ± SD from 3 samples of each group where each sample is composed of two livers. *Values are significantly different ($P < 0.05$).

Table 7.2.4. Fatty acid compositions of individual phospholipids and triacylglycerol from livers of wild and farmed Atlantic salmon parr (Values are weight %).

	Wild	Farmed	Wild	Farmed	Wild	Farmed	Wild	Farmed	Wild	Farmed
Fatty acid	PC		PS		PI		PE		TAG	
14:0	1.1	1.2	0.1	0.2	0.3	0.2	0.1	0.3	1.2	2.8
16:0	27.9	25.6	14.6	14.1	10.2	9.6	11.4	11.4	11.1	9.1
18:0	1.7	1.0	16.7	18.1	21.9	26.0	3.7	2.4	6.3	2.9
Total sats.[1]	31.5	28.4	32.4	33.1	33.1	36.5	15.6	14.4	19.9	15.1
16:1n-7	3.9	2.7	0.1	t	0.9	0.4	1.0	0.9	8.4	6.0
18:1n-9	6.6	7.3	2.2	3.6	3.9	5.9	8.1	10.4	17.1	29.2
18:1n-7	2.6	1.3	2.6	1.4	2.4	1.5	5.9	3.9	8.3	4.7
20:1n-9	0.1	1.5	0.8	3.6	0.2	2.5	0.4	6.1	1.1	13.7
22:1n-11	-	0.4	-	0.4	-	0.4	-	1.1	-	6.5
24:1	0.6	1.2	0.6	0.9	t	0.5	t	0.1	t	0.5
Total monos.[2]	13.8	14.4	6.3	11.7	7.4	11.2	15.7	22.8	34.9	60.6
18:2n-6	2.6	1.2	1.0	0.8	2.9	0.7	5.9	3.4	8.8	4.3
18:3n-6	0.3	0.1	0.2	t	0.2	0.1	0.3	0.1	0.9	t
20:2n-6	0.1	0.1	0.1	t	0.4	0.2	0.4	0.4	0.6	0.5
20:3n-6	0.5	0.2	0.3	t	0.7	1.9	0.9	0.3	0.5	0.4
20:4n-6	2.0	0.6	1.0	0.8	27.9	22.2	4.0	0.8	0.9	0.5
22:5n-6	0.3	0.2	0.6	t	-	0.1	0.5	0.3	-	-
Total n-6[3]	5.9	2.4	3.4	1.6	32.1	25.2	12.1	5.3	11.7	5.7
18:3n-3	3.8	0.4	0.9	t	4.4	0.4	4.6	0.8	11.9	0.7
18:4n-3	0.8	0.4	0.3	t	t	t	0.1	0.1	2.0	1.2
20:3n-3	0.2	t	0.3	t	0.3	-	0.5	0.1	0.7	t
20:4n-3	0.5	0.6	0.1	t	0.4	0.4	0.5	0.8	1.2	1.7
20:5n-3	11.3	7.5	2.2	0.8	4.2	7.1	12.6	6.1	6.8	2.4
22:5n-3	3.5	1.3	4.3	1.4	2.6	1.2	3.5	1.2	2.7	2.1
22:6n-3	26.2	42.4	46.5	45.0	14.6	14.8	31.9	45.3	3.9	6.1
Total n-3	46.3	52.6	54.6	47.2	26.5	23.9	53.7	54.4	29.2	13.3
Total PUFA	52.2	55.0	58.0	48.8	58.6	49.1	65.8	59.7	40.9	19.0
n-3/n-6	7.9	21.9	16.1	29.5	0.8	1.0	4.4	10.3	2.5	2.3
Total DMA	0.2	0.1	-	-	-	-	0.6	0.2	-	-
20:4/20:5	0.2	0.1	0.5	1.0	6.6	3.1	0.3	0.1	0.1	0.2

Values were obtained from pooled samples of 6 livers. [1]Includes 15:0, 17:0 and 20:0. [2]Includes 16:1n-9, 20:1n-11, 20:1n-7 and 22:1n-9. [3]Includes 22:4n-6. t = trace value < 0.05. - = not detected.

7.3 Lipid compositions of hearts and livers from wild and farmed Atlantic salmon pre-smolts.

The lipid class compositions of hearts from wild and farmed pre-smolts are shown in Table 7.3.1. Although the mean value for triacylglycerol content of wild hearts was considerably lower than that for farmed fish the large variation in this class in the four wild hearts sampled meant that most of the lipid class percentages were not significantly different between wild and farmed fish. However, levels of PS, PI and phosphatidic acid/cardiolipin were significantly greater while triacylglycerol values were significantly lower in wild hearts compared to farmed. The lower accumulation of triacylglycerol in wild hearts suggests that the lipid content of commercial parr feeds is higher than the lipid content of the natural diet.

The fatty acid compositions of the individual phospholipids and TAG in pre-smolt hearts are shown in Table 7.3.2. In PC the levels of total saturates and total monoenes were similar although farmed fish contained more 20:1n-9 and 22:1n-11 and less 16:1n-7 and 18:1n-9 compared to wild pre-smolts. The levels of 18:2n-6, 20:2n-6, 20:3n-6, AA and consequently total n-6 PUFA were increased in PC from wild fish compared to their farmed counterparts. Among the n-3 PUFA, levels of 18:3n-3, 20:4n-3, EPA and 22:5n-3 were increased in wild pre-smolts compared to farmed equivalents although the total n-3 PUFA levels were similar largely due to the high level of DHA in heart PC of farmed fish. In heart PE total saturate levels were elevated in farmed pre-smolts compared to wild pre-smolts. While levels of total monoenes were similar in PE, farmed fish contained higher levels of 16:1n-7, 20:1n-9 and 22:1n-11 but lower levels of 18:1n-7, and 24:1 compared to farmed pre-smolts. The changes in PE n-6 PUFA composition were largely similar to those in PC but the levels of AA and 22:5n-6 were considerably higher in wild fish compared to farmed. The n-3 PUFA composition of heart PE from wild and farmed pre-smolts was broadly similar to that in PC although total n-3 PUFA and total PUFA were

considerably higher in PE from wild fish compared to farmed. The total dimethylacetal content was greater in wild fish compared to farmed. The fatty acid compositions of heart PS showed similar differences between farmed and wild pre-smolts to those in PC and PE. However, 16:0 and 18:0, and consequently total saturates were much higher in heart PS from wild fish compared to farmed. The fatty acid compositions of PI from wild and farmed pre-smolts were very similar with the only notable differences in the n-3 PUFA where wild fish contained higher levels of 18:3n-3 and 22:5n-3 but lower levels of DHA compared to their farmed counterparts. The total saturated fatty acid level in TAG was similar in hearts from wild and farmed presmolts although wild fish contained considerably more 18:0 but much less 14:0 compared to farmed fish. The total monoenes in heart TAG were higher in farmed fish largely due to greater levels of 20:1n-9 and 22:1n-11 but wild fish did contain appreciably more 18:1n-9. Wild pre-smolt TAG contained more total n-6 PUFA than their farmed counterparts largely due to the former containing more 18:2n-6, 18:3n-6 and 20:3n-6. While levels of total n-3 PUFA were similar in heart TAG from both groups farmed pre-smolts contained more DHA, 22:5n-3 and EPA but much less 18:3n-3 compared to wild fish.

The lipid class compositions of livers from wild and farmed pre-smolts did not show any significant differences, both compositions being very similar to those of farmed parr (Table 7.2.3). The fatty acid compositions of individual phospholipids and TAG from livers of wild and farmed pre-smolts are shown in Table 7.3.3. The differences between the fatty acid compositions of the individual phospholipids in livers from wild and farmed pre-smolts were very similar to those in pre-smolt hearts. In PC, PE and PS from wild fish liver, levels of 18:2n-6, AA, 18:3n-3, EPA and 22:5n-3 were higher than in farmed fish. In all liver phospholipid classes farmed pre-smolts contained considerably more DHA than in their wild counterparts. As in pre-smolt hearts the fatty acid compositions of liver PI were very similar in both wild and

farmed fish. The fatty acid compositions of liver TAG from wild and farmed pre-smolts were broadly similar to those in heart.

Table 7.3.1. Lipid class compositions of hearts from wild and farmed Atlantic salmon pre-smolts. (Values are weight %)

Lipid class	Wild pre-smolts	Farmed pre-smolts
Sphingomyelin	2.5 ± 0.1	1.9 ± 0.3
Phosphatidylcholine	24.4 ± 7.6	16.6 ± 0.5
Phosphatidylserine	3.9 ± 0.9	2.5 ± 0.1*
Phosphatidylinositol	4.9 ± 1.1	3.0 ± 0.1*
Phosphatidic acid/cardiolipin	10.5 ± 2.0	7.3 ± 1.0*
Phosphatidylethanolamine	21.1 ± 8.1	13.3 ± 1.0
Total polar lipid	67.2 ± 19.4	44.5 ± 2.8
Cholesterol	11.8 ± 5.5	12.9 ± 1.0
Free fatty acid	1.6 ± 0.3	1.8 ± 0.1
Triacylglycerol	17.9 ± 12.5	38.6 ± 2.0*
Total neutral lipid	31.3 ± 18.0	53.3 ± 3.0

Values are mean ± SD for 4 samples per treatment where each sample comprised two individual hearts. *Values are significantly different ($P < 0.05$).

Table 7.3.2. Fatty acid compositions of individual phospholipids and triacylglycerol from hearts of wild and farmed Atlantic salmon pre-smolts. (Values are weight %).

Fatty acid	Wild PC	Farmed PC	Wild PS	Farmed PS	Wild PI	Farmed PI	Wild PE	Farmed PE	Wild TAG	Farmed TAG
14:0	3.5	27.3	1.1	1.0	0.5	0.5	2.3	4.7	0.9	6.8
16:0	27.3	25.5	13.2	9.3	7.0	7.8	9.8	13.5	16.0	15.3
18:0	6.6	7.4	23.1	16.9	29.5	30.1	7.7	8.1	11.0	4.3
Total saturates[1]	29.8	28.9	34.4	33.9	36.6	36.3	14.3	13.8	30.3	26.1
16:1n-7	3.4	2.4	1.5	1.3	0.7	0.5	0.9	1.7	3.1	5.0
18:1n-9	10.5	7.7	6.4	5.6	4.7	4.6	6.5	6.9	23.6	16.1
18:1n-7	1.4	1.2	2.3	1.8	1.8	1.6	4.5	1.4	2.9	3.0
20:1n-9	0.4	1.7	5.7	7.2	5.6	4.2	0.2	3.0	0.5	7.3
22:1n-11	t	0.8	0.4	1.1	0.2	0.2	t	0.9	0.4	7.1
24:1	0.4	0.8	1.5	0.5	1.2	1.1	0.7	0.2	0.2	0.8
Total monoenes[2]	16.1	15.6	19.1	17.5	14.4	12.2	12.8	14.9	19.1	17.5
18:2n-6	2.3	1.5	1.2	0.9	0.9	0.4	2.6	2.3	7.4	4.5
18:3n-6	t	0.2	1.1	0.7	1.0	0.5	t	t	0.5	t
20:2n-6	0.3	0.1	0.2	0.3	0.1	t	0.3	0.2	0.4	0.4
20:3n-6	0.4	t	0.3	t	0.4	0.2	0.5	0.1	0.4	t
20:4n-6	3.4	1.0	1.2	1.3	19.9	19.6	4.7	1.1	0.6	0.5
22:5n-6	0.4	0.2	0.7	0.6	0.4	0.3	0.8	0.6	0.7	t
Total n-6[3]	6.8	3.4	5.1	4.5	23.1	20.9	9.5	4.3	9.3	5.4
18:3n-3	3.1	0.4	0.8	0.3	1.7	0.4	3.6	0.1	13.4	1.9
18:4n-3	0.4	0.8	0.5	0.7	0.4	0.4	0.8	0.7	2.4	2.3
20:4n-3	0.8	0.3	0.4	0.5	0.3	0.3	1.5	0.6	1.1	1.2
20:5n-3	8.8	6.1	1.5	1.1	7.9	7.9	6.9	3.0	1.5	3.1
22:5n-3	1.1	0.6	1.5	1.1	1.6	0.6	2.8	1.8	0.7	1.5
22:6n-3	16.4	23.4	21.3	35.6	9.2	14.0	31.3	34.2	2.7	14.4
Total n-3	30.6	31.6	27.7	40.1	21.1	23.6	46.9	40.6	22.6	24.4
Total PUFA	37.4	35.0	32.8	44.6	44.2	44.5	56.4	44.9	31.9	29.8
n-3/n-6	4.5	9.3	5.4	8.9	0.9	1.1	4.9	9.4	2.4	4.5

Values were obtained from pooled samples of eight hearts from wild and farmed fish. t = trace value < 0.05%. - = not detected. [1]Includes 15:0, 17:0 and 20:0. [2]Includes 16:1n-9, 20:1n-11, 20:1n-7 and 22:1n-9. [3]Includes 18:3n-6, 20:3n-6 and 22:5n- 6.[4]Includes 18:4n-3 and 20:3n-3.

Table 7.3.3. Fatty acid compositions of individual phospholipids and triacylglycerol from livers of wild and farmed Atlantic salmon pre-smolts. (Values are Weight %)

Fatty acid	Wild PC	Farmed PC	Wild PS	Farmed PS	Wild PI	Farmed PI	Wild PE	Farmed PE	Wild TAG	Farmed TAG
14:0	1.9	2.7	1.3	0.5	1.5	1.0	0.5	0.6	3.3	3.2
16:0	25.4	24.2	14.1	15.6	11.4	12.4	10.8	17.6	14.3	12.3
18:0	1.7	1.5	17.6	15.6	22.2	21.8	2.5	2.3	10.4	7.6
Total saturates[1]	29.8	28.9	34.4	33.9	36.6	36.3	14.3	13.8	30.3	26.1
16:1n-7	3.5	2.7	1.1	0.6	2.2	1.4	1.4	1.2	6.3	5.5
18:1n-9	6.8	7.4	3.3	4.2	7.4	7.4	10.6	11.4	25.5	27.6
18:1n-7	1.2	1.1	1.1	1.1	1.7	1.6	3.4	2.8	3.9	3.2
20:1n-9	0.6	0.9	5.7	3.0	1.6	2.3	2.0	4.7	1.7	3.7
22:1n-11	0.3	0.3	0.2	0.4	1.3	0.4	0.4	0.8	0.6	1.7
24:1	0.8	0.5	1.0	0.8	0.7	0.7	0.1	0.1	1.1	1.5
Total monoenes[2]	13.3	13.1	13.4	10.9	14.7	14.0	18.1	21.3	40.6	45.4
18:2n-6	1.6	1.5	0.5	0.4	1.3	1.8	3.8	3.0	4.1	4.6
18:3n-6	0.1	-	0.6	0.3	-	-	0.2	0.1	0.4	-
20:2n-6	0.1	0.2	0.2	0.2	0.2	0.3	0.5	0.4	0.3	0.3
20:3n-6	0.4	0.3	0.6	0.1	1.7	1.0	0.6	0.4	0.2	t
20:4n-6	1.7	0.7	1.0	0.4	20.7	18.7	3.1	0.9	0.5	0.3
22:5n-6	0.4	0.2	0.7	0.6	0.4	0.3	0.8	0.6	0.7	t
Total n-6[3]	4.4	2.9	3.6	2.1	24.3	22.3	5.4	3.6	6.2	5.2
18:3n-3	2.5	1.3	0.6	0.4	1.1	0.8	0.8	0.6	3.9	2.1
18:4n-3	0.6	0.5	0.2	0.2	0.6	0.3	0.2	0.1	0.9	4.3
20:4n-3	0.9	0.7	0.9	0.1	0.3	0.4	1.2	0.8	0.6	0.3
20:5n-3	8.4	6.5	1.6	0.5	3.7	5.6	8.5	5.5	1.0	1.0
22:5n-3	1.7	1.2	1.7	1.3	1.1	1.1	1.8	1.1	0.4	0.6
22:6n-3	30.6	39.1	34.6	44.0	9.8	17.9	38.4	45.7	3.2	3.8
Total n-3	44.7	49.3	39.6	46.5	16.6	26.1	54.6	54.8	10.0	12.1
Total PUFA	49.1	52.2	43.2	48.6	40.9	48.4	63.7	60.2	16.2	17.3
n-3/n-6	10.2	17.0	11.0	22.1	0.7	1.2	6.0	10.2	1.6	2.3

Values were obtained from pooled samples of eight livers from wild and farmed fish. t = trace value < 0.05%. - = not detected. [1]Includes 15:0, 17:0 and 20:0. [2]Includes 16:1n-9, 20:1n-11, 20:1n-7 and 22:1n-9. [3]Includes 18:3n-6, 20:3n-6 and 22:5n- 6. [4]Includes 18:4n-3 and 20:3n-3.

7.4 Summary

1. Triacylglycerol levels were significantly greater in hearts of both wild Atlantic salmon parr and pre-smolts compared to their farmed counterparts.
2. The fatty acid compositions of triacylglycerols and phospholipids in hearts and livers of both farmed parr and pre-smolts contained considerably more monoenoic fatty acids, largely due to increased levels of 20:1n-9 and 22:1n-11, compared to their wild counterparts.
3. Triacylglycerol, PC and PE contained greater amounts of 18:2n-6 and 20:4n-6 in tissues of wild fish compared to farmed, although heart phospholipids (except PI) generally contained more AA than livers.
4. Linolenic acid was considerably greater in all tissue lipid classes examined in wild fish compared to farmed fish while the opposite was generally true for 18:4n-3. EPA and 22:5n-3 were generally higher in PC, PS and PE from heart and liver of wild fish compared to farmed fish.
5. DHA levels were considerably higher in tissues of farmed fish, particularly in heart PC, PS and TAG, although similar trends were apparent in liver. High DHA levels in farmed parr would tend to inhibit Δ6-desaturation of 18:2n-6 (and 18:3n-3) and contribute to the lower AA levels found in farmed parr.
6. In general, the fatty acid compositions of PI were most conserved between wild and farmed fish, especially with respect to AA and EPA concentrations.
7. The n-3/n-6 PUFA ratios were generally lower in heart than in liver and were lower in wild compared to farmed fish. This effect was largely due to increased levels of n-6 PUFA in wild fish, and in particular of AA.
8. The results from this study, which highlight the differences in lipid composition between wild and farmed parr, suggest that the diet formulations currently used by the aquaculture industry may be less than ideal and may explain the problems of seawater adaptation which are often encountered.

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Section 8

General Discussion

Section 8.

8.1 General Discussion

In the relatively small number of fish species which have been studied to date evidence suggests that freshwater fish can elongate and desaturate C_{18} PUFA to C_{22} PUFA, whereas marine fish require C_{20} and probably C_{22} PUFA to be supplied in the diet, due to the very low Δ5 and Δ4 desaturation activities in the latter. In anadromous fish like Atlantic salmon, which start life in freshwater before migrating to seawater following smoltification, the situation is less clear. Evidence suggests that salmon parr can convert C_{18} PUFA to C_{22} PUFA and that this proceeds rapidly prior to parr-smolt transformation (smoltification) as part of the physiological adaptation to the marine environment (Sheridan *et al.* 1985; Li and Yamada, 1992). The fatty acid composition data from the present study, using wild and farmed salmon parr, support the capability to elongate and desaturate C_{18} PUFA to C_{22} PUFA in this species. Studies suggest that the freshwater invertebrate species which comprise the major dietary input of Atlantic salmon parr contain 18:2n-6, 18:3n-3 and EPA as their principle PUFA, but contain only trace amounts of DHA (Hanson *et al.* 1985; Bell *et al.* 1994a). The presence of high levels of DHA, and also of AA, in the tissue phospholipids of wild salmon parr suggests they are capable of converting C_{18} to C_{22} PUFA.

However, while parr are thought to express fully the enzymes required to perform these conversions, the ability of salmon undergoing smoltification, or salmon adapted to the marine environment to perform these desaturation and elongation reactions is not clear (Ackman and Takeuchi, 1986). The wild Atlantic salmon parr used by Ackman and Takeuchi (1986) contained much higher levels of AA compared to their farmed counterparts, despite the latter containing higher levels of 18:2n-6 as a result of dietary input. This suggested that desaturation of 18:2n-6 to AA was impaired in farmed parr.

In the present studies, feeding diets containing increased levels of 18:2n-6 (> 10% of dietary fatty acids), due to the presence of sunflower oil (SO), resulted in increased incorporation of this fatty acid into the membrane phospholipids of all tissues studied. The incorporation of increased levels of 20:3n-6 and AA in phospholipids from fish fed SO confirms the presence of active Δ6- and Δ5-desaturation and elongation of C_{18} PUFA in post-smolt Atlantic salmon. The increased incorporation of 22:5n-6, in fish fed SO compared to those fed FO, particularly into liver PE and PS (Tables 5.6.1-5.6.3), indicates that post-smolt Atlantic salmon are also capable of Δ4-desaturation (The "Sprecher shunt"). In addition, fish fed linseed oil (LO) contained levels of EPA in tissue phospholipids which were greater than or equal to those from fish fed fish oil (FO) (e.g. Tables 5.5.2., 5.5.3., 5.5.4 etc), despite dietary levels of EPA being lower compared to both FO and SO-fed fish. This further supports the evidence for active Δ6- and Δ5-desaturation being present in post-smolts. The high activity of Δ6-desaturase is particularly apparent in the liver where the elongation and desaturation of 18:2n-6 results in an accumulation of 20:3n-6 such that this fatty acid becomes the most abundant C_{20} PUFA in PC, PS and PE of SO-fed fish. Although AA was increased, particularly in PC and PE, in fish fed SO the level of 20:3n-6 was increased up to 25-fold in liver, compared to fish fed FO. This accumulation of 20:3n-6 suggests that, while salmon possess high Δ6-desaturase and elongase activities the activity of Δ5-desaturase is somewhat lower. A low Δ5-desaturase activity has been observed previously by Hagve *et al.* (1986) in isolated hepatocytes from a related salmonid, the rainbow trout.

In dietary trial C there were no significant differences in the final weights of salmon fed the experimental diets. However, in dietary trial B, where fish were fed linoleic acid at 10, 25 and 45% of dietary fatty acids, there were statistically significant differences in the final weights between dietary treatments. The results suggest that a moderate dietary input of linoleic acid (25%) may stimulate growth rate. In a study

with juvenile turbot (*Scophthalmus maximus)* Castell *et al.* (1994) observed increased growth rate with increasing dietary level of AA, which is the product of Δ6 and Δ5-desaturation and elongation of linoleic acid. While the precise mechanism of growth promotion is not clear, studies with mammals have indicated that skeletal muscle synthesis is stimulated by PGI_2 and inhibited by PGE_2 (McLennan, 1991). While PGI_2 (or its stable metabolite 6-keto$PGF_{1\alpha}$) and PGE_2 were not measured in muscle during dietary trial B it is possible that the ratio of these two prostanoids was altered by the dietary treatments and, thus, had an influence on the relative rates of protein synthesis and degradation.

The increase in 18:3n-6 and 20:3n-6 on supplementation with 18:2n-6 and the increase in 18:4n-3 and 20:4n-3 on supplementation with 18:3n-3 clearly indicates the presence of Δ6-desaturase activity in CHH-1 cells. Although conversion of both 18:2n-6 and 18:3n-3 to AA and EPA respectively, was observed, in general the accumulation of 20:3n-6 and 20:4n-3 suggested a lower Δ5-desaturase activity in CHH-1 cells compared to both RTG-2 (rainbow trout gonad) and AS (Atlantic salmon, whole body) cell lines (Tocher, 1990; Tocher and Dick, 1990). CHH-1 cells, like other salmonid-derived established cell lines, appear to possess both Δ6- and Δ5-desaturase activities, while Δ4-desaturation appears to be absent. Whether the full desaturase complement was lost as a result of continuous culture or whether the original cells were never capable of Δ4-desaturation is not known. However, a number of cell lines are known to lose their ability to express desaturase activities after long-term culture. Among six mammalian cultured cell lines investigated, Δ6-desaturation was absent in three, although in two of those Δ5-desaturation was present. However, Δ5-desaturation was only present in two cell lines out of six, while Δ4-desaturation was only present in one (Maeda *et al.* 1978; Robert *et al.* 1978). Recent evidence suggests that Δ4-desaturation of EPA to DHA proceeds via initial elongation of EPA to 24:5n-3 followed by Δ6-desaturation to 24:6n-3 and chain-shortening to give DHA (The "Sprecher Shunt") (Voss *et al.* 1991). Since the chain

shortening step requires peroxisomal oxidation it is possible that impaired peroxisomal function after long-term culture could explain the apparent loss of Δ4-desaturase activity in some established cell lines.

The considerable accumulation of C_{22} PUFA in comparison to other fish cell lines studied (Tocher, 1990; Tocher and MacKinlay, 1990; Tocher and Dick, 1990) suggests that appreciable elongase activity is present in CHH-1 cells. The large concentration of elongation products arising from supplementation with both AA and EPA suggests an important functional role for long-chain PUFA in these cells. DHA is a major component of rat cardiac phospholipids (Swanson and Kinsella, 1986) as well as Atlantic salmon cardiac phospholipids, as observed in this study. Interestingly, the ability of CHH-1 cells to elongate extensively both AA and EPA is contrary to the situation in rat heart and isolated cardiac myocytes which are unable to perform elongation (Mohammed *et al.* 1990) or desaturation reactions (Hagve and Sprecher, 1989).

Both essential fatty acids, 18:2n-6 and 18:3n-3, are metabolised by the same enzyme systems of sequential desaturation and elongation which result in the production of long-chain PUFA of the n-6 and n-3 series (Sprecher, 1981). The first two dietary experiments in this study suggest that feeding diets rich in 18:2n-6 results in increased AA in the membrane phospholipids. However, an important consideration in such dietary experiments concerns the influence which one type of fatty acid can have on the metabolism of the other (Garcia and Holman, 1965). Thus, an excess of n-6 PUFA may inhibit the metabolism of 18:3n-3 although, in general, the n-3 PUFA are more efficient in inhibiting the metabolism of n-6 PUFA than vice versa (Horrobin, 1991). Feeding both SO and LO to post-smolt Atlantic salmon results in increased levels of 18:2n-6, 20:2n-6 and 20:3n-6 (the product of Δ6-desaturation and elongation) into tissue phospholipids compared to fish fed FO (p113, 116, 117, 118-126). However, in most phospholipid classes, the incorporation of AA (the product of Δ5-desaturation) was increased when SO was fed but reduced

when LO was the dietary lipid source, compared to fish fed FO. Similarly 22:5n-6, the product of Δ4-desaturation and elongation of AA was increased in fish fed SO but decreased in fish fed LO compared to fish fed FO (pages 113-126). Therefore, it appears that dietary LO, which is rich in 18:2n-6 but much richer in 18:3n-3, has an inhibitory effect on the Δ5-desaturase enzyme responsible for converting 20:3n-6 to AA. Since there is more n-3 PUFA than n-6 PUFA in both C_{18} and C_{20} fatty acids in LO-fed fish compared to SO-fed fish, it is inevitable that 18:2n-6 is metabolised to AA in SO-fed fish whereas the same does not occur in LO-fed fish to the same extent. That is, in LO-fed fish, there is competition at the Δ6-desaturase between 18:2n-6 and 18:3n-3 and also at the Δ5-desaturase between 20:3n-6 and 20:4n-3 the net result of which is an apparent inhibition of Δ5-desaturation of 20:3n-6. Similar results regarding the ability of 18:3n-3 to inhibit conversion of 18:2n-6 to AA have been observed in mammals (Brenner, 1981). Similar competitive effects presumably operate to increase or reduce 22:5n-6 levels in SO- and LO-fed fish respectively.

The apparent inability of farmed parr undergoing smoltification to convert 18:2n-6 to AA, as described by Ackman and Takeuchi (1986), may be explained by competitive interactions between fatty acids for the desaturase binding sites. The increased PUFA levels in cell membranes of salmonids, particularly EPA and DHA, of fish undergoing smoltification are well documented and are believed to occur in freshwater prior to seawater entry (Sheridan *et al.* 1985; Li and Yamada, 1992). The importance of long-chain PUFA in adaptation to changes in salinity has been observed in essential fatty acid deficient trout, which had reduced ionic permeability in brush border membranes (Di Constanzo *et al.* 1983) while a similar EFA-deficiency caused morphological changes in gills of turbot (Bell *et al.* 1985a). In addition, membrane PUFA composition is known to affect the activity of a number of ion pumps and membrane-bound enzymes including Na^+, K^+-ATPase (Spector and Yorek, 1985; Gerbi *et al.* 1994). The recent identification of "the Sprecher shunt"

demonstrates that Δ4-desaturation of EPA to DHA actually proceeds via Δ6-desaturation of 24:5n-3 to 24:6n-3, followed by chain shortening to DHA (Voss *et al.* 1991). Thus, given the preference of n-3 over n-6 PUFA at the Δ6-desaturase binding site (Horrobin, 1991) both in mammals, and in fish (Hagve *et al.* 1986; Henderson and Tocher, 1987), the apparent inability of salmon undergoing smoltification to convert 18:2n-6 to AA can be accounted for. In addition, in farmed salmon fed diets containing marine fish oils the high levels of DHA will further prevent conversion of 18:2n-6 to AA by end-product feedback inhibition of Δ6-desaturase (Garcia and Holman, 1965).

In a previous study using rainbow trout no increase in elongated and desaturated products of 18:3n-3 were observed in the polar lipid fraction of fish fed an 18:3n-3 enriched diet (Sowizral *et al.* 1990). However, in the present study with Atlantic salmon, although dietary EPA levels were similar in both LO and SO diets, the fatty acid compositions of tissue phospholipid classes showed decreased EPA in SO-fed fish, but LO-fed fish had EPA values greater than or equal to those of FO-fed fish (pages 113, 116, 121, 122, 125, 126). These results suggest that salmon can synthesise EPA from 18:3n-3 utilising the pathways of Δ6- and Δ5-desaturation and elongation. Although some phospholipid classes from liver of LO-fed fish had elevated 22:5n-3, there was no apparent increase in DHA compared to fish fed FO. Previous studies with salmonid parr suggest that synthesis of DHA from 18:3n-3 should be possible (Henderson and Tocher, 1987) but in the larger post-smolts used in the present study Δ4-desaturation is either very low or, more likely, the requirement for DHA is met by dietary input (derived from fish meal; page 36).

In CHH-1 cells the relative percentages of the Δ6-desaturation products of 18:2n-6 and 18:3n-3 indicated that there was no specificity for either substrate. This result is similar to that of Tocher *et al.* (1989) with RTG-2 and turbot fin (TF) cells, but differed from AS cells which showed a preference for the n-3 substrate (Tocher and Dick, 1990). However, the Δ5-desaturase in CHH-1 cells showed a preference towards

the n-3 substrate since supplementation with 18:3n-3 resulted in increased EPA while 18:2n-6 supplementation resulted in minimal AA production (pages 135-136). This does not reflect a specific adaptation of fish cells since similar substrate preferences have also been observed in mammalian cells (Stubbs and Smith, 1984) and may be enhanced by the n-3 PUFA deficiency which exists in cells routinely cultured in foetal calf serum (FCS). Interestingly, culture of CHH-1 cells in medium supplemented with 1% FCS and 25μM DHA resulted in phospholipid fatty acid compositions similar to those in intact salmon hearts. The ability to return these cells to a lipid composition approaching that of the salmonid heart makes them a useful model system to study the effects of membrane fatty acid composition on a number of biochemical and physiological functions, including eicosanoid production and membrane-bound enzyme activities etc.

While considerable data have been accumulated, from both mammals and fish, on the effects of different dietary lipids on tissue fatty acid compositions, the effects on lipid class composition are less well documented. In dietary trial C, salmon fed LO had significantly reduced levels of heart PC compared to both other dietary treatments resulting in a PC:PE ratio less than unity. In the vast majority of fish species and tissues studied PC is the dominant phospholipid class (Henderson and Tocher, 1987), although, as in mammals, some fish brain tissue has PE in excess of PC Tocher and Harvie, 1988). Acclimation to low environmental temperature can decrease the PC:PE ratio in fish tissues (Miller *et al.* 1976) which is accompanied by increased incorporation of n-3 PUFA, principally DHA, into membrane phospholipids especially PE. In the present series of experiments DHA was not significantly increased in heart membrane phospholipids although there was an overall increase in n-3 PUFA in PE largely as a result of elevated 18:3n-3. While the exact reason for the decreased PC:PE ratio in fish fed LO is unclear, the consequences in terms of membrane physiology and biochemistry could be important in, for example, homeoviscous adaptation and function of membrane-

associated proteins including enzymes, ion channels and receptors (Spector and Yorek, 1985; Tocher, 1995).

Nutritional cardiomyopathy has been recorded in a number of fish species, particularly in intensive culture, and especially associated with vitamin E and selenium deficiences (Ferguson, 1989). Focal aggregation and infiltration of leucocytes on or in a myotome is often the pathological manifestation of an inflammatory stimulation. In the absence of detectable infectious agents or other antigenic sources in the affected areas, it is reasonable to conclude that such lesions are a direct result of local tissue damage (i.e. necrosis). Loss of individual myotome integrity and loss of muscle striation and occurrence of necrotic debris was confirmed in a sufficient number of lesions in dietary trials A and C to confirm that this was the primary cause of the inflammation. These latter lesions are difficult to photograph properly with the light microscope as they require a variety of different focal planes and even different lighting conditions (e.g. phase) to be detectable. Similar myopathies have been observed in mammals, including man, which can be attributed to deficiencies of selenium and/or vitamin E (Halliwell and Gutteridge, 1985; Porter and Whelan, 1983), but no cardiomyopathy has been specifically associated with n-6 fatty acid intake. Fjolstad and Heyeraas (1985) found muscular and myocardial degeneration in their study on Hitra disease in Norwegian farmed salmon and suggested a relationship to dietary deficiency of vitamin E and/or selenium. An extensive degenerative cardiomyopathy studied by Ferguson *et al.* (1986) in association with pancreas disease (PD) of farmed salmon was also attributed by these authors to vitamin E and/or selenium deficiency. Although these authors considered the lesions of the pancreas to be linked to those of heart and muscle, McVicar (1987) gave evidence that heart and muscle lesions are not consistently or primarily associated with PD. In addition, Bell *et al.* (1987) considered that the depletion of vitamin E in PD-affected salmon was a consequence, rather than a cause of the disease.

In the three dietary studies the oil source was the only difference between the experimental diets. The high proportion of damaged hearts (page 58, 108) associated with the SO diet in comparison with the rarity of the condition in FO-fed fish indicates that the damage was a consequence of the dietary lipids. Vitamin E is probably not involved in the cardiomyopathy because, although high amounts of PUFA are present in the SO diet, dietary vitamin E was included in the diets at or above a threefold excess of requirement (U.S. National Research Council, 1981). In addition measurement of heart vitamin E content showed no significant differences between dietary treatments (15.4 ± 7.5, FO diet; 17.0 ± 4.0, SO diet; 16.6 ± 2.3, LO diet, mean (μg/g tissue) ± SD, n =4) and these values were more than 7-fold higher than those recorded in a study by Raynard *et al.* (1991) in which no cardiac lesions were observed. In dietary trial C focal cardiac myopathy was observed in fish fed SO and while some degeneration of cardiac tissue was also observed in fish fed FO these were much less severe. Cardiac myopathy was virtually absent in fish fed LO suggesting that dietary 18:3n-3 may have an attenuating effect on development of the pathological lesion.

Although the exact cause of the cardiac lesion is not known a number of aspects deserve comment. The first is the similarity, in some ways, to the fainting or shock syndrome described by Sinnhuber (1969) and Castell *et al.* (1972) in trout fed a diet deficient in n-3 PUFA. This syndrome was similar to a transportation shock syndrome originally described by Black and Barrett (1957). In rainbow trout given 18:2n-6 and lauric acid (14:0) as their only dietary lipids, Castell *et al.* (1972) observed an unusual heart condition, described as local myocarditis, with some degeneration and hypertrophy. A similar myopathy was observed in the present study, although the ventricular swellings observed in rainbow trout did not occur in SO-fed salmon. However, the extent to which either the shock syndrome described by Sinnhuber (1969) or the transportation induced mortalities described in this work involved cardiac or cardiovascular malfunction is not known. Clearly the SO-fed fish

in dietary trials A and C were not deficient in n-3 PUFA on an absolute basis, but they could be considered to be relatively deficient in n-3 PUFA in the sense that their diet contained a considerable excess of n-6 compared with n-3 PUFA.

This raises concern as to the level at which the dietary ratio of n-6/n-3 PUFA becomes high enough to be pathological to fish. As far as I are aware, there is no evidence in the literature to suggest that a high ratio of these lipids is deleterious to fish, despite the now well established fact that high dietary ratios of n-6/n-3 PUFA can lead to the development of pathological conditions in humans (Lands, 1986; Crawford, 1987). Indeed, lowering the high dietary ratio of n-6/n-3 PUFA by taking n-3 PUFA-rich fish oil concentrates is known to be beneficial in alleviating a number of human disorders, including cardiac disease (Lands, 1986; Budowski, 1988).

The experiments with cardiac-derived CHH-1 cells showed that cell cultures grew well when supplemented with 1% FCS and fatty acids, attached to BSA, added at a concentration of 25 μM. The exception was those cells which were supplemented with 20:3n-6 which did not achieve confluence and began to detach from the flask after 6-7 days. The experiment was repeated twice using the same fatty acid concentration, with two different sources of 20:3n-6 and the same deleterious effect on growth was recorded. While the exact cause of the growth inhibition remains unclear, it seems unlikely that it was due to the presence of toxic lipid oxidation products since measurement of thiobarbituric acid reactive material in the PUFA-BSA complexes indicated the greatest levels of oxidation products were present in the EPA and DHA supplements which demonstrated no growth inhibition (Results not shown). While CHH-1 cells supplemented with 20:3n-6 had a considerably reduced n-3/n-6 PUFA ratio, similar to the cardiac tissue from fish fed a diet containing SO, it is unlikely that this was the only explanation for the growth inhibition since supplementation with other n-6 PUFA, including AA, at the same concentration did not affect cell growth. However, it is important to note that in cardiac tissue from SO-fed fish, some of which developed cardiac lesions, 20:3n-6

showed the biggest percentage increase of any C_{20} PUFA when compared to fish fed FO.

Alterations in the dietary n-3/n-6 PUFA ratio can alter the physico-chemical properties of the membranes and consequently the activities of membrane-associated enzymes. Examples include mitochondrial ATPase (Robblee and Clandinin, 1984), sarcolemmal 5' -nucleotidase (Awad and Chattopadhyay, 1983), ornithine decarboxylase (Bunce and Abou-El-Ela, 1990) and sarcoplasmic reticulum (SR) Ca^{2+}-Mg^{2+} ATPase (Swanson *et al.* 1989). The latter enzyme is an assymetric integral transmembrane protein (Scales and Inesi, 1976) of the SR which controls calcium flux in excitable tissues e.g. cardiac myocytes. Swanson *et al.* (1989) showed that a relatively small increase in the n-3/n-6 PUFA ratio in the SR phospholipid could cause a large decrease (around 6-fold) in murine Ca^{2+}-Mg^{2+} ATPase activity. In the present study with salmon heart, considerable changes in the n-3/n-6 PUFA ratio in cardiac phospholipids caused no significant alteration in SR Ca^{2+}-Mg^{2+} ATPase activity. The major alteration in the fatty acid composition of murine SR was an increase in DHA, particularly in PC of mice given menhaden oil, whereas the present study showed no significant dietary-induced alteration in phospholipid DHA. Rabbits given menhaden oil showed no incorporation of DHA but some incorporation of EPA into skeletal muscle SR and consequently, no alteration of skeletal muscle SR Ca^{2+}-Mg^{2+}ATPase was observed (Gould *et al.* 1987). Thus, it appears that DHA may be essential in the control of the SR calcium pump. While the work of Swanson *et al.* (1989) indicates decreased activity of the SR calcium pump in mice fed menhaden oil, a study by Karmazyn *et al.* (1987) observed enhanced uptake of Ca^{2+} in cardiac myocytes from rats fed cod-liver oil. Infante (1987) suggests that low dietary n-3 (or high n-6) PUFA may encourage uncoupling of the SR Ca^{2+}ATPase, such that ATP hydrolysis may be occurring in

the absence of calcium translocation. This possibility was not examined in the present work. The importance of the dietary n-3/n-6 PUFA ratio on the microenvironment of the Ca^{2+}-Mg^{2+}ATPase and thus, the control of Ca^{2+}/ATP coupling is an obvious area for further study.

A recent study has shown that alterations in the membrane n-3/n-6 PUFA ratio in cultured rat ventricular myocytes can influence the activity of enzymes involved in lipid metabolism. Nalbone *et al.* (1990) demonstrated that cardiomyocyte cultures supplemented with n-6 PUFA had a higher phospholipase A activity and lower lysophospholipase activity compared to those supplemented with n-3 PUFA. In this study the activity of cardiac phospholipase A was significantly increased in fish fed 18:2n-6-rich SO compared to fish fed either FO or LO. Increased phospholipase activity towards endogenous phospholipids could increase turnover of membrane fatty acids, provide increased substrate for eicosanoid synthesis and generally have a destabilising effect on membrane integrity. Although lysophospholipase activity was not measured in the present study increased hydrolysis of PC could lead to increased production of lyso PC which is a potent cytolytic agent (Choy *et al.* 1989).

The term eicosanoid was first used by Corey *et al.* (1980) to describe a number of biologically active metabolites of eicosapolyenoic fatty acids which include prostaglandins and thromboxanes (collectively called prostanoids) and leukotrienes (Johnson *et al.* 1983). Eicosanoids are produced in response to various extracellular stimuli by two main dioxygenase enzymes, cyclooxygenase and lipoxygenase, and their products have been identified in almost all mammalian tissues and fluids (Horrobin, 1978). Eicosanoids have been found in a large range of freshwater and marine fish (Mustafa and Srivastava, 1989) and virtually all fish tissues studied, including spleen, kidney, gill, heart, liver, stomach, intestine, muscle, brain, skin, reproductive organs and blood cells have shown cyclooxygenase activity (Tocher, 1995).

Many of the beneficial effects of fish oil supplements in attenuating a number of

atherothrombotic, autoimmune and inflammatory conditions are, in part, due to the modulation of AA-derived eicosanoids by EPA and DHA (Weber, 1990). Suppression of AA-derived eicosanoids by dietary 18:3n-3 has also been observed in mammals (Marshall and Johnson, 1982). The enzymatic pathway producing eicosanoids is often called the 'arachidonic acid cascade', since AA, which yields 2-series prostaglandins, is the principal precursor in mammals (Horrobin, 1983). However, 20:3n-6 and EPA are also eicosanoid precursors, yielding 1- and 3-series prostaglandins, respectively, although both are poorer substrates than AA for prostaglandin synthetase (Crawford, 1983). While both 20:3n-6 and EPA are inferior to AA as substrates for prostaglandin production they can act as competitive inhibitors of AA for the enzyme binding sites (Willis, 1981). In addition, 1- and 3-series prostanoids generally have lower biological activity compared to their 2-series homologues and thus dietary supplementation with 20:3n-6, EPA or their C_{18} precursors can attenuate production and efficacy of AA-derived eicosanoids (Willis, 1981; Weber, 1990).

Feeding salmon increasing levels of 18:2n-6 resulted in moderately increased phospholipid AA with simultaneously reduced phospholipid EPA and thereby substantially increased the 20:4/20:5 eicosanoid precursor ratio in membrane phospholipids (page 83-86, 88-92, 94-97). In dietary experiment C feeding LO resulted in decreased AA and increased EPA in tissue phospholipids. Thus, the feeding of dietary SO or LO might be expected to alter the spectrum of eicosanoids produced by these fish compared to those fed FO. This effect is well illustrated by comparing the fatty acid compositions of liver PI from the third dietary experiment (page 126). AA was increased from 28% in FO-fed fish to 42% in SO-fed fish while the value in LO-fed fish was reduced to 21%. EPA was reduced from 4.9% in FO-fed fish to 0.7% in SO-fed fish but increased to 10.1% in fish fed LO. The tendency for the PI fraction to accumulate C_{20} PUFA in fish has resulted in the hypothesis that this phospholipid might be the source of precursor fatty acids for eicosanoid

production (Bell *et al.* 1983, German and Hu,1990).

Given the changes in eicosanoid precursor availability resulting from feeding dietary SO, the possibility exists that the cardiac myopathy observed in the present study arises from an over production of AA-derived eicosanoids. Cardiac-derived prostaglandins are important modulators of myocardial function (Karmazyn and Dhalla, 1983) with prostacyclin (PGI_2) being the primary cyclooxygenase-derived product in mammalian heart (Karmazyn *et al.* 1987). In this study, the production of 6-keto $PGF_{1\alpha}$, the stable metabolite of PGI_2, and TXB_2, the stable metabolite of thromboxane (TXA_2), were both increased in stimulated cardiac myocytes from salmon fed high 18:2n-6 compared to those fed FO. Production of PGE_2 by stimulated cardiac myocytes was increased in fish fed both SO and LO compared to those fed FO, although the antibody used to measure PGE_2 is equally reactive with PGE_3 and thus the precise effect on the AA-derived homologue could not be determined. Therefore, the results presented in the present studies should really be interpreted as total PGE and total TXB, although evidence suggests that the majority of these will be AA-derived (Anderson *et al.* 1981; Bell *et al.* 1994b). Thromboxane is an important mediator of platelet aggregation, vascular tone and intracellular calcium concentration (Weber, 1990). Studies investigating skeletal muscle synthesis have shown that PGI_2 is stimulatory whereas PGE_2 is inhibitory to muscle fibre formation (McLennan, 1991). PGE_2 can also stimulate protein catabolism (Barnett and Ellis, 1987). If similar effects were active in salmon cardiac myocytes it may explain the apparent loss of musculature which occurred in fish fed SO.

In addition to eicosanoids produced by cardiac myocytes themselves heart tissue is also subjected to eicosanoids produced in the bloodstream. Fish which developed the histopathological cardiac lesion showed extensive leucocyte infiltration at the lesion site. Thus, increased production of highly bioactive AA-derived eicosanoids by leucocytes may be responsible for the severity of lesion development. For these reasons the levels of plasma eicosanoids and those

produced by blood stimulated by the calcium ionophore, A23187, were measured. Stimulated whole blood was used in these studies, instead of isolated leucocytes, to study eicosanoid production because this more closely resembles the *in vivo* situation where interactions between different cell types are known to have profound effects on the spectrum of eicosanoids produced (Lagarde *et al.* 1989). Studies with rainbow trout have indicated that leucocytes are the only blood cells which are directly capable of eicosanoid production and that no eicosanoids are generated by ionophore-stimulated erythrocytes (Pettitt *et al.* 1989). The changes in leucocyte membrane fatty acid composition in the present study are reflected in the nature of the eicosanoids produced on stimulation with A23187. Salmon given SO showed significantly increased levels of plasma 6-keto $PGF_{1\alpha}$ and TXB_2 while LTB_4 was increased and LTB_5 decreased after stimulation of blood cells with A23187 compared to fish fed FO. LTB_4 is a particularly powerful chemotactic agent for neutrophils and it is also a potent agonist in stimulating cellular uptake of calcium (Strasser *et al.* 1985). EPA-derived LTB_5, however, is up to 30 times less potent in mammals (Lee *et al.* 1984) and one study in fish suggests that rainbow trout leucocytes are indeed less sensitive to this isomer (Secombes *et al.* 1994). Levels of the 12-lipoxygenase product, 12-HEPE derived from EPA were significantly increased in stimulated blood cells, while levels of AA-derived 12-HETE were significantly reduced in fish fed FO compared to those fed SO.

In addition to cardiac myocytes and blood cells it was decided to look at eicosanoid production in gill cells since they are known to possess a highly active 12-lipoxygenase (German *et al.* 1986) and also contain significant cyclooxygenase activity (Tocher, 1995). While trout gill homogenates have a 12-lipoxygenase capable of converting exogenous radiolabeled AA and EPA to their 12-hydroxy metabolites (German *et al.* 1986), there has been little information on the production of these metabolites from endogenous precursor fatty acids derived from gill phospholipids. Dietary study A (page 72) demonstrates that in Atlantic salmon given

FO the major product from gill cells is EPA-derived 12-HEPE. This is in contrast to a study using gill homogenates from rainbow trout which demonstrated that AA was the major fatty acid released from membrane phospholipids and that 12-HETE was the predominant metabolite (German and Hu, 1990). While the diet used by German and Hu (1990) is not known, in the present study only in salmon given the highest level of dietary SO did the ratio of 12-HETE/12-HEPE increase towards unity. Stimulated gill cells from fish fed LO produced a level of 12-HEPE greater than SO-fed but lower than FO-fed fish and the 12-HETE/12-HEPE ratio was not significantly different to that in FO-fed fish.

While feeding SO caused a reduction in the ratio of EPA:AA-derived lipoxygenase products in gills, it also caused a dramatic reduction in total lipoxygenase metabolites produced from both AA and EPA. Similarly, there was a decrease in 6-keto $PGF_{1\alpha}$ but no significant decrease in TXB_2 produced by gill cells from fish fed SO. It is difficult to account for the marked decrease in eicosanoid products in gills in terms of altered levels of their substrate fatty acids esterified in phospholipids. However, it may be notable that the amount of 20:3n-6 was increased up to 7-fold in fish fed SO and this fatty acid is known to inhibit production of AA-derived lipoxygenase metabolites (Miller *et al.* 1990) and prostaglandins (Bell *et al.* 1994b). PGE_1, which is derived from 20:3n-6, can cause increased synthesis of cAMP, a potent inhibitor of AA mobilisation (Horrobin, 1980). Recent studies with rats have shown that a decrease in dietary n-3/n-6 PUFA ratio can affect the activity of phospholipase A_2, being either decreased in gastric mucosa (Grataroli *et al.* 1988) or increased in cardiac myocytes (Nalbone *et al.* 1990). In a very recent study the activity of gill phospholipase A activity was reduced in salmon fed SO (66%) and LO (40%) compared to fish fed FO (Bell *et al.* 1996). A decrease in phospholipase activity in the gills of salmon fed SO in the present studies would result in less available substrate for lipoxygenase and cyclooxygenase and could explain the diminished eicosanoid production which were observed in gills. However, in dietary

trial C the reduction in gill lipoxygenase products was accompanied by increased production of prostanoids, suggesting that PUFA substrates which were not utilised by lipoxygenase were metabolised via cyclooxygenase. Clearly, the spectrum of eicosanoids produced by a particular cell is dependent on a more complex mechanistic process than the concentration or ratios of substrate PUFA esterified in membrane phospholipids alone.

Previous studies with rainbow trout leucocytes (Pettitt and Rowley, 1990) and with rat platelets and guinea-pig epidermis (Careaga-Houck and Sprecher, 1989; Miller *et al.* 1990) have failed to detect DHA-derived lipoxygenase metabolites from endogenous DHA, although human platelets could synthesise 14-HDHE from exogenous [1-^{14}C]-DHA (Aveldano and Sprecher, 1983). In dietary study B, (page 100) gill cells from fish fed FO produced greater amounts of 14-HDHE than 12-HETE and only marginally less than 12-HEPE. Feeding SO resulted in reduced production of 14-HDHE as well as 12-HEPE and 12-HETE. While the production of 14-HDHE, derived from endogenous DHA, has been observed in homogenates of trout gill (German and Hu, 1990), the present work shows that 12-lipoxygenase products of DHA are generated, from endogenous precursors, by intact gill cells. The ability of gills to synthesise 14-HDHE has also been observed in rainbow trout (*Oncorhynchus mykiss)* (Knight *et al.* 1995).

The tendency for PI to accumulate C_{20} PUFA, especially AA, in fish which otherwise contain large amounts of DHA and EPA, has suggested that this lipid class may be a potential source of eicosanoid precursors (Bell *et al.* 1983; German and Hu, 1990). However, the ratio of 12-HETE/12-HEPE produced by stimulated gill cells in the current experiments was most similar to the AA/EPA ratio present in gill PC (0.20, 0.61 and 0.20 for the FO, SO and LO diets respectively). Comparison of the precursor fatty acid ratios of both PC and PI would make the former class the more likely source of lipoxygenase substrate fatty acids. PC-specific phospholipases have been identified in mammalian platelets and cultured umbilical

cells (Fischer *et al.* 1984; Takayama *et al.* 1989). In contrast, stimulated whole blood cells (Dietary experiment 2) showed average values for 12-HETE/12-HEPE and LTB_4/LTB_5 ratios which most closely resemble the AA:EPA ratio in leucocyte PE. It is possible that PI may represent a slowly turning over pool of eicosanoid precursors whereas in a rapid turnover situation, such as stimulation with A23187, the precursors are derived from the major phospholipid classes, PC and PE.

Although few specific physiological roles have been identified for lipoxygenase products a number of recent studies have implicated 12-HETE as a modulator of ion-channels (Buttner *et al.* 1989; Massferrer *et al.* 1990). Clearly this activity would be particularly important in gills which, along with the posterior kidney are vital in controlling osmoregulation in fish. The ability of fish kidney and gill to synthesise prostaglandins has been established for some time although their functions were largely unknown (Ogata *et al.* 1978; Christ and van Dorp, 1972). However, PGE_2 and PGE_1 are both active antiduiretic agents in trout and eel kidney (Wales and Gaunt, 1986; Brown *et al.* 1991) while PGE_2 inhibits ion transport in gill epithelia (Van Praag *et al.* 1987). Clearly diet-induced changes in gill (and kidney) eicosanoid products might therefore affect the ability of salmon to adapt to salinity changes which would be especially important in salmon parr about to undergo transition to seawater. Prostaglandins can reduce the activity of Na^+/K^+-ATPase in kidney tubules allowing more Na^+ to be lost via urinary excretion (Jabs *et al.* 1989) and can function in conjunction with hormones to control osmoregulation (Mustafa and Srivastava, 1989). In addition, leukotrienes and HETES, products of lipoxygenases, can inhibit Na^+, K^+ and Cl^- absorption by the kidney (Egan *et al.* 1992; Satoh *et al.* 1993). The gills and gut epithelium are other important sites of osmoregulation in fish (Girard *et al.* 1977; Di Constanzo *et al.* 1983) and both tissues had increased levels of AA in masu salmon (*Oncorhynchus masou)* undergoing parr-smolt transformation (Li and Yamada, 1992). Wild Atlantic salmon

parr contain much higher levels of AA than their farmed counterparts, and this factor could be especially important during smoltification and transition to seawater, where the functionality of eicosanoids in osmoregulatory tissues would be vital to achieve physiological adaptation to increased salinity.

8.2 Overall summary

1. Practical-type diets containing either fish oil (FO), sunflower oil (SO) or linseed oil (LO) as their lipid component were fed to Atlantic salmon post-smolts for up to 16 weeks. All diets contained levels of n-3 PUFA sufficient to satisfy growth and development. No differences in final weights between the different dietary treatments were observed in trials A and C. However, in trial B fish fed the intermediate level of linoleic acid (25%) had significantly higher final weights compared to both other treatments while fish fed the highest level of linoleic acid (45%) had significantly lower final weights compared to both other treatments (page 80).

2. A number of fish fed SO developed severe cardiac lesions which caused thinning of the ventricular wall and necrosis. In addition the fish fed SO suffered a transportation-induced shock syndrome which resulted in mortalities in excess of 30%. A small number of fish fed FO developed a similar cardiac lesion, albeit in a less severe form, whereas the lesion was virtually absent in fish fed LO.

3. Incorporation of linoleic acid into membrane phospholipids was increased in response to dietary intake. In addition to linoleic acid, tissue phospholipids from fish fed SO showed increased 20:2n-6 (up to 4-fold), 20:3n-6 (up to 7-fold) and AA (up to 3-fold), and decreased 20:5n-3 (up to 2-fold). The ratio of n-3/n-6 PUFA in fish fed SO decreased (up to 4-fold) and the AA/EPA ratio increased (up to 9-fold) in tissue phospholipids.

4. The tissue phospholipids from fish fed LO had increased levels of 18:2n-6, 20:2n-6 and 20:3n-6 but AA, 22:4n-6 and 22:5n-6 were similar, or significantly reduced, compared to fish fed FO. Tissue phospholipids from fish fed LO also had increased 18:3n-3 and 20:4n-3 compared to both other treatments while in certain tissues and phospholipid classes EPA was increased compared to fish fed FO. In addition, fish fed LO had reduced levels of PC in cardiac membranes, compared to fish fed FO or SO, which resulted in a PC:PE ratio of less than unity.

5. These changes in eicosanoid precursor ratios were reflected in altered eicosanoid production. 12-HEPE, derived from EPA, was the major 12-lipoxygenase product produced by gill cells, from FO-fed fish, stimulated with A23187. Production of 12-HEPE and also 12-HETE, 14-HDHE and TXB_2 were all significantly decreased in fish fed both SO and LO compared to fish fed FO. However, the ratio of 12-HETE/12-HEPE generally reflected phospholipid AA/EPA ratios.

6. Blood leucocytes stimulated with A23187 showed increased production of LTB_4, 12-HETE and TXB_2, and reduced production of LTB_5 and 12-HEPE in fish fed SO compared to fish fed FO. Stimulated blood leucocytes from fish fed LO produced less TXB_2 compared to those fed SO and less PGE_2 compared to those fed both SO and FO.

7. In isolated cardiac myocytes stimulated with A23187, TXB_2 production was significantly increased in fish fed the highest dietary level of 18:2n-6 (45% of total fatty acids) compared to fish fed the lower levels of dietary linoleic acid (10 and 25% of total fatty acids). The activity of heart sarcoplasmic reticulum Ca^{2+}-Mg^{2+} ATPase was not affected by dietary lipid.

8. In an established cell line derived from chum salmon hearts (CHH-1) $\Delta 6$ desaturase activity was considerable but showed no preference for n-3 substrates over n-6. CHH-1 cells also exhibited significant $\Delta 5$ desaturase activity which showed a preference towards n-3 PUFA. No $\Delta 4$ desaturation activity was observed. Elongation of C_{20} PUFA was particularly active in CHH-1 cells with C_{22} PUFA being incorporated specifically into PE and PS.

9. CHH-1 cells supplemented with 20:3n-6 showed reduced growth rate, cell death and unusual pycnotic appearance, compared to those supplemented with other PUFA.

10. The fatty acid compositions of triacylglycerols (TAG) and phospholipids in hearts and livers of both farmed parr and pre-smolts contained greater amounts of monoenoic fatty acids, largely due to increased 20:1n-9 and 22:1n-11, compared to

their wild counterparts. TAG, PC and PE from heart and liver of wild fish contained more 18:2n-6 and AA compared to farmed fish. Linolenic acid was increased in all lipid classes of wild fish while EPA and 22:5n-3 were increased in hearts and livers of wild fish compared to farmed. DHA levels were considerably higher in both tissues from farmed fish but particularly in heart PC, PS and TAG. The n-3/n-6 PUFA ratios were generally lower in hearts compared to livers and were lower in wild compared to farmed fish, largely due to increased n-6 PUFA in wild fish, and in particular AA.

8.3 Future studies

The development of a pronounced cardiac lesion in salmon fed diets containing high levels of 18:2n-6 (and having a low n-3/n-6 PUFA ratio) was unexpected and previously unrecorded in the literature. The accompanying increase in AA-derived eicosanoids in fish fed SO suggests that over production of series 2 prostaglandins and series 4 leukotrienes may be implicated in the lesion development. This suggestion is supported by the apparent ability of LO, which significantly reduces phospholipid AA but maintains or increases EPA compared to fish fed FO, to reduce the incidence of cardiac lesions. It would therefore be interesting to see if a similar suppression of the lesion occurred on feeding an oil enriched in EPA, for example a southern hemisphere fish oil like sardine oil.

Supplementation of the cardiac-derived cell line, CHH-1, with 20:3n-6 resulted in reduced growth rate, cell death and unusual cellular appearance. Interestingly, feeding diets rich in 18:2n-6 resulted in 20:3n-6 being increased, in percentage terms, to a greater degree than any other C_{20} PUFA. Thus, increased levels of 20:3n-6 in salmon membrane phospholipids may also be involved in the cellular damage to cardiac tissue recorded in fish fed SO. A dietary trial utilising borage oil or evening primrose oil would be useful because they are rich in 18:3n-6 which should lead to an accumulation of 20:3n-6 since the activity of the C_{18} elongase is considerably greater than that of the $\Delta 5$ desaturase. A trial with an 18:3n-6-rich oil

would be doubly interesting since these oils are generally regarded as anti-inflammatory, at least in mammals, due to their ability to reduce AA levels and the ability of 20:3n-6 to act as a competitive inhibitor of cyclooxygenase and lipoxygenase. Thus, 20:3n-6 can reduce the amount of AA-derived eicosanoids produced, in a similar manner to EPA, and also forms series 1 prostaglandins which, in mammals at least, are less biologically potent compared to their series 2 homologues. Indeed the reduced production of eicosanoids in gills of salmon fed SO may be attributed to the inhibitory actions of series 1 prostaglandins, especially PGE_1, on the hydrolysis of AA from membrane phospholipids. PGE_1 has a range of pharmacological activities in mammals, including stimulation of cAMP formation which in turn can inhibit phospholipase A_2 and thus the mobilisation of AA in a manner similar to steroids (Horrobin, 1992).

In the present study the prostaglandins measured were restricted to those of the 2 series i.e. derived from AA. Recent developments in our laboratory, coupled with the availability of standards, have allowed measurement of prostaglandins of the 3-, 2- and 1-series from the same sample. These techniques would be applied in future dietary trials to ascertain the effects of diets which increase membrane EPA and 20:3n-6 on concentrations of prostaglandins derived from all three C_{20} precursors.

The CHH-1 established cell line proved useful in studying the incorporation and metabolism of polyunsaturated fatty acids in cardiac-derived cells from a salmonid fish. In addition, it would be useful to prepare primary cultures of salmon cardiac myocytes, as myocytes can be routinely cultured from mammals, to further investigate lipid metabolism in heart cells and also to test the effects of supplementaion with 20:3n-6, AA and EPA on cell growth and eicosanoid production in vitro.

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High Dietary Linoleic Acid Affects the Fatty Acid Compositions of Individual Phospholipids from Tissues of Atlantic Salmon (*Salmo salar*): Association with Stress Susceptibility and Cardiac Lesion

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ABSTRACT For 16 wk Atlantic salmon (*Salmo salar*) post-smolts were fed practical-type diets that contained either fish oil (FO) or sunflower oil (SO) as the lipid component. Both diets contained adequate (*n*-3) polyunsaturated fatty acids (PUFA). All the phospholipids of heart and liver from SO-fed fish had increased levels of 18:2(*n*-6), 20:2(*n*-6) and 20:3(*n*-6); phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) also had increased 20:4(*n*-6). There was a general decrease in 20:5(*n*-3) in the phospholipids, reflected in an increase in the 20:4(*n*-6)/20:5(*n*-3) ratio, especially in PC and PE. The fatty acid compositions of phospholipids from brain and retina were much less affected by dietary linoleate than those of heart and liver. Fish fed SO developed severe heart lesions that caused thinning of the ventricular wall and muscle necrosis. The fish fed SO also were susceptible to a transportation-induced shock syndrome that caused 30% mortality. These results establish that a diet with a low (*n*-3)/(*n*-6) ratio can cause changes in fatty acid metabolism that are deleterious to the health of salmonid fish, especially when subjected to stress. J. Nutr. 121: 1163–1172, 1991.

INDEXING KEY WORDS:

• *linoleic acid* • *fish* • *stress*
• *heart* • *phospholipids*

Freshwater fish (e.g., rainbow trout, eel, ayu) can metabolize linoleic acid [18:2(*n*-6)] by Δ-6-desaturation to gammalinolenic acid [18:3(*n*-6)], which can undergo elongation to dihomogammalinolenic acid [20:3(*n*-6)] and subsequently be converted to arachidonic acid [20:4(*n*-6)] by Δ-5-desaturation (1). Marine fish such as turbot (*Scopthalmus maximus*) seem to be deficient in Δ-5-desaturase and therefore require the polyunsaturated fatty acids (PUFA)[1] eicosapentaenoic acid [20:5(*n*-3)], docosahexaenoic acid [22:6(*n*-3)] and probably 20:4(*n*-6) to be supplied by the diet (2, 3). Studies with an anadramous fish, Atlantic salmon (*Salmo salar*), suggest an inability to desaturate and elongate 18:2(*n*-6) during smoltification (4), but this inability is not apparent during the post-smolt seawater phase of the salmon life cycle (5). In this study Atlantic salmon post-smolts were fed diets in which the dietary lipid was supplied either as fish oil (FO) or sunflower oil (SO), although the latter diet contained adequate (*n*-3) PUFA (6) derived from dietary fish meal. Our objective was to alter the (*n*-3)/(*n*-6) fatty acid ratio in salmon by feeding a diet high in (*n*-6) fatty acids and to specifically investigate the incorporation of 18:2(*n*-6) and its metabolites into the individual phospholipids of heart, liver, brain and retina. Heart and liver were chosen because of their known high metabolic activity, whereas brain and retina were investigated because they are especially rich in (*n*-3) PUFA (7). The (*n*-3) PUFA are reported to have an important functional role in both brain and retina (8), which could be seriously impaired if significant incorporation of (*n*-6) PUFA were to occur. This supposition has now been established for primates (9).

[1]Abbreviations used: BHT, butylated hydroxytoluene; FO, fish oil; PC, phosphatidyl choline; PD, pancreas disease; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PS, phosphatidyl serine; PUFA, polyunsaturated fatty acid; SO, sunflower oil.

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TABLE 1

Composition of the basal diet

Ingredient	Amount
	g/kg
Fishmeal	650
Starch[1]	150
Oil[2]	100
Vitamin mix[3]	10
Mineral mix[4]	24
α-Cellulose	61.5
Antioxidant mix[5]	0.4
Choline chloride	4

[1]Passelli WA4, Tunnel Avebe Starches, Gillingham, U.K.

[2]Fosol, Seven Seas, Hull, U.K. or sunflower oil, Tesco, Cheshunt, U.K.

[3]Supplied (mg/kg diet): all-*rac*-α-tocopheryl acetate, 40; menadione, 10; ascorbic acid, 1000; thiamine hydrochloride, 10; riboflavin, 20; pyridoxine hydrochloride, 12; calcium pantothenate, 44; nicotinic acid, 150; biotin, 1; folic acid, 5; cyanocobalamin, 0.02; myo-inositol, 400; retinyl acetate, 7.3; cholecalciferol, 0.06.

[4]Supplied (per kg diet): KH_2PO_4, 22 g; $FeSO_4 \cdot 7H_2O$, 1.0 g; $ZnSO_4 \cdot 7H_2O$, 0.13 g; $MnSO_4 \cdot 4H_2O$, 52.8 mg; $CuSO_4 \cdot 5H_2O$, 12 mg; $CoSO_4 \cdot 7H_2O$, 2 mg; KI, 2 mg.

[5]Dissolved in propylene glycol and contained (g/L): butylated hydroxy anisole, 60; propyl gallate, 60; citric acid, 40.

TABLE 2

Fatty acid composition of diets

Fatty acid	Fish oil diet	Sunflower oil diet
	weight %	
14:0	5.7	2.0
16:0	13.8	9.7
18:0	2.5	3.4
16:1(*n*-7)	5.2	2.2
18:1(*n*-9)	12.0	14.7
18:1(*n*-7)	2.3	2.3
20:1(*n*-9)	8.9	3.2
22:1(*n*-11)	13.8	4.0
24:1	1.4	0.4
18:2(*n*-6)	1.4	42.1
18:3(*n*-6)	0.2	1.1
20:2(*n*-6)	0.2	<0.1
20:3(*n*-6)	0.2	0.1
20:4(*n*-6)	0.6	0.4
18:3(*n*-3)	1.3	0.4
18:4(*n*-3)	2.9	0.7
20:5(*n*-3)	7.2	3.4
22:5(*n*-3)	1.2	0.5
22:6(*n*-3)	10.4	4.6
total (*n*-6)	2.5	43.6
total (*n*-3)	23.8	9.9
(*n*-3)/(*n*-6)	9.4	0.2
Unsaturation index[1]	173.5	168.3

[1]Unsaturation index = wt % × number of double bonds.

MATERIALS AND METHODS

Animals and diets. Three hundred Atlantic salmon S1 smolts (initial mean weight, ~51 g) were obtained from the D.A.F.S. Fish Cultivation Unit (Aultbea, Wester Ross, Scotland) and transferred to seawater tanks at the same facility. The fish were transported from the Fish Cultivation Unit to the laboratory aquarium in sealed black polythene bags. Each bag contained ~12 L of seawater and 2 kg biomass of fish and was sealed after inflation with 25 L of oxygen. The initial water temperature was 13°C, and the final temperature was 15°C. The salmon were randomly distributed into two tanks of 2000-L capacity each; these tanks were supplied with seawater at a rate of 26 L/min. The tanks were subjected to natural photoperiod, and the temperature over the experimental period (June–October) was 9–13°C. The diets were supplied by automatic feeders, which were activated for a few seconds every 15 min during daylight hours and adjusted to provide 20 g/kg biomass each day. Fish were bulk weighed every 28 d, and the ration was adjusted accordingly. Initial sampling was performed after 16 wk of the feeding experiment. The experiment was conducted in accordance with the British Home Office guidelines regarding research on experimental animals.

The fish meal–based diets were formulated to meet the nutritional requirements of salmon (10) and contained 46% protein and 17% lipid (Table 1). Salmonid fish require ~1% of their dry diet as (*n*-3) lipid (11), which was supplied by both the FO and SO diets. The fatty acid compositions of the two diets are shown in Table 2.

Sampling procedure. Fish were sampled from both dietary treatments at 16, 18 and 20 wk after the start of the experiment. In total 21 fish fed the FO diet and 24 fish fed the SO diet were sampled. Fish were starved for 2 wk before the final sampling. The fish were killed by decapitation. The liver, heart, brain and eyes were quickly dissected and placed in liquid N_2. The cardiosomatic index was determined by dividing the heart weight in milligrams by the total body weight in grams. Samples were stored at −20°C prior to lipid analysis. Immediately before lipid extraction, the eyes were partly defrosted and cut in half, and the frozen lens and humor were removed. The whole retina, including the pigment layer, was dissected from the underlying tissues and placed in ice-cold chloroform:methanol (2:1, v/v).

Lipid extraction and analysis. Total lipid was extracted from tissues and diets by the method of Folch et al. (12). After homogenization in approximately 10 volumes of ice-cold chloroform:methanol (2:1, v/v), the homogenates were filtered, 0.2 volume of 8.8 g KCl/L was added and the solutions were mixed on a vortex mixer. After separation the chloroform layer was evaporated under a stream of nitrogen. The lipids were dried by vacuum dessication before weighing.

TABLE 3

Prevalence of heart muscle necrosis in salmon post-smolts fed diets containing fish oil or sunflower oil

Sampling time (wk)	Fish oil diet			Sunflower oil diet		
	Total sample	Normal fish	Fish with necrosis	Total sample	Normal fish	Fish with necrosis
16	5	5	0	5	1	4
18	6	6	0	10	1	9
20	10	9	1	9	5	4
Total	21	20	1	24	7	17

The extracts were redissolved in chloroform: methanol (2:1, v/v) at a final concentration of 100 g lipid/L and stored at –20°C. All solvents contained 50 mg of butylated hydroxytoluene (BHT) per liter as an antioxidant. Lipid extracts were separated into phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and phosphatidyl inositol (PI) fractions by TLC using methyl acetate: isopropanol:chloroform:methanol:2.5 g KCl/L (aqueous) (25:25:25:10:9, v/v/v/v/v) as developing solvent as described by Vitiello and Zanetta (13). The plates were sprayed with 1 g 2,7-dichlorofluorescein/L prepared in 970 mL/L methanol containing 50 mg BHT/L, and the lipid bands were visualized under UV light. The phospholipid classes were scraped from the plates and converted to fatty acid methyl esters by acid-catalyzed transmethylation performed overnight at 50°C according to Christie (14). The fatty acid methyl esters were separated and quantified by gas-liquid chromatography (Carlo Erba Fractovap 4160, Fisons, Crawley, U.K.) using a 50-m capillary column coated with free fatty acid phase (S.G.E., Milton Keynes, U.K.). Hydrogen was used as carrier gas and temperature programming was from 150°C at 5°C/min to 200°C (20 min) and then to 220°C at 10°C/min for 20 min. Individual methyl esters were identified by comparison with known standards and by reference to published data (15, 16).

Histology. Samples of liver, pancreas, kidney, spleen, heart and gills were fixed in 20% buffered formol saline and wax embedded, and sections were stained with hematoxylin and eosin. Pathological assessment was initially made on slides in sequence; but, when significant pathology was observed, code numbers were concealed and slides were randomized before final assessment of results to eliminate any bias in interpretation.

Materials. The TLC plates (20 cm × 20 cm × 0.25 mm), precoated with silica gel 60, were obtained from Merck (Darmstadt, Germany). All solvents were of HPLC grade and were obtained from Rathburn Chemicals (Walkerburn, U.K.).

Statistical analysis. Significance of difference ($P < 0.05$) between dietary treatments was determined by Student's t test. Data identified as nonhomogeneous (by means of an F test) were subjected to arcsin square root transformation before applying the t test. Data that were nonhomogeneous after transformation were analyzed by the Kruskal-Wallis test.

RESULTS

Good growth was achieved with both dietary treatments over the 16-wk experimental period (46 to 243 g, FO diet; 55 to 241 g, SO diet), and mortalities were low with both diets. However, when 30 fish per treatment were transported to the University aquarium at Stirling from the experimental unit (a 4-h journey), 30% of the SO-fed fish died, whereas all of the FO-fed fish survived. Although no fish were dead on arrival, several of the SO-fed fish were in a distressed condition, i.e., lying upside down and breathing with difficulty. The tanks were immediately bubbled with oxygen (for 30 min), but within 24 h 30% were dead. These symptoms are similar to the shock syndrome described by Castell et al. (17) in essential fatty acid–deficient rainbow trout.

The cardiosomatic index (measured in nonstressed fish prior to transportation) was significantly reduced ($P < 0.05$) in the SO-fed fish compared with the FO-fed fish (0.51 ± 0.10 and 0.74 ± 0.03; values are means ± SD for three fish per treatment) although the total lipid (percent by weight) values were similar. No other gross pathologies were evident in fish from either dietary regimen.

No significant histopathology was observed in the pancreas, kidney, spleen, heart and gills of the salmon fed the FO diet. However, one fish fed the SO diet showed areas of focal degenerative necrosis in the liver, and another fish had a similar lesion in the spleen and kidney. These organs were normal in all other fish sampled from this group; therefore, little significance probably can be attributed to these observations. However, a high proportion of fish fed the SO diet showed heart ventricle lesions ranging from mild to severe (Table 3). At low magnifications 45% of all hearts from SO-fed fish showed a marked depletion in

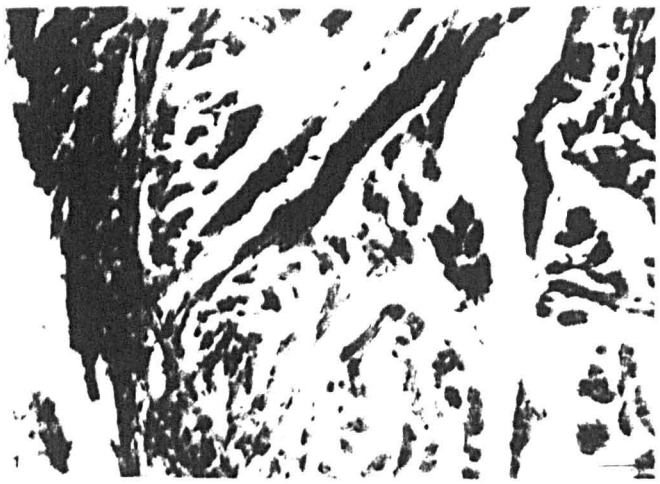

FIGURE 1 Light micrograph of heart tissue from Atlantic salmon fed the sunflower oil–containing diet. Micrograph indicates the presence of focal necrosis (arrow) and depletion in the amount of muscle present in both spongy and compact layers, resulting in thinning of the heart wall. (Scale = 20 μm).

the amount of muscle present in both the spongy and compact layers, with (in extreme instances) the heart wall becoming exceedingly thin (Fig. 1) compared with the normal organ (Fig. 2). Although it is not possible to quantify accurately heart wall thickness from sections because of differences in muscle contraction at fixation, this observation can be directly associated with the observed differences in cardiosomatic indices described earlier. Closer histological examination of affected hearts, as well as some not showing obvious muscle depletion, typically showed active focal myodegeneration, particularly in the spongy tissue (Fig. 1). The foci of degeneration ranged from single small lesions to multiple extensive areas. Similar lesions also were present in atrial muscle (Fig. 3). The observed gross wasting of the heart may have been a result of this active cardiomyopathy persisting over a prolonged period; although relatively few damaged areas may be seen in one section, the effect may be cumulative.

The fatty acid compositions of heart phospholipid classes are shown in Tables 4 and 5. In PC from SO-fed fish there were significant increases in 18:2(*n*-6), 18:3(*n*-6), 20:2(*n*-6), 20:3(*n*-6) and 20:4(*n*-6) compared with FO-fed fish. The SO-fed fish also showed significantly reduced 20:5(*n*-3) and total monoenes. In PE the same pattern of changes was observed, and in both classes the (*n*-3)/(*n*-6) ratio was significantly reduced in the SO-fed fish. A significant decrease in total saturates was also seen in PE. In PS of SO-fed fish there were increases in the (*n*-6) PUFA mentioned above, except for 20:4(*n*-6), which was unchanged. The levels of 18:3(*n*-3), 20:5(*n*-3) and 22:5(*n*-3) were significantly reduced as were total monoenes. Total saturates were significantly increased in the heart of SO-fed fish compared with those fed FO. In PI of hearts from SO-fed fish, there were increases in 18:2(*n*-6), 20:2(*n*-6), 20:3(*n*-6) and 20:4(*n*-6), whereas 20:5(*n*-3) and 22:6(*n*-3) were significantly reduced. The 20:4(*n*-6)/20:5(*n*-3) ratio was significantly increased in all phospholipid classes of fish fed SO. The (*n*-3)/(*n*-6) ratio was significantly reduced in both PS and PI of SO-fed fish.

The fatty acid composition of the liver phospholipid classes are shown in Tables 6 and 7. Liver PC from SO-fed fish showed significantly increased 18:2(*n*-6), 18:3(*n*-6), 20:2(*n*-6) and 20:3(*n*-6); the latter was elevated ~25 times that in FO-fed fish. Significant decreases in 20:5(*n*-3) and 22:5(*n*-3) were observed in the liver PC of SO-fed fish. Similar changes were apparent in liver PE, but, in addition, 20:4(*n*-6) and

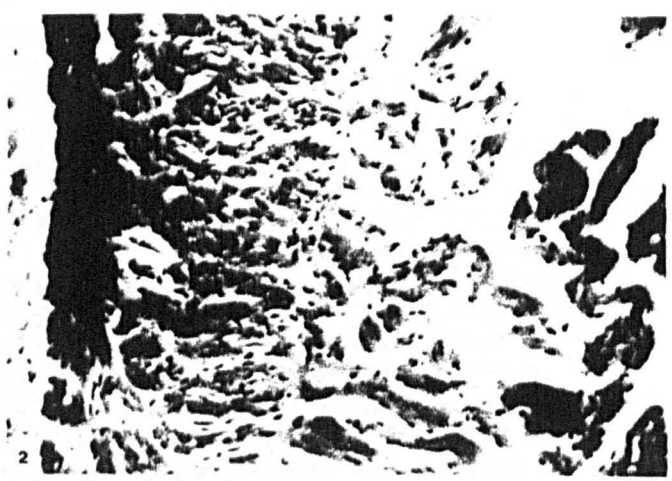

FIGURE 2 Light micrograph of heart tissue from Atlantic salmon fed the fish oil-containing diet. (Scale = 20 μm), showing normal appearance of compact and spongy muscle layers.

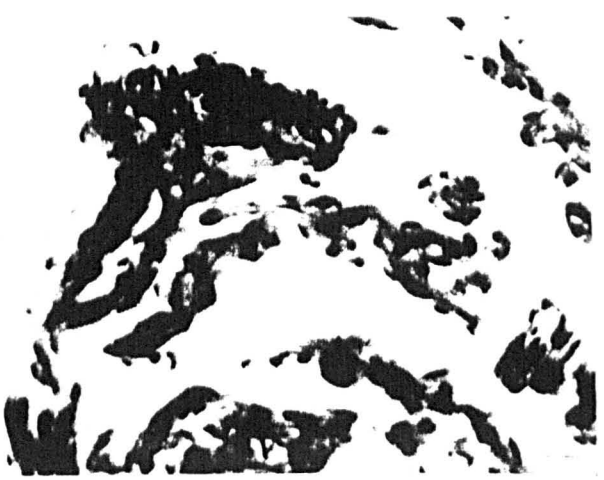

FIGURE 3 Light micrograph of heart atrium from Atlantic salmon fed the sunflower oil-containing diet. Micrograph indicates active focal myodegeneration in the muscle (Scale = 10 μm).

TABLE 4

Fatty acid composition of phosphatidyl choline and phosphatidyl ethanolamine from hearts of salmon fed diets containing fish oil (FO) or sunflower oil (SO)[1]

Fatty acid	Phosphatidyl choline		Phosphatidyl ethanolamine	
	FO diet	SO diet	FO diet	SO diet
	weight %			
Total saturates	40.8 ± 2.3	40.6 ± 1.3	24.0 ± 0.5*	20.8 ± 0.7
Total monoenes	18.3 ± 1.1*	14.8 ± 1.0	10.5 ± 0.6*	8.3 ± 0.7
18:2(*n*-6)	0.4 ± 0.1*	6.6 ± 0.5	0.7 ± 0.2*	6.7 ± 0.4
18:3(*n*-6)	0.1 ± 0.0*	0.3 ± 0.1	0.2 ± 0.0*	0.1 ± 0.0
20:2(*n*-6)	0.1 ± 0.0*	0.6 ± 0.1	0.1 ± 0.0*	0.7 ± 0.1
20:3(*n*-6)	0.1 ± 0.0*	0.9 ± 0.2	0.1 ± 0.0*	0.8 ± 0.2
20:4(*n*-6)	1.6 ± 0.1*	2.6 ± 0.4	1.5 ± 0.2*	2.9 ± 0.5
Total (*n*-6)[2]	2.8 ± 0.2*	11.3 ± 0.2	3.6 ± 0.1*	12.1 ± 0.4
18:3(*n*-3)	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0
20:5(*n*-3)	6.4 ± 0.2*	4.2 ± 0.3	3.9 ± 0.4*	2.5 ± 0.1
22:5(*n*-3)	0.9 ± 0.1	0.9 ± 0.0	2.7 ± 0.0	2.5 ± 0.2
22:6(*n*-3)	24.2 ± 1.9	22.6 ± 2.1	42.1 ± 1.4	43.5 ± 1.6
Total (*n*-3)[3]	32.1 ± 1.6	28.0 ± 2.0	49.4 ± 1.6	48.8 ± 1.6
Total PUFA[4]	34.9 ± 1.8	39.3 ± 1.9	53.0 ± 1.6*	61.0 ± 1.8
(*n*-3)/(*n*-6)	11.5 ± 0.4*	2.5 ± 0.2	13.5 ± 0.5*	4.0 ± 0.2
20:4/20:5	0.2 ± 0.1*	0.6 ± 0.2	0.4 ± 0.0**	1.1 ± 0.3

[1]Values are means ± SD from three salmon. *Values are significantly different ($P < 0.05$) as determined by Student's *t* test. **Values are significantly different ($P < 0.05$) as determined by Kruskal-Wallis analysis. SD < 0.05 are tabulated as 0.0.
[2]Includes 22:4(*n*-6) and 22:5(*n*-6).
[3]Includes 18:4(*n*-3) and 20:4(*n*-3).
[4]PUFA = polyunsaturated fatty acids.

TABLE 5

Fatty acid composition of phosphatidyl serine and phosphatidyl inositol from hearts of salmon fed diets containing fish oil (FO) or sunflower oil (SO)[1]

Fatty acid	Phosphatidyl serine		Phosphatidyl inositol	
	FO diet	SO diet	FO diet	SO diet
	weight %			
Total saturates	30.6 ± 1.4*	34.9 ± 1.7	31.2 ± 0.6	34.6 ± 3.0
Total monoenes	13.3 ± 0.7*	8.0 ± 0.1	9.4 ± 0.1	7.8 ± 1.2
18:2(*n*-6)	0.9 ± 0.1*	8.0 ± 0.1	0.6 ± 0.1*	4.2 ± 0.4
18:3(*n*-6)	0.7 ± 0.1*	0.4 ± 0.1	0.3 ± 0.0*	0.2 ± 0.0
20:2(*n*-6)	0.2 ± 0.0*	0.9 ± 0.2	trace*	0.3 ± 0.1
20:3(*n*-6)	0.1 ± 0.0*	1.0 ± 0.1	0.1 ± 0.1*	0.8 ± 0.2
20:4(*n*-6)	1.8 ± 0.8	1.9 ± 0.3	13.5 ± 0.1**	16.1 ± 2.1
Total (*n*-6)[2]	4.4 ± 0.8*	12.8 ± 0.4	15.0 ± 0.3*	22.0 ± 1.9
18:3(*n*-3)	0.6 ± 0.1*	0.1 ± 0.1	0.3 ± 0.0*	trace
20:5(*n*-3)	1.8 ± 0.3*	0.9 ± 0.2	7.6 ± 0.8*	3.6 ± 0.3
22:5(*n*-3)	3.2 ± 0.3*	2.1 ± 0.4	1.1 ± 0.2	1.4 ± 0.5
22:6(*n*-3)	37.3 ± 0.7	35.4 ± 2.1	28.9 ± 0.8*	22.6 ± 2.5
Total (*n*-3)[3]	43.4 ± 0.8*	36.9 ± 1.5	38.3 ± 0.2**	28.0 ± 2.8
Total PUFA[4]	47.8 ± 1.0	49.8 ± 1.9	53.3 ± 0.3*	50.0 ± 4.7
(*n*-3)/(*n*-6)	10.1 ± 2.0**	2.9 ± 0.1	2.6 ± 0.1*	1.3 ± 0.0
20:4/20:5	1.0 ± 0.4*	2.1 ± 0.3	1.8 ± 0.2*	4.5 ± 0.9

[1]Values are means ± SD from three salmon. *Values are significantly different ($P < 0.05$) as determined by Student's *t* test. **Values are significantly different ($P < 0.05$) as determined by Kruskal-Wallis analysis. Trace, values <0.1%. SD < 0.05 are tabulated as 0.0.
[2]Includes 22:4(*n*-6) and 22:5(*n*-6).
[3]Includes 18:4(*n*-3) and 20:4(*n*-3).
[4]PUFA = polyunsaturated fatty acids.

TABLE 6

Fatty acid composition of phosphatidyl choline and phosphatidyl ethanolamine from liver of salmon fed diets containing fish oil (FO) or sunflower oil (SO)[1]

Fatty acid	Phosphatidyl choline		Phosphatidyl ethanolamine	
	FO diet	SO diet	FO diet	SO diet
	weight %			
Total saturates	41.1 ± 4.3	38.2 ± 2.9	25.6 ± 2.7	22.9 ± 2.6
Total monoenes	18.3 ± 2.7	16.5 ± 3.5	23.2 ± 0.7*	15.1 ± 1.9
18:2(*n*-6)	0.6 ± 0.1*	11.9 ± 2.9	0.8 ± 0.1**	12.1 ± 2.1
18:3(*n*-6)	trace*	0.6 ± 0.4	trace	trace
20:2(*n*-6)	0.3 ± 0.2*	2.1 ± 1.1	0.3 ± 0.1*	2.8 ± 0.9
20:3(*n*-6)	0.2 ± 0.1*	5.1 ± 1.2	0.2 ± 0.1*	4.3 ± 0.2
20:4(*n*-6)	1.3 ± 1.0	2.7 ± 1.6	1.8 ± 0.2*	4.2 ± 0.2
Total (*n*-6)[2]	2.9 ± 1.5*	22.8 ± 1.6	4.0 ± 0.5*	24.1 ± 2.4
18:3(*n*-3)	0.1 ± 0.0	trace	0.2 ± 0.1*	trace
20:5(*n*-3)	4.8 ± 0.4*	1.9 ± 0.2	5.2 ± 0.4*	2.7 ± 0.4
22:5(*n*-3)	1.3 ± 0.1*	0.9 ± 0.1	1.6 ± 0.3	1.0 ± 0.3
22:6(*n*-3)	21.2 ± 3.3	15.0 ± 3.1	32.0 ± 1.6	30.1 ± 2.0
Total (*n*-3)[3]	27.6 ± 3.6*	17.8 ± 2.9	39.8 ± 1.7*	33.8 ± 1.7
Total PUFA[4]	30.5 ± 4.7	40.6 ± 4.1	43.7 ± 1.5*	57.9 ± 3.4
(*n*-3)/(*n*-6)	10.7 ± 3.8**	0.8 ± 0.1	10.1 ± 1.5*	1.4 ± 0.1
20:4/20:5	0.3 ± 0.2	1.5 ± 0.9	0.4 ± 0.1*	1.6 ± 0.2

[1]Values are means ± SD from three salmon. *Values are significantly different ($P < 0.05$) as determined by Student's *t* test. **Values are significantly different ($P < 0.05$) as determined by Kruskal-Wallis analysis. Trace, values <0.1%. SD < 0.05 are tabulated as 0.0.

[2]Includes 22:4(*n*-6) and 22:5(*n*-6).

[3]Includes 18:4(*n*-3) and 20:4(*n*-3).

[4]PUFA = polyunsaturated fatty acids.

TABLE 7

Fatty acid composition of phosphatidyl serine and phosphatidyl inositol from liver of salmon fed diets containing fish oil (FO) or sunflower oil (SO)[1]

Fatty acid	Phosphatidyl serine		Phosphatidyl inositol	
	FO diet	SO diet	FO diet	SO diet
	weight %			
Total saturates	34.9 ± 1.0	34.8 ± 3.8	36.5 ± 3.9	35.1 ± 3.2
Total monoenes	25.9 ± 4.5	24.3 ± 9.2	13.3 ± 1.1	10.4 ± 2.8
18:2(*n*-6)	1.2 ± 0.3*	9.2 ± 3.9	0.5 ± 0.0**	3.4 ± 1.2
18:3(*n*-6)	0.9 ± 0.3	0.4 ± 0.2	0.6 ± 0.1*	0.2 ± 0.1
20:2(*n*-6)	0.3 ± 0.2	1.1 ± 0.5	0.2 ± 0.1*	1.1 ± 0.3
20:3(*n*-6)	0.1 ± 0.1*	2.0 ± 0.3	0.7 ± 0.2*	3.9 ± 1.2
20:4(*n*-6)	0.4 ± 0.1	1.0 ± 0.5	19.5 ± 1.7	20.6 ± 4.2
Total (*n*-6)[2]	2.7 ± 0.2*	13.9 ± 3.9	21.8 ± 1.6*	29.5 ± 2.9
18:3(*n*-3)	0.3 ± 0.1*	trace	0.2 ± 0.1*	trace
20:5(*n*-3)	1.4 ± 0.6	0.7 ± 0.1	3.2 ± 0.3	2.6 ± 0.7
22:5(*n*-3)	1.1 ± 0.2	1.5 ± 0.5	1.5 ± 0.5	0.9 ± 0.2
22:6(*n*-3)	20.5 ± 3.8	13.1 ± 2.9	14.2 ± 1.2	13.1 ± 3.7
Total (*n*-3)[3]	24.0 ± 4.6	14.7 ± 3.3	19.4 ± 2.0	16.6 ± 4.2
Total PUFA[4]	26.7 ± 4.7	28.5 ± 5.4	41.2 ± 0.4	46.2 ± 3.0
(*n*-3)/(*n*-6)	8.7 ± 1.4*	1.1 ± 0.4	0.9 ± 0.2	0.6 ± 0.2
20:4/20:5	0.3 ± 0.1	1.4 ± 0.7	6.2 ± 1.2	8.3 ± 3.0

[1]Values are means ± SD from three salmon. *Values are significantly different ($P < 0.05$) as determined by Student's *t* test. **Values are significantly different ($P < 0.05$) as determined by Kruskal-Wallis analysis. Trace, values <0.1%. SD < 0.05 are tabulated as 0.0.

[2]Includes 22:4(*n*-6) and 22:5(*n*-6).

[3]Includes 18:4(*n*-3) and 20:4(*n*-3).

[4]PUFA = polyunsaturated fatty acids.

TABLE 8

Fatty acid composition of phosphatidyl choline and phosphatidyl ethanolamine from retina of salmon fed diets containing fish oil (FO) or sunflower oil (SO)[1]

Fatty acid	Phosphatidyl choline		Phosphatidyl ethanolamine	
	FO diet	SO diet	FO diet	SO diet
		weight %		
Total saturates	35.4 ± 1.1	33.3 ± 0.4	14.7 ± 0.9	14.6 ± 1.7
Total monoenes	20.3 ± 0.2	20.8 ± 0.4	18.8 ± 2.9	22.1 ± 3.4
18:2(*n*-6)	0.4 ± 0.1*	5.6 ± 0.1	0.5 ± 0.1**	2.8 ± 0.9
18:3(*n*-6)	trace	0.1 ± 0.0	0.1 ± 0.1	trace
20:2(*n*-6)	trace*	0.8 ± 0.1	trace*	0.5 ± 0.1
20:3(*n*-6)	trace*	0.9 ± 0.1	0.2 ± 0.1*	0.6 ± 0.1
20:4(*n*-6)	0.9 ± 0.0*	1.1 ± 0.0	1.7 ± 0.0	2.3 ± 0.6
Total (*n*-6)[2]	1.6 ± 0.1*	8.7 ± 0.2	2.7 ± 0.3*	6.5 ± 0.6
18:3(*n*-3)	0.1 ± 0.0	trace	0.2 ± 0.1	0.2 ± 0.1
20:5(*n*-3)	4.4 ± 0.1*	3.9 ± 0.2	3.0 ± 0.3*	2.4 ± 0.1
22:5(*n*-3)	0.8 ± 0.1	0.8 ± 0.1	1.9 ± 0.7	1.4 ± 0.1
22:6(*n*-3)	31.2 ± 2.6**	26.2 ± 0.6	43.6 ± 0.8	39.9 ± 5.5
Total (*n*-3)[3]	36.6 ± 2.2**	31.2 ± 0.5	48.5 ± 0.9	41.8 ± 5.6
Total PUFA[4]	38.4 ± 1.7	39.6 ± 0.3	50.8 ± 0.9	48.3 ± 5.9
(*n*-3)/(*n*-6)	23.4 ± 1.8*	3.6 ± 0.2	18.1 ± 1.5*	6.5 ± 0.8
20:4/20:5	0.2 ± 0.0*	0.3 ± 0.0	0.6 ± 0.1	1.0 ± 0.3

[1]Values are means ± SD from three salmon. *Values are significantly different ($P < 0.05$) as determined by Student's *t* test. **Values are significantly different ($P < 0.05$) as determined by Kruskal-Wallis analysis. Trace, values <0.1%. SD < 0.05 are tabulated as 0.0.

[2]Includes 22:4(*n*-6) and 22:5(*n*-6).

[3]Includes 18:4(*n*-3) and 20:4(*n*-3).

[4]PUFA = polyunsaturated fatty acids.

total PUFA were significantly increased and 18:3(*n*-3), 20:5(*n*-3) and total monoenes were significantly decreased in SO-fed fish. In liver PS, 18:2(*n*-6) and 20:3(*n*-6) were significantly elevated and 18:3(*n*-3) was significantly decreased in SO-fed fish. In liver PI, values for 18:2(*n*-6), 20:2(*n*-6) and 20:3(*n*-6) were significantly increased in SO-fed fish. The (*n*-3)/(*n*-6) ratio in liver phospholipid classes was significantly reduced in SO-fed fish, except in PI. The 20:4(*n*-6)/20:5(*n*-3) ratio was generally increased in fish fed SO, although this was only significant in PE. No changes were observed in the amount of 22:4(*n*-6) and 22:5(*n*-6) in both heart and liver (data not shown).

The fatty acid compositions of the phospholipid classes from brain and retina were much less affected by dietary lipid composition than those of heart and liver. Changes generally were confined to PC and PE, and these fatty acid compositions are shown in Tables 8 and 9. In retinal PC and PE, significant increases were observed in 18:2(*n*-6), 20:2(*n*-6) and 20:3(*n*-6). In SO-fed fish, 20:4(*n*-6) was increased in PC but not PE. In both PC and PE 20:5(*n*-3) was decreased, and 22:6(*n*-3) was decreased in PC but not PE. In retinal PI, 22:6(*n*-3) also was significantly decreased (23.0% ± 1.6 to 15.2% ± 0.9). A similar pattern was observed in brain PC and PE where 18:2(*n*-6), 20:2(*n*-6) and 20:3(*n*-6) were increased in SO-fed fish but 20:5(*n*-3) was significantly decreased only in PC.

DISCUSSION

The presence of increased levels of 20-carbon (*n*-6) fatty acids in the individual phospholipid classes of heart and liver in SO-fed fish could be accounted for by appreciable Δ-6- and Δ-5-desaturase activity in these tissues. The high activity of the Δ-6-desaturase is particularly apparent in the liver where the elongation and desaturation of 18:2(*n*-6) could cause an accumulation of 20:3(*n*-6) and result in this fatty acid being the most abundant 20-carbon species in PC, PE and PS. Although 20:4(*n*-6) was increased in SO-fed fish, particularly in PC and PE of heart and liver, the accumulation of 20:3(*n*-6) is compatible with a rather low Δ-5-desaturase activity in Atlantic salmon. In fact, a low Δ-5-desaturase activity already has been observed by Hagve et al. (18) in isolated hepatocytes of rainbow trout. Salmon fed SO showed depressed levels of 20:5(*n*-3) in most phospholipid classes of heart and liver; subsequently, the 20:4(*n*-6)/20:5(*n*-3) ratio was considerably increased in these fish. Although both these fatty acids (after release from membrane phospholipids by phospholipase A_2) are substrates for both cyclooxygenase and lipoxygenase, mammalian studies indicate that 20:5(*n*-3)-derived eicosanoids tend to be of lower efficacy compared with

TABLE 9

Fatty acid composition of phosphatidyl choline and phosphatidyl ethanolamine from brain of salmon fed diets containing fish oil (FO) or sunflower oil (SO)[1]

Fatty acid	Phosphatidyl choline		Phosphatidyl ethanolamine	
	FO diet	SO diet	FO diet	SO diet
	weight %			
Total saturates	33.2 ± 2.6	31.3 ± 1.1	16.9 ± 0.2	17.1 ± 0.2
Total monoenes	47.6 ± 2.0	46.5 ± 0.4	30.9 ± 0.3	31.1 ± 0.4
18:2(*n*-6)	0.3 ± 0.0*	1.2 ± 0.3	0.3 ± 0.0*	1.1 ± 0.2
18:3(*n*-6)	trace	trace	0.1 ± 0.1	trace
20:2(*n*-6)	trace*	0.3 ± 0.1	trace*	0.4 ± 0.1
20:3(*n*-6)	trace*	0.3 ± 0.0	trace*	0.3 ± 0.1
20:4(*n*-6)	0.4 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	1.1 ± 0.2
Total (*n*-6)[2]	0.9 ± 0.0*	2.7 ± 0.3	1.2 ± 0.2*	3.0 ± 0.1
18:3(*n*-3)	trace	trace	trace	trace
20:5(*n*-3)	2.9 ± 0.1*	2.5 ± 0.1	4.8 ± 0.2	4.4 ± 0.1
22:5(*n*-3)	0.9 ± 0.1	1.0 ± 0.4	1.8 ± 0.2	1.6 ± 0.1
22:6(*n*-3)	11.5 ± 1.3	12.7 ± 0.7	20.9 ± 1.8	21.5 ± 0.8
Total (*n*-3)[3]	15.4 ± 1.5	16.3 ± 0.4	27.8 ± 2.0	27.7 ± 0.8
Total PUFA[4]	16.3 ± 1.5	18.9 ± 0.1	29.0 ± 1.9	30.6 ± 0.8
(*n*-3)/(*n*-6)	17.2 ± 1.6*	6.2 ± 1.0	22.9 ± 4.3**	9.3 ± 0.1
20:4/20:5	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1

[1]Values are means ± SD from three salmon. *Values are significantly different ($P < 0.05$) as determined by Student's *t* test. **Values are significantly different ($P < 0.05$) as determined by Kruskal-Wallis analysis. Trace, values <0.1%. SD < 0.05 are tabulated as 0.0.

[2]Includes 22:4(*n*-6) and 22:5(*n*-6).

[3]Includes 18:4(*n*-3) and 20:4(*n*-3).

[4]PUFA = polyunsaturated fatty acids.

their 20:4(*n*-6)–derived homologues (19, 20). Thus eicosanoids generated by heart and liver of SO-fed fish would tend to be of higher biological activity than those from FO-fed fish. However, 20:3(*n*-6) also is a substrate for cyclooxygenase and lipoxygenase, and the resulting eicosanoids are antagonistic to their 20:4(*n*-6)–derived counterparts (21). The eicosanoid status of the fish in this study, particularly after being subjected to stress, is an obvious area for further study. The fatty acid composition of phospholipids from brain and retina show only minor alterations as a result of dietary treatment. This probably reflects the slow turnover of fatty acids in these tissues as well as the relatively short period of the experiment. Interestingly, 22:6(*n*-3) was reduced in retinal PI of SO-fed fish (data not shown), a fact also observed in rat neural tissues when animals were fed FO or SO (22). Possibly 22:6(*n*-3) is redistributed into PS and PE [63 and 44% 22:6(*n*-3), respectively; data not shown in tables] when the dietary supply of this fatty acid is limited.

Nutritional cardiomyopathy has been recorded in a number of fish, particularly in intensive culture and especially associated with vitamin E and selenium deficiencies (23), but no cardiomyopathy has been specifically associated with (*n*-6) fatty acid intake. Fjolstad and Heyeraas (24) found muscular and myocardial degeneration in their study on Hitra disease in Norwegian farmed salmon and suggested a relationship to dietary deficiency of vitamin E and/or selenium. An extensive degenerative cardiomyopathy studied by Ferguson et al. (25) in association with pancreas disease (PD) of farmed salmon also was attributed by these authors to vitamin E and/or selenium deficiency. Although these authors considered the lesions of the pancreas to be linked to those of heart and muscle, McVicar (26) gave evidence that heart and muscle lesions are not consistently or primarily associated with PD. In addition, Bell et al. (27) considered that the depletion of vitamin E in PD-affected salmon was a consequence, rather than the cause, of the disease.

In the present study the oil source was the only difference between the two experimental diets. The high proportion of damaged hearts associated with the SO diet in comparison to the rarity of the condition in FO-fed fish indicates that the damage was a consequence of the dietary lipids. The apparent recovery from the cardiomyopathy by a proportion of the SO-fed fish observed in the study between wk 18 and 20, during which time no food was provided to the fish, tends to support this conclusion. Vitamin E probably is not involved in the cardiomyopathy because, although high amounts of PUFA are present in the SO

diet, dietary vitamin E was included in the diet at or above a threefold excess of requirement (10).

Although the exact cause of the cardiac lesion was not defined, two aspects deserve comment. The first is the similarity in some ways to the fainting or shock syndrome described by Sinnhuber (28) and Castell et al. (17) in trout fed a diet deficient in (*n*-3) PUFA. This syndrome was similar to a transportation shock syndrome originally described by Black and Barrett (29). In rainbow trout given linoleic [18:2(*n*-6)] and lauric acids (14:0) as their only dietary lipids, Castell et al. (17) observed an unusual heart condition, described as local myocarditis, with some degeneration and hypertrophy. A similar myopathy was observed in our present experiment, although the ventricular swellings observed in rainbow trout did not occur in SO-fed salmon. However, the extent to which either the shock syndrome described by Sinnhuber (28) or the transportation syndrome in the present study involved cardiac or cardiovascular malfunction is not known. Clearly the SO-fed fish in the present study were not deficient in (*n*-3) PUFA on an absolute basis, but they could be considered to be relatively deficient in (*n*-3) PUFA in the sense that their diet contained a considerable excess of (*n*-6) compared with (*n*-3) PUFA.

The second aspect concerns the level at which the dietary ratio of (*n*-6)/(*n*-3) PUFA becomes high enough to be pathological to fish. As far as we are aware, there is no evidence in the literature to suggest that a high ratio of these lipids is deleterious to fish, despite the now well-established fact that high dietary ratios of (*n*-6)/(*n*-3) PUFA can be deleterious to humans (30, 31). Indeed, lowering the high dietary ratio of (*n*-6)/(*n*-3) PUFA by taking (*n*-3) PUFA–rich fish oil concentrates is known to be beneficial in alleviating a wide range of human disorders, including cardiac disease (30, 32). Furthermore, beneficial effects stem at least in part from reduced production of arachidonic acid [20:4(*n*-6)]–derived 2-series prostaglandins and 4-series leukotrienes. Possibly the cardiac myopathy observed in the present study of salmon arises from an overproduction of (*n*-6) PUFA–derived eicosanoids, which are known to regulate protein biosynthesis in mammals (33).

These considerations, albeit speculative, are important in terms of human health because the high (*n*-3)/(*n*-6) PUFA ratio in fish lipid is particularly useful in counteracting the excesses of (*n*-6) PUFA currently common in Western diets. Therefore, oversupplementing fish food formulations with (*n*-6) PUFA–rich vegetable oils to generate fish rich in (*n*-6) PUFA may be undesirable.

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The Effect of Dietary Linoleic Acid on the Fatty Acid Composition of Individual Phospholipids and Lipoxygenase Products from Gills and Leucocytes of Atlantic Salmon (*Salmo salar*)

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Diets containing either fish oil or sunflower oil, both of which supplied the minimum required level of n-3 fatty acids, were given to Atlantic salmon *(Salmo salar)* post-smolts for a period of 16 weeks. In fish fed sunflower oil, the phospholipids of gills showed increased 18:2n-6 (2-13-fold), 20:2n-6 (4.5-12-fold) and 20:3n-6 (2-8-fold). In addition, phosphatidylethanolamine had increased 20:4n-6 (1.5-fold). Changes of a similar magnitude were observed in the phospholipids of blood leucocytes except that, in addition, 20:4n-6 was elevated in phosphatidylserine (1.7-fold) and phosphatidylinositol (1.4-fold). Both tissues showed a general decrease in phospholipid 20:5n-3 (up to 3-fold), which caused an increase in 20:4n-6/20:5n-3 ratio (1.3-6-fold). The elongation and desaturation products of 20:4n-6, 22:4n-6 and 22:5n-6 were not increased as a result of feeding sunflower oil. When isolated gill cells were stimulated with the calcium ionophore A23187, 12-hydroxy-8,10,14,17-eicosapentaenoic acid (12-HEPE) was the major lipoxygenase product from salmon given fish oil. 12-HEPE was significantly reduced in salmon given sunflower oil. When stimulated with A23187, the lipoxygenase products derived from whole blood of fish given sunflower oil showed decreased levels of leukotriene B_5, 12-HEPE and 12-hydroxy-5,8,10,14-eicosatetraenoic acid. *Lipids 26,* 445-450 (1991).

Most mammals can metabolize linoleic acid (LA), 18:2n-6 by Δ6 desaturation to gamma-linolenic acid 18:3n-6 which, after elongation to dihomogamma-linolenic acid 20:3n-6, can subsequently undergo Δ5 desaturation to arachidonic acid (AA), 20:4n-6 (1). Generally freshwater fish are capable of utilizing the same pathway (2), whereas marine species, such as turbot *(Scophthalmus maximums)*, apparently lack Δ5 desaturase and thus require their diet to supply the highly unsaturated fatty acids (HUFA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and probably AA (3,4). Studies with an anadramous species, Atlantic salmon *(Salmo salar)*, indicated an apparent inability to metabolize LA to AA during smolting (the transition from freshwater to seawater) (5), although post-smolts given linoleic acid as corn oil showed increased AA in the phospholipids of liver and muscle (6).

AA and EPA can be metabolized by cyclooxygenase and lipoxygenase enzymes to yield a group of biologically active products known collectively as eicosanoids. While the AA-derived eicosanoids are powerful agonists in a number of pathophysiological conditions such as psoriasis and cardiovascular disease (7,8), the EPA-derived eicosanoids are generally of decreased efficacy, and thus their generation *in vivo* may allow them to function as anti-inflammatory mediators (9). This fact underlies the use of n-3 HUFA-rich fish oils in the treatment of a number of inflammatory conditions in humans (10).

Fish are an ideal model system for studying the production and interaction of AA- and EPA-derived eicosanoids because their tissues are naturally rich in n-3 HUFA. In this study Atlantic salmon post-smolts were given diets in which the lipid was supplied either as fish oil or sunflower oil. Although the latter diet had a markedly reduced n-3/n-6 ratio, it contained sufficient n-3 HUFA derived from the dietary fish meal to meet the normally recommended requirements of salmon (11). Our aims were i) to study the incorporation of LA and its metabolic products into the individual phospholipids of gills and leucocytes. These tissues are highly active in eicosanoid production (12-14). ii) To determine lipoxygenase products from endogenous fatty acid precursors in isolated gill cells and whole blood challenged with the calcium ionophore A23187.

MATERIALS AND METHODS

Materials. Leukotriene B_5 (LTB_5), 12-hydroxyeicosatetraenoic acid (12(R,S)-HETE) and 12-hydroxyeicosapentaenoic acid (12(S)-HEPE) were purchased from Cascade Biochem Ltd. (Reading, U.K.). Leukotriene B_4 (LTB_4), A23187 and "Sigmacote" were from Sigma Chemical Co. Ltd. (Poole, U.K.). 15-Hydroxyeicosatetraenoic acid (15(S)-HETE) and 5-hydroxyeicosatetraenoic acid (5(R,S)-HETE) were purchased from Novabiochem Ltd. (Nottingham, U.K.).

Thin-layer chromatography (TLC) plates (20 × 20 cm × 0.25 mm) precoated with Silica Gel 60 were obtained from Merck (Darmstadt, Germany). All solvents were of high-performance liquid chr tography (HPLC) grade and were obtained from Rathb n Chemicals Ltd. (Walkerburn, U.K.).

Animals and diets. Three hundred Atlantic salmon S1 smolts (Salmon undergoing transition to seawater in one year, mean wt. *ca.* 51 g) were obtained from the D.A.F.S. Fish Cultivation Unit (Aultbea, Wester Ross, Scotland) and transferred to sea water tanks at the same unit. The diets were supplied by automatic feeders which were adjusted to provide 20 g/kg biomass per day, 7 days each week. Fish were bulk weighed every 28 days and the ration adjusted accordingly. Samples for analytical measurements were collected after 16 weeks of the feeding experiment. The fish meal-based diets were formulated to meet the nutritional requirements of salmon (15) and contained 46% crude protein and 17% lipid (Table 1). The

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Abbreviations: AA, arachidonic acid; BHT, butylated hydroxytoluene; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; HEPE, hydroxy-5,8,10,14,17-eicosapentaenoic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); HETE, hydroxy-5,8,10,14-eicosatetraenoic acid; HPLC, high-performance liquid chromatography; HUFA, highly unsaturated fatty acid; LA, linoleic acid; LTB_4, leukotriene B_4; LTB_5, leukotriene B_5; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SFO, sunflower oil; TLC, thin-layer chromatography.

TABLE 1

Composition of the Basal Diet

Constituent	Amount (g/kg)
Fishmeal	650
Starch[a]	150
Oil[b]	100
Vitamin mix[c]	10
Mineral mix[d]	24
α-Cellulose	65.5
Antioxidant mix[e]	0.4
Choline chloride (40% w/v)	10 mL

[a] Passelli WA4, Tunnel Avebe Starches Ltd., Gillingham, U.K.

[b] Fosol, Seven Seas Ltd. (Hull, U.K.) or Sunflower oil (Tesco Ltd. U.K.).

[c] Supplied (mg/kg): α-tocopherol acetate, 40; menadione, 10; ascorbic acid, 1000; thiamin hydrochloride, 10; riboflavin, 20; pyridoxine hydrochloride, 12; calcium pantothenate, 44; nicotinic acid, 150; biotin, 1; folic acid, 5; cyanocobalamin, 0.02; myo-inositol, 400; retinyl acetate, 2500 I.U.; and cholicalciferol, 2400I.U.

[d] Supplied per kg diet: KH_2PO_4, 22 g; $FeSO_4.7H_2O$, 1.0 g; $ZnSO_4.7H_2O$, 0.13 g; $MnSO_4.4H_2O$, 52.8 mg; $CuSO_4.5H_2O$, 12 mg; $CoSO_4.7H_2O$, 2 mg; and KI, 2 mg.

[e] Contained (g/L): Butylated hydroxy anisole, 60; propyl gallate, 60; citric acid, 40; dissolved in proylene glycol.

TABLE 2

Fatty Acid Composition of Diets

Fatty acids	Fish oil diet (weight %)	Sunflower oil diet (weight %)
14:0	5.7	2.0
16:0	13.8	9.7
18:0	2.5	3.4
16:1n-7	5.2	2.2
18:1n-9	12.0	14.7
20:1n-9	8.9	3.2
22:1n-11	13.8	4.0
24:1	1.4	0.4
18:2n-6	1.4	42.1
18:3n-6	0.2	1.1
20:2n-6	0.2	<0.1
20:3n-6	0.2	0.1
20:4n-6	0.6	0.4
18:3n-3	1.3	0.4
18:4n-3	2.9	0.7
20:5n-3	7.2	3.4
22:5n-3	1.2	0.5
22:6n-3	10.4	4.6
Total n-6	2.5	43.6
Total n-3	23.8	9.9
n-3/n-6	9.4	0.2

dietary lipid was supplied either as fish oil (FO) or sunflower oil (SFO), and both diets fulfilled the requirement for n-3 fatty acids to be *ca.* 1% of the dry diet. The fatty acid compositions of the two diets are shown in Table 2.

Leucocyte preparation and lipid extraction. Five mL of blood were collected in heparinized syringes from the caudal vein of six fish from each dietary treatment. The blood was diluted with two volumes of phosphate buffered saline (PBS). Four mL of the diluted blood were layered onto 6 mL of lymphocyte separation medium (Gibco Ltd., Paisley, Scotland) and centrifuged at 400 × *g* for 30 min at 4°C. The cells around the interface of the erythrocytes were harvested and washed in PBS. If erythrocyte contamination was greater than 1%, the gradient centrifugation was repeated. The leucocytes obtained from six samples per dietary treatment were pooled and the lipids extracted by the method of Folch *et al.* (16). The extracts were stored at −20°C prior to further analysis. All solvents contained 0.05% butylated hydroxytoluene (BHT) as an antioxidant.

Extraction of gill lipids. Intact gill arches were dissected from three fish per treatment, blotted on paper tissue to remove excess blood and immediately frozen in liquid nitrogen. Samples were stored at −20°C prior to lipid extraction. After thawing, the gill filaments were dissected from the gill arches, weighed and homogenized in chloroform/methanol (2:1, v/v) using a "Polytron" tissue macerator. The remaining procedures were as described for leucocyte lipid extraction. Lipids were extracted from 1.0 g samples of the diets by a similar procedure.

Lipid class separation and fatty acid analysis. Lipid extracts were separated into phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) fractions by TLC as described by Vitiello and Zanetta (17). The plates were sprayed with 0.1% 2',7'-dichlorofluorescein in 97% methanol containing 0.05% BHT, and the lipid bands were visualized under UV light. Acid-catalyzed transmethylation was performed overnight at 50°C by the method of Christie (18). The fatty acid methyl esters were separated and quantified by gas-liquid chromatography (Carlo Erba Fractovap 4160, Fisons Ltd., Crawley, U.K.) using a capillary column (50 m × 0.22 mm i.d.) coated with free fatty acid phase (S.G.E. Ltd., Milton Keynes, U.K.). Individual methyl esters were identified by comparison with known standards and by reference to published data (19,20).

Preparation of isolated gill cells. Fish were killed by a blow to the head. The pericardium was opened and the bulbus arteriosus was perfused with 20 mL of ice-cold 0.9% NaCl (w/v) to flush blood from the gills. The gill arches were then removed and placed in ice-cold Hank's medium. This is a calcium-free medium which is specially modified for use with salmonid fish as described by Moon *et al.* (21). The composition was: 10.29 g/L NaCl, 0.4 g/L KCl, 0.2 g/L $MgSO_4.7H_2O$, 0.06 g/L KH_2PO_4, 0.13 g/L $Na_2HPO_4.12H_2O$, 0.5 g/L $NaHCO_3$, and 2.38 g/L HEPES. The arches were then blotted on tissue and the epithelial layer scraped with a blunt scalpel blade to remove cells. The cells were then suspended in ice-cold Ca^{2+}-free Hank's medium, gently separated by stirring with a magnetic stirrer and filtered through a 100 μm nylon gauze. The cells were collected by centrifugation at 100 × *g* for 5 min.

Ionophore challenge. Glass test tubes (15 mL) used in the incubations were pre-coated with "Sigmacote" prior to use. Gill cells were suspended in modified Hank's medium (containing 2 mM $CaCl_2$) at a concentration of 100 mg wet weight per mL. An aliquot of the suspension was retained for protein determination (22). One mL of whole blood was mixed with an equal volume of modified Hank's medium and both blood and gill cell suspensions were incubated in a shaking water bath at 15°C for

10 min. Calcium ionophore A23187 was added in 5 μL dimethylsulfoxide at a final concentration of 20 μM.

Eicosanoid extraction. After 30 min incubation, the samples were centrifuged at 12,000 × *g* for 2 min and the eicosanoids extracted by the method of Pettitt *et al.* (14).

High-performance liquid chromatography. The profiles of the hydroxy fatty acids and leukotrienes from the gill and blood extracts were determined by reverse phase high-performance liquid chromatography (HPLC) using a Spherisorb 5 μm octadecylsilica (ODS2) column (25 cm × 4.6 mm i.d., Scotlab, Bellshill, Scotland). The chromatographic system was equipped with Waters Model M-45 pumps and 680 Automated Gradient Controller and the effluent was monitored at 280 nm and 235 nm to detect leukotrienes and hydroxy fatty acids, respectively, using a Pye Unicam LC-UV detector. Two isocratic solvent systems were used at a flow rate of 1 mL/min. To separate the monohydroxy acids, solvent A containing methanol/water/acetic acid (80:20:0.05, v/v/v) was used. To separate leukotrienes and monohydroxy acids, solvent B containing acetonitrile/methanol/water/acetic acid/phosphoric acid/ammonia (40:29:30:0.5:0.3:0.2, by vol) was used. Quantitations were based on external standards of 12-HEPE, 12-HETE, LTB_5 and LTB_4. A representative chromatogram of leukotriene and hydroxy fatty acid standards is shown in Figure 1.

Preparation and chromatography of hydroxy acid methyl esters. Methyl esters of standard hydroxy acids and peaks collected from HPLC were methylated using diazomethane as described by Schwartz and Bright (23). The methyl esters were purified using HPLC (Solvent A) and analyzed by gas-liquid chromatography (Philips PU 4500 chromatograph) using a CP Sil 5 column (50 m × 0.32 mm i.d.; Chrompak, Middelberg, The Netherlands). Hydrogen was used as carrier gas and the temperature was programmed from 50°C to 260°C at 4°C/min.

Statistical analysis. Significance of difference ($P<0.05$) between dietary treatments was determined by Student's *t*-test.

RESULTS

The fatty acid compositions of leucocyte phospholipid classes are shown in Tables 3 and 4. In PC from SFO fish there were increases in 18:2n-6, 18:3n-6, 20:2n-6 and 20:3n-6 as compared to FO fish. The SFO fish also showed decreased 20:5n-3, 22:6n-3, total saturated acids and total monoenes. In PE a similar pattern of changes was observed, although 20:4n-6 was also increased in SFO fish as compared to FO fish. In both lipid classes of SFO fish the n-3/n-6 ratio was reduced. In PS of SFO fish the n-6 PUFA were also elevated compared to the FO fish, and 20:5n-3 and 22:6n-3 were reduced. In PI 20:3n-6 and 20:4n-6 were increased in SFO fish but the n-3 HUFA were also elevated. In general, PI was the least altered by dietary lipid and this was reflected by little change in either the n-3/n-6 ratio or the 20:4n-6/20:5n-3 ratio. In PS, as with PC and PE, the n-3/n-6 ratio was decreased and the 20:4n-6/20:5n-3 ratio was increased.

The fatty acid compositions of gill phospholipid classes are shown in Tables 5 and 6. PC from SFO fish showed significantly increased 18:2n-6, 20:2n-6 and 20:3n-6. The SFO fish also had decreased 20:5n-3 and total n-3 PUFA, but increased total PUFA as compared to FO fish. Similar

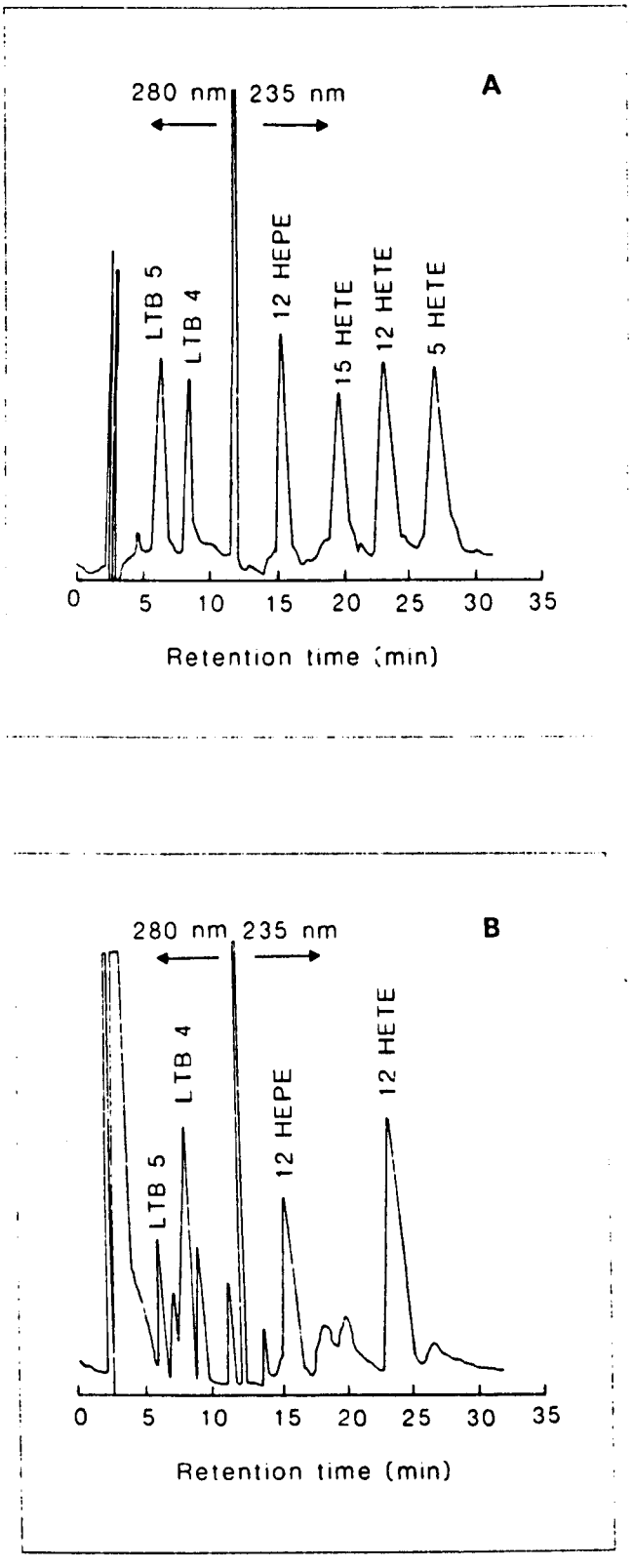

FIG. 1. (A) A typical HPLC chromatogram of leukotriene and hydroxy acid standards separated using solvent system described in Materials and Methods. (B) A typical HPLC chromatogram of lipoxygenase products obtained after stimulation of whole blood with A23187 (20 μM). Experimental conditions are described in Materials and Methods.

TABLE 3

Fatty Acid Compositions of Phosphatidylcholine and Phosphatidylethanolamine from Salmon Leucocytes[a]

Fatty acids (weight %)	PC		PE	
	FO diet	SFO diet	FO diet	SFO diet
Total saturated	34.8	32.6	22.5	18.2
Total monoenoic	25.0	20.6	25.4	14.2
18:2n-6	1.0	8.8	2.4	12.2
18:3n-6	0.2	0.5	0.1	0.6
20:2n-6	0.2	1.6	0.3	2.0
20:3n-6	0.2	1.6	0.2	1.5
20:4n-6	2.8	2.8	2.8	4.2
Total n-6[b]	4.9	15.5	6.5	20.9
18:3n-3	0.2	0.2	0.3	0.2
20:5n-3	8.8	5.1	4.1	2.8
22:5n-3	1.1	0.9	1.0	1.8
22:6n-3	18.6	14.6	29.1	31.1
Total n-3[c]	29.3	20.9	35.5	36.2
Total PUFA	34.2	36.4	42.0	57.0
n-3/n-6	6.0	1.4	5.4	1.7
20:4/20:5	0.3	0.7	0.6	1.5

[a] Results are from pooled leucocyte samples from six salmon.
[b] Includes 22:4n-6 and 22:5n-6.
[c] Includes 18:4n-3 and 20:4n-3.

TABLE 4

Fatty Acid Compositions of Phosphatidylserine and Phosphatidylinositol from Salmon Leucocytes[a]

Fatty acids (weight %)	PS		PI	
	FO diet	SFO diet	FO diet	SFO diet
Total saturated	31.9	34.9	26.7	22.9
Total monoenoic	11.4	7.1	12.5	6.6
18:2n-6	1.0	3.8	1.2	1.4
18:3n-6	<0.1	0.2	0.3	0.2
20:2n-6	<0.1	1.0	0.2	0.3
20:3n-6	<0.1	0.9	0.4	0.8
20:4n-6	1.6	2.7	13.0	17.8
Total n-6[b]	2.8	9.1	15.2	21.1
18:3n-3	0.2	0.3	0.3	<0.1
20:5n-3	2.1	1.7	2.4	2.7
22:5n-3	1.7	1.9	1.8	1.8
22:6n-3	41.8	38.4	25.3	30.4
Total n-3[c]	46.0	42.4	31.7	34.9
Total PUFA	48.7	51.4	47.4	56.0
n-3/n-6	16.7	4.7	2.1	1.7
20:4/20:5	0.8	1.6	5.4	6.6

Footnotes as for Table 3.

TABLE 5

Fatty Acid Compositions of Phosphatidylcholine and Phosphatidylethanolamine from Salmon Gill[a]

Fatty acids (weight %)	PC		PE	
	FO diet	SFO diet	FO diet	SFO diet
Total saturated	40.6 ± 2.3	40.5 ± 1.1	19.1 ± 0.7	21.5 ± 4.1
Total monoenoic	24.9 ± 1.5	22.3 ± 0.9	14.8 ± 1.3	16.2 ± 4.1
18:2n-6	0.6 ± 0.1[b]	7.9 ± 1.0	0.9 ± 0.2[b]	4.8 ± 0.8
18:3n-6	0.2 ± 0.1	0.2 ± 0.2	0.4 ± 0.2	0.2 ± 0.1
20:2n-6	0.1 ± 0.0[b]	1.2 ± 0.2	0.2 ± 0.1[b]	0.9 ± 0.1
20:3n-6	0.1 ± 0.0[b]	1.6 ± 0.3	0.1 ± 0.0[b]	0.7 ± 0.3
20:4n-6	1.4 ± 0.2	2.2 ± 0.9	2.8 ± 0.4	4.2 ± 2.3
Total n-6[c]	2.6 ± 0.4[b]	13.4 ± 0.6	5.0 ± 0.6[b]	11.4 ± 2.6
18:3n-3	<0.1	<0.1	0.1 ± 0.0	<0.1
20:5n-3	4.9 ± 0.6[b]	2.6 ± 0.2	4.3 ± 0.5[b]	2.2 ± 0.2
22:5n-3	0.8 ± 0.3	0.5 ± 0.1	1.2 ± 0.2	0.8 ± 0.3
22:6n-3	15.3 ± 1.3	13.8 ± 0.6	33.7 ± 0.6	26.9 ± 5.2
Total n-3[d]	21.3 ± 2.0[b]	16.9 ± 0.7	39.4 ± 0.6	30.4 ± 5.2
Total PUFA	23.9 ± 2.4[b]	30.3 ± 1.3	44.5 ± 0.4	41.8 ± 7.7
n-3/n-6	8.1 ± 0.5[b]	1.3 ± 0.0	8.0 ± 0.9[b]	2.7 ± 0.3
20:4/20:5	0.3 ± 0.1	0.9 ± 0.4	0.7 ± 0.2	1.9 ± 0.9

[a] Results are % by weight ± standard deviation from three salmon.
[b] Values are significantly different ($P<0.05$).
[c] Includes 22:4n-6 and 22:5n-6.
[d] Includes 18:4n-3 and 20:4n-3.

TABLE 6

Fatty Acid Compositions of Phosphatidylserine and Phosphatidylinositol from Salmon Gills[a]

Fatty acids (weight %)	PS		PI	
	FO diet	SFO diet	FO diet	SFO diet
Total saturated	36.1 ± 0.5[b]	45.8 ± 1.5	36.0 ± 4.8	40.3 ± 4.5
Total monoenoic	15.0 ± 0.7[b]	10.7 ± 1.2	14.6 ± 0.6	14.6 ± 0.3
18:2n-6	0.8 ± 0.3[b]	2.8 ± 0.5	1.6 ± 0.1[b]	3.9 ± 0.4
18:3n-6	0.8 ± 0.1	0.6 ± 0.2	1.0 ± 0.5	0.6 ± 0.2
20:2n-6	0.1[b]	0.9 ± 0.1	<0.1[b]	0.5 ± 0.0
20:3n-6	<0.1[b]	0.9 ± 0.1	<0.4[b]	1.2 ± 0.1
20:4n-6	0.8 ± 0.1	1.1 ± 0.2	9.1 ± 0.8	8.7 ± 2.1
Total n-6[c]	3.3 ± 0.4[b]	6.9 ± 0.3	12.1 ± 0.5	15.4 ± 1.9
18:3n-3	0.1 ± 0.0	<0.1	0.5	0.2 ± 0.1
20:5n-3	1.3 ± 0.3[b]	0.4 ± 0.1	2.5 ± 0.3[b]	1.3 ± 0.1
22:5n-3	1.7 ± 0.1[b]	1.2 ± 0.2	1.0 ± 0.2	0.6 ± 0.1
22:6n-3	27.6 ± 1.0	25.2 ± 1.4	21.5 ± 2.9	17.6 ± 0.8
Total n-3[d]	30.8 ± 1.2	26.9 ± 1.7	25.4 ± 3.3	19.7 ± 0.7
Total PUFA	34.2 ± 1.0	33.8 ± 1.7	37.5 ± 3.8	35.1 ± 2.5
n-3/n-6	9.3 ± 1.4[b]	3.9 ± 0.4	2.1 ± 0.3[b]	1.3 ± 0.2
20:4/20:5	0.7 ± 0.2[b]	2.5 ± 0.7	3.7 ± 0.3	6.9 ± 1.9

Footnotes as described in Table 5.

changes were apprent in gill PE. In gill PS, 18:2n-6, 20:2n-6 and 20:3n-6 were significantly elevated in SFO fish, while significant decreases were observed in 20:5n-3 and 22:5n-3. Similar changes were observed in gill PI. The n-3/n-6 ratio in all gill phospholipids was reduced in fish given the SFO diet. The mean value of the ratio 20:4n-6/20:5n-3 was generally increased in SFO fish but, due to large standard deviations, the increase was only significant in PS. In the phospholipids from both leucocytes and gills, no changes in the very small amounts of 22:4n-6 and 22:5n-6 detected were observed (data not shown).

The major lipoxygenase products from gill and blood are shown in Tables 7 and 8 and a typical HPLC chromatogram

TABLE 7

Lipoxygenase Products from Gill of Salmon Given Diets Containing Either Fish Oil or Sunflower Oil[a]

Eicosanoid	FO diet (ng/mg protein)	SFO diet (ng/mg protein)
12-HEPE	108.3 ± 7.2[b]	43.1 ± 1.7
12-HETE	54.8 ± 10.9	52.7 ± 7.4
12-HETE/12-HEPE	0.5 ± 0.1[b]	1.3 ± 0.2

[a] Values are mean ± standard error for five fish per treatment.
[b] Values are significantly different ($P<0.05$).

TABLE 8

Lipoxygenase Products from Blood of Salmon Given Diets Containing Either Fish Oil or Sunflower Oil[a]

Eicosanoid	FO diet (ng/mL blood)	SFO diet (ng/mL blood)
LTB_4	23.2 ± 2.2	18.1 ± 7.4
LTB_5	35.2 ± 10.2[b]	9.6 ± 1.6
LTB_4/LTB_5	0.7 ± 0.2[b]	1.9 ± 0.6
12-HEPE	109.0 ± 25.5[b]	38.1 ± 4.5
12-HETE	185.0 ± 17.3[b]	97.2 ± 14.8
12-HETE/12-HEPE	2.1 ± 0.3	2.7 ± 0.2

[a] Values are mean ± standard error for three fish per treatment.
[b] Values are significantly different ($P<0.05$).

of the products produced by incubation of blood with A23187 is shown in Figure 1. Peak identity was confirmed by high resolution capillary GC of methyl esters prepared from hydroxy fatty acids recovered from the HPLC separation. No HPLC peaks were detected when blood was incubated with dimethylsulfoxide alone (data not shown).

Gill cells from both dietary treatments produced substantial amounts of both 12-HEPE and 12-HETE. The amount of 12-HETE produced did not vary with dietary n-3/n-6 ratio but the amount of 12-HEPE produced was significantly reduced in fish given SFO. No LTB_4 or LTB_5 were detected in gill cell extracts. A reduction in the dietary n-3/n-6 ratio did not alter production of leucocyte LTB_4, but the production of LTB_5 was significantly reduced in fish given SFO. However, SFO significantly reduced the formation of both 12-HEPE and 12-HETE as compared to leucocytes from fish given FO.

DISCUSSION

The increased levels of 20-carbon n-6 fatty acids present in the individual phospholipids of gill and leucocytes from fish given SFO indicate that both Δ6 and Δ5 desaturation are occurring in these tissues. In gill cells Δ5 desaturase appears less active than Δ6 desaturase. This is confirmed by the accumulation of 20:3n-6 in all phospholipids, whereas 20:4n-6 is only increased in PE. There was a similar accumulation of 20:3n-6 in leucocytes, although 20:4n-6 was also increased in PE, PS and PI. This relatively low Δ5 desaturase activity also was observed in isolated hepatocytes from a related salmonid, the rainbow trout (24).

While trout gill homogenates have a 12-lipoxygenase capable of converting exogenous radiolabeled AA and EPA to their 12-hydroxy metabolites (25), there has been no information on the production of these metabolites from endogenous precursor fatty acids derived from gill phospholipids. This study demonstrates that in Atlantic salmon given a FO containing diet, the major 12-lipoxygenase product from gill cells is EPA-derived 12-HEPE. The reduced production of 12-HEPE in gills of SFO fish corresponds with a reduction of EPA and an increase in the AA/EPA ratio in the gill phospholipids. The ratio of 12-HETE/12-HEPE produced by gill cells and the ratio of the precursor fatty acids in gill PC suggest a possible relationship between the levels of these fatty acids in PC and their metabolism by gill lipoxygenase. A similar relationship has also been suggested by Fischer *et al.* (26) and Takayama *et al.* (27) for mammalian platelets and cultured mammalian umbilical tissue, respectively.

The precise physiological role of 12-HETE is not presently well established, although recent studies have postulated a possible role for this eicosanoid in the control of ion channels (28,29). This role would be particularly important in gills which, along with renal tissue, are responsible for osmoregulation in fish. In general, EPA-derived eicosanoids have less biopotency relative to their AA-derived counterparts (30,31). Therefore, the alteration of 12-HETE/12-HEPE ratio as a result of SFO intake could have a profound effect on gill physiology and function.

The quantity of lipoxygenase products derived from endogenous precursors in salmon gill was very substantial in that the recorded value of approximately 100 ng 12-HEPE/mg tissue protein represents 0.3 mg 12-HEPE/g tissue lipid or 0.7% of total precursor fatty acid metabolized by lipoxygenase. The significance of this high lipoxygenase activity is not clear.

We used whole blood, instead of isolated leucocytes, to study eicosanoid production because this most closely resembled the *in vivo* situation where interactions between different cell types are known to have profound influences on eicosanoid production (32). Studies with rainbow trout have indicated that leucocytes are the only blood cells which are directly capable of eicosanoid production and that no eicosanoids are generated by ionophore-stimulated erythrocytes (14). Salmon given SFO showed decreased production of LTB_5 and, consequently, an increased LTB_4/LTB_5 ratio as compared to those given FO. LTB_4 is an extremely powerful chemotactic agent and is a potent agonist in increasing intracellular calcium (33). EPA-derived LTB_5, however, is up to 30 times less potent than LTB_4 (34). Clearly the changes induced by high n-6 PUFA intake could have significant consequences for immune function.

Both 12-HEPE and 12-HETE production were reduced in fish given SFO even though AA was slightly increased in their phospholipids. However, 20:3n-6 was increased from 1.5% (in FO fish) to 13% (in SFO fish) of the total 20-carbon PUFA in leucocyte total phospholipids (Tables 3 and 4) and this fatty acid, which is also a substrate for lipoxygenase, is known to inhibit production of AA-derived eicosanoids in guinea-pig epidermis (7). In trout, endogenous 20:3n-6 was not utilized directly for prostaglandin production, but it was an effective inhibitor of AA-derived prostaglandin formation (35). Changes in dietary

n-3/n-6 PUFA ratio can increase (36) or decrease (37) the activity of phospholipase A_2 depending on the tissue. A decrease in phospholipase A_2 activity, which would result in less available substrate for lipoxygenase, could explain the decline in both 12-HETE and 12-HEPE production in leucocytes of fish given SFO. Under our experimental conditions, no lipoxygenase products of 22:6n-3 were detected in the eicosanoid extracts from either gill cells or leucocytes. Similar studies with rainbow trout (38) and mammals supplemented with 22:6n-3 (7,39) also found no lipoxygenase-derived products of this fatty acid. While this may be surprising in cells containing large amounts of 22:6n-3, it may reflect a selective conservation mechaism for a fatty acid considered vital in maintaining membrane structure and function (40,41).

In general, therefore, the inclusion of linoleic acid in the diets of salmon results in alterations in the phospholipid fatty acid compositions in gills and leucocytes paralleled by an altered eicosanoid spectrum in these tissues. The biological consequences of these changes remain to be assessed. However, the very high biological activity of AA-derived eicosanoids suggest that caution is required in over-supplementing commercial fish oils with n-6 rich vegetable oils in fish feeds, especially under conditions where the fish are likely to be stressed.

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COMPARATIVE NUTRITION PAPERS

DIETARY LINOLEIC ACID AFFECTS PHOSPHOLIPID FATTY ACID COMPOSITION IN HEART AND EICOSANOID PRODUCTION BY CARDIOMYOCYTES FROM ATLANTIC SALMON (*SALMO SALAR*)

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Abstract—1. Atlantic salmon post-smolts were fed practical-type diets containing linoleic acid at 10, 25 or 45% of total dietary fatty acids for a period of 20 weeks.

2. As dietary linoleic acid was increased, individual phospholipids of heart contained increased levels of 18:2n-6, 20:2n-6, 20:3n-6 and 20:4n-6 and reduced levels of 20:5n-3. The ratio of n-3/n-6 polyunsaturated fatty acids in heart phospholipids decreased and the ratio of 20:4n-6/20:5n-3 increased.

3. An increased production of thromboxane B_2 occurred in isolated cardiac myocytes from fish given the highest dietary linoleic acid but the production of 6-keto prostaglandin $F_{1\alpha}$ was not significantly affected, nor was the activity of heart sarcoplasmic reticulum Ca^{2+}–Mg^{2+} ATPase (EC 3.6.1.4).

INTRODUCTION

A number of mammalian studies have demonstrated that the polyunsaturated fatty acid (PUFA) composition of heart membrane lipids can be influenced by modifying dietary fat intake (Swanson and Kinsella, 1986; Leonardi *et al.*, 1987). In a previous study with Atlantic salmon (*Salmo salar*), we demonstrated that the fatty acid composition of the membrane phospholipids of heart could be altered profoundly by feeding diets in which the fat component was either fish oil or sunflower oil (Bell *et al.*, 1991). Fish given sunflower oil also developed a severe cardiomyopathy which was absent in fish given fish oil. The lesion caused extensive thinning of the ventricular and atrial wall, as well as active muscle necrosis.

The desaturation and elongation of linoleic acid (LA; 18:2n-6) produces arachidonic acid (AA; 20:4n-6) which can be metabolised by cyclooxygenase and lipoxygenase enzymes to yield a wide range of biologically active eicosanoids (Samuelsson, 1983). Eicosapentaenoic acid (EPA; 20:5n-3), a PUFA ubiquitous in fish tissues (Henderson and Tocher, 1987; Ackman, 1980), is also a precursor for eicosanoid synthesis although the biological potency of the compounds formed in mammals is considerably less than those derived from AA (Lands, 1989). Additionally, both EPA and docosahexaenoic acid (DHA; 22:6n-3), the major PUFA in fish tissues (Henderson and Tocher, 1987; Ackman, 1980) can directly inhibit the activity of cyclooxygenase (Terano *et al.*, 1986; Corey *et al.*, 1983), leading to decreased production of prostaglandins, thromboxanes and prostacyclins. Prostaglandins are important mediators of myocardial function (Karmazyn, 1985) and are also involved in the control of muscle protein synthesis (Palmer, 1990).

Alterations in cell membrane phospholipid fatty acid composition can also have direct effects on the activities of membrane-bound enzymes and the control of ion permeability (Stubbs and Smith, 1984). In particular, the activity of cardiac sarcoplasmic reticulum (SR) Ca^{2+}–Mg^{2+} ATPase (EC 3.6.1.4) can be influenced greatly by the membrane n-3 and n-6-PUFA composition (Swanson *et al.*, 1989). Thus, in mice fed diets containing either menhaden oil or corn oil, Ca^{2+}–Mg^{2+} ATPase activity in SR was increased 6.5-fold in the latter dietary treatment. The SR regulates intracellular calcium concentration during the contraction–relaxation cycle of the myocardium and the calcium-dependent ATPase within the SR membrane is fundamental in this regulatory process. Therefore, dietary modifications which influence the enzyme and thereby alter intracellular Ca^{2+} concentration can affect the severity of cardiac necrosis resulting from ischemia and reperfusion (Dhalla *et al.*, 1978).

Our objectives in the present study were to investigate the effects of different dietary levels of linoleic acid on the fatty acid compositions of individual phospholipids in Atlantic salmon hearts, and to measure both Ca^{2+}–Mg^{2+} ATPase activity in heart SR and eicosanoid production by stimulated cardiomyocytes.

MATERIALS AND METHODS

Animals and diets

Three hundred Atlantic salmon S1 smolts (initial mean weight *ca* 36.4 g) were obtained from the S.O.A.F.D. Fish Cultivation Unit, Aultbea, Wester Ross, U.K. The salmon

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were randomly distributed into three tanks of 1 m diameter containing 500 l of sea-water which was supplied at a rate of 10 l/min. The tanks were subjected to natural photoperiod and the water temperature over the experimental period (May–November) varied from 9 to 14°C. Diets were supplied by automatic feeders which were activated for a few seconds every 15 min (during daylight hours) and were adjusted to provide 20 g/kg biomass per day, 7 days each week. Fish were bulk weighed every 28 days and the feed ration adjusted accordingly.

The practical-type diets using fish meal as protein source were formulated to meet the nutritional requirements of salmon (U.S. National Research Council, 1981) and contained 46% protein and 16% lipid (Table 1). The lipid component was formulated to supply linoleic acid at 10, 25 and 45% of dietary lipid for the three dietary treatments. All diets satisfied the minimum requirement of salmonids for n-3PUFA (Kanazawa, 1985). The fatty acid compositions of the three diets are given in Table 2.

Sampling procedure

Samples for lipid analysis were collected after 20 and 24 weeks of the dietary treatment. Fish were killed by a blow to the head and immediately weighed, after which the heart (including the bulbous arteriosus) was dissected, weighed and frozen in liquid nitrogen. Samples were stored at −20°C prior to lipid analysis. Cardio-somatic index was calculated as heart weight in mg/body weight in g.

Lipid extraction and analysis

Total lipid was extracted from hearts and diets by the method of Folch *et al.* (1957). The procedures employed have been described in detail previously (Bell *et al.*, 1991). All solvents contained 0.05% butylated hydroxytoluene (BHT) as antioxidant. Total lipid extracts were separated into phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidyl inositol (PI) fractions by thin layer chromatography using methyl acetate:isopropanol:chloroform:methanol:0.25% aqueous KCl (25:25:25:10:9, v/v/v/v/v) as developing solvent, as described by Vitiello and Zanetta (1978). The plates were sprayed with 0.1% 2,7-dichlorofluorescein dissolved in 97% methanol with 0.05% BHT as antioxidant and the lipid bands visualised under UV light. The phospholipids were scraped from the plates and converted to fatty acid methyl esters by acid-catalysed transmethylation carried out overnight at 50°C according to Christie (1982). The fatty acid methyl esters were separated and quantified by gas–liquid chromatography, the details of which have been described previously (Bell *et al.*, 1991). Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman, 1980; Bell *et al.*, 1983).

Table 1. Composition of experimental diets

Ingredient	Diet 1	Diet 2 (g/kg)	Diet 3
Fishmeal	650	650	650
Starch (pre-cooked)	150	150	150
Fish oil	78	40	—
Sunflower oil	22	60	—
Vitelma oil*	—	—	100
Mineral mix†	24	24	24
Vitamin mix†	10	10	10
α-Cellulose	61.6	61.6	61.6
Choline chloride	4	4	4
Antioxidant mix†	0.4	0.4	0.4

*Vitelma is a blend of sunflower, safflower and grapeseed oil supplied by Vandemoortele (UK) Ltd., Hounslow, Middlesex, U.K.
†The composition of these pre-mixes have been given in detail previously (Bell *et al.*, 1991).

Table 2. Fatty acid composition of diets

Fatty acid	Diet 1	Diet 2 (weight %)	Diet 3
14:0	4.9	3.7	2.1
16:0	12.6	11.9	9.8
18:0	2.6	3.3	3.5
16:1(n-7)	4.0	3.3	2.4
18:1(n-9)	12.1	15.0	14.1
20:1(n-9)	7.2	5.5	2.8
22:1(n-11)	9.9	6.7	3.0
24:1	0.9	0.7	0.4
18:2(n-6)	10.4	25.3	46.3
20:2(n-6)	0.2	0.2	trace
20:3(n-6)	trace	trace	trace
20:4(n-6)	0.7	0.6	0.5
18:3(n-3)	1.0	0.8	0.5
18:4(n-3)	2.8	2.1	1.1
20:5(n-3)	6.4	5.1	3.5
22:5(n-3)	1.3	0.8	0.5
22:6(n-3)	9.4	6.5	4.7
Total (n-6)	11.9	26.4	46.8
Total (n-3)	21.8	15.8	10.6
(n-3)/(n-6)	1.8	0.6	0.2

Trace = value < 0.05%.

Isolation of cardiac sarcoplasmic reticulum

Salmon were killed by a blow to the head and the hearts quickly removed. The procedure used was largely that described by Swanson *et al.* (1989). Hearts from two fish were pooled (300–500 mg wet weight) and homogenised in 20 volumes of ice-cold 20 mM Tris–HCl (pH 7.4) buffer containing 30 mM L-histidine and 250 mM sucrose using a Polytron tissue homogeniser. The homogenate was centrifuged at 1600 *g* for 10 min at 4°C to remove nuclei and cell debris. The supernatant was retained and the pellet rehomogenised and centrifuged as described above. The combined supernatants were then centrifuged at 14,000 *g* for 15 min at 4°C to remove mitochondria and lysosomes. The pellet was discarded and the supernatant centrifuged at 45,000 *g* for 50 min at 4°C. The pellet was resuspended in 20 ml homogenisation buffer and recentrifuged at 48,000 *g* for 50 min at 4°C. The final SR pellet was resuspended in 0.25 ml of homogenisation buffer prior to Ca^{2+}–Mg^{2+} ATPase assay.

Ca^{2+}–Mg^{2+} ATPase assay

The assay was performed largely as described by Bonis *et al.* (1985). SR vesicles (25–50 μg protein) were incubated in a 2.0 ml reaction medium containing 50 mM Tris–HCl (pH 7.1), 5 mM potassium oxalate, 60 μM $CaCl_2$, 30 mM KCl and 5 mM $MgCl_2$ for 5 min at 20°C. The reaction was initiated by addition of 2 mM disodium-ATP and the incubation was continued for a further 45 min before the reaction was terminated by addition of 1.0 ml of 5% (w/v) trichloroacetic acid. Total ATPase activity was determined by measurement of inorganic phosphorous released from ATP by the method of Stanton (1968), using a diagnostic kit (Sigma Chemical Co., Poole, U.K.). Calcium-dependent ATPase activity was determined by subtracting the basal value obtained in the presence of 0.1 mM [ethylenebis (oxyethylene nitrilo)] tetraacetic acid and the absence of calcium from values obtained with 60 μM $CaCl_2$. Protein was determined by the method of Lowry *et al.* (1951).

Isolation of cardiac myocytes

The procedure for enzymatic isolation of cardiac myocytes is based on the method of Powell *et al.* (1980). Hearts from two salmon were cannulated via the bulbous arteriosus and perfused with modified Hanks' medium (Moon *et al.*, 1985) (concentrations in g/l; NaCl, 10.29; KCl, 0.4; $MgSO_4 \cdot 7H_2O$, 0.2; KH_2PO_4, 0.06; $Na_2HPO_4 \cdot 12H_2O$, 0.13; $NaHCO_3$, 0.5; HEPES, 2.38), which was continuously gassed with 95% O_2, 5% CO_2. After 15 min, the system was

changed to recirculation and the medium changed to modified Hanks' containing 0.6% collagenase. The perfusion was continued for a further 45 min, after which the hearts were removed and chopped finely in perfusion medium. This material was filtered through 190 μm nylon gauze and the myocytes collected by centrifugation (100 g, 2 min). The myocytes were washed twice in Hanks' medium (without collagenase) and finally, resuspended in 1.0 ml of the same medium containing 1 mM $CaCl_2$. The suspensions routinely contained 60–85% of the total cells present as rod-shaped, cardiac myocytes. Fifty microlitres of suspension were retained for protein determination.

Stimulation with calcium ionophore and extraction of eicosanoids

The myocytes were incubated in a shaking water bath at 18°C for 10 min before addition of calcium ionophore (A23187, final concentration 20 μM in 5 μl dimethyl sulphoxide). The incubation was continued for 20 min, after which the cells were sedimented by centrifugation (12,000 g, 2 min). The prostanoids were extracted from the supernatant largely by the method of Powell (1982). Absolute ethanol was added (final concentration, 15% v/v) and the supernatant adjusted to pH 3.5–4.0 by addition of 2 M formic acid (50 μl/ml). The samples were mixed and centrifuged at 12,000 g for 2 min. The supernatant was loaded on to a 'Sep-Pak' C18 column (Millipore, Watford, U.K.) which had been pre-washed with 5 ml methanol and 10 ml distilled water. The sample was washed with 10 ml of distilled water followed by 5 ml hexane:chloroform (65:35, v/v) to remove non-esterified fatty acids and hydroxy acids, before elution of prostanoids with 10 ml of ethyl acetate. The ethyl acetate extract was dried under nitrogen and the eicosanoids dissolved in 100 μl of methanol and stored at −20°C prior to radioimmunoassay. Determination of 6-ketoprostaglandin $F_{1\alpha}$, thromboxane B_2, 6ketoPGF$_{1\alpha}$ and TXB_2 were measured by radioimmunoassay using the tritium isotope kits supplied by Amersham (U.K.) Ltd.

Materials

TLC plates (20 × 20 cm × 0.25 mm) pre-coated with silica gel 60 were obtained from Merck, Darmstadt, Germany. Disodium ATP, A23187 (hemicalcium salt) and collagenase (type IV) were obtained from Sigma. All solvents were of HPLC grade and were obtained from Rathburn Chemicals, Walkerburn, U.K.

Statistical analysis

Significance of difference ($P < 0.05$) between dietary treatments was determined by analysis of variance. Data which were non-homogeneous (identified by application of an F-test) were subjected to arc sine square root transformation before analysis. Differences between means were calculated using Duncan's multiple range test (Duncan, 1960).

RESULTS

Good growth was achieved with all three dietary treatments over the 20-week experimental period, although mortalities were increased in the fish fed the two highest levels of LA (16 and 17% mortality), compared with those fed the lowest LA (10% mortality). The cardio-somatic index was significantly reduced in fish fed the highest concentration of dietary LA (Table 3), compared with those fed the lowest level. The amounts of total cardiac lipid were not significantly altered by dietary lipid neither was the activity of the sarcoplasmic reticulum (SR) Ca^{2+}–Mg^{2+} ATPase (Table 3).

The fatty acid compositions of heart phospholipids are shown in Tables 4–7. In PC (Table 4), 18:2n-6, 20:2n-6, 20:3n-6 and total n-6PUFA all increased significantly with increasing dietary intake of LA. Arachidonic acid was significantly increased in fish fed the highest dietary LA (45%), compared with those fed the lowest LA (10%). The fish fed the highest LA also had significantly reduced EPA, 22:5n-3 and total n-3PUFA compared with those fed the lowest and intermediate (25%) dietary LA. Total PUFAs were significantly increased in fish fed the intermediate and highest level of LA compared with those fed the lowest LA. The n-3/n-6PUFA ratio decreased significantly with increasing level of dietary LA and the 20:4/20:5 ratio was significantly increased in fish fed the highest dietary LA.

The fatty acid compositions of heart PE are shown in Table 5. Total monoenes were significantly reduced in fish fed the highest dietary LA compared with those fed the lowest LA. The changes in n-6PUFA were similar to those observed in PC but EPA was significantly reduced with each increase in dietary LA. 22:5n-3 was significantly reduced in fish fed the highest dietary LA compared with those fed the lowest LA. The n-3/n-6 ratio decreased significantly with increasing dietary LA, but the 20:4/20:5 ratio was significantly increased only in fish given the highest dietary LA.

The fatty acid compositions of heart PS are shown in Table 6. Total saturates and 18:2n-6 were significantly increased in fish fed the intermediate and highest levels of LA compared with those fed the lowest LA. 20:2n-6, 20:3n-6 and total n-6PUFA were all significantly increased, while EPA was significantly decreased with increasing dietary LA. 22:5n-3 was significantly decreased in fish fed the intermediate and highest dietary LA and 22:6n-3 was significantly decreased in fish given the highest LA compared with those given the lowest. The n-3/n-6PUFA ratio was significantly decreased in fish fed the intermediate and highest dietary LA, but the 20:4/20:5 ratio was only increased in those fed the highest dietary LA.

Table 3. Cardio-somatic index, heart total lipid and sarcoplasmic reticulum Ca^{2+}–Mg^{2+} ATPase activity in salmon fed diets containing increasing levels of linoleic acid

	Diet 1	Diet 2	Diet 3
Cardio-somatic index*	1.34 ± 0.17§	1.30 ± 0.16§‖	1.17 ± 0.15‖
Heart total lipid†	1.97 ± 0.28	2.07 ± 0.11	2.16 ± 0.08
Ca^{2+}–Mg^{2+} ATPase activity‡	650.7 ± 161.3	366.8 ± 100.7	488.9 ± 173.1

*Details described in Materials and Methods, values represent mean ± SD for 20 fish per treatment.

†Total lipid (weight %) measured as described in Materials and Methods, values represent mean ± SD for four fish per treatment.

‡nmol P_i released/min.mg protein, values represent mean ± SD for four sarcoplasmic reticula samples per treatment. Values in the same row with different symbols are significantly different ($P < 0.05$).

Table 4. Fatty acid compositions of heart phosphatidylcholine from salmon fed increasing levels of linoleic acid

Fatty acid	Diet 1	Diet 2 (weight %)	Diet 3
Total saturates	45.9 ± 4.8	43.2 ± 2.9	47.3 ± 2.3
Total monoenes	17.2 ± 2.0	16.2 ± 0.9	14.7 ± 1.3
18:2(n-6)	1.7 ± 0.2‡	4.0 ± 0.3§	7.2 ± 0.6‖
20:2(n-6)	0.2 ± 0.1‡	0.4 ± 0.1§	0.6 ± 0.1‖
20:3(n-6)	0.2 ± 0.0‡	0.5 ± 0.1§	1.1 ± 0.1‖
20:4(n-6)	1.3 ± 0.2‡	1.6 ± 0.1‡‖	2.0 ± 0.4§
Total (n-6)*	3.8 ± 0.6‡	7.2 ± 0.2§	11.6 ± 0.4‖
20:5(n-3)	4.4 ± 1.1‡	3.9 ± 0.4‡	2.2 ± 0.3§
22:5(n-3)	0.6 ± 0.1‡	0.5 ± 0.1‡	0.4 ± 0.1§
22:6(n-3)	20.9 ± 1.1	22.6 ± 3.1	17.8 ± 0.7
Total (n-3)†	25.5 ± 1.8‡	27.2 ± 3.5‡	20.8 ± 0.6§
Total PUFA	29.1 ± 2.0‡	34.5 ± 3.4§	33.1 ± 1.0§
n-3/n-6	7.5 ± 1.2‡	4.0 ± 0.7§	1.7 ± 0.2‖
20:4/20:5	0.3 ± 0.1‡	0.4 ± 0.0‡	0.9 ± 0.3§

Results are mean ± SD for four fish per treatment.
*Includes 18:3(n-6), 22:4(n-6) and 22:5(n-6).
†Includes 18:3(n-3), 18:4(n-3) and 20:4(n-3).
Values in the same row with different symbols are significantly different ($P < 0.05$).

The fatty acid compositions of heart PI are shown in Table 7. 18:2n-6, 20:3n-6 and total n-6PUFA were all significantly increased and 20:5n-3 and n-3/n-6PUFA ratio were both significantly decreased with increasing dietary LA. 20:2n-6 was significantly increased in fish fed the intermediate and highest dietary LA compared with those fed the lowest LA. Total n-3PUFA were significantly decreased and both AA and the 20:4/20:5 ratio significantly increased in fish given the highest dietary LA.

The amounts of 6-ketoPGF$_{1\alpha}$ and TXB$_2$ produced by isolated cardiac myocytes stimulated with A23187 are given in Table 8. 6-ketoPGF$_{1\alpha}$ production was increased, but not significantly so, by isolated cardiac myocytes as a result of increasing dietary LA. TXB$_2$ production was also increased with increasing dietary levels of LA, but the increase (more than two-fold) was only significant at the highest level of dietary LA.

DISCUSSION

The results indicate that increasing dietary LA is reflected in increased incorporation of this fatty acid into all membrane phospholipid classes. In conjunction, there is a concomitant increase in 20:3n-6 and, to a lesser extent, 20:4n-6. These results confirm previous findings which suggest appreciable Δ-6- and Δ-5-desaturase activity in salmon and incorporation of the n-6 products into cardiac tissue (Bell *et al.*, 1991). Increasing dietary LA caused a decrease in phospholipid EPA and also a small (but not statistically significant) decrease in docosahexaenoic acid (DHA; 22:6n-3) which, combined with the increase in n-6PUFA, resulted in a lowered n-3/n-6PUFA ratio and an increased 20:4/20:5 eicosanoid precursor ratio.

Alterations in the dietary n-3/n-6PUFA ratio can alter the physico-chemical properties of membranes and the activities of associated enzymes. Examples include mitochondrial ATPase (Robblee and Clandinin, 1984), sarcolemmal 5′-nucleotidase (Awad and Chattopadhyay, 1983), ornithine decarboxylase (Bunce and Abou-El-Ela, 1990) and sarcoplasmic reticulum Ca^{2+}–Mg^{2+} ATPase (Swanson *et al.*, 1989). The latter enzyme is an asymmetric integral transmembrane protein (Scales and Inesi, 1976) of the SR which controls calcium flux in excitable tissues, e.g. cardiac myocytes. Swanson *et al.* (1989) showed that a relatively small increase in the n-3/n-6PUFA ratio in SR phospholipid could cause a large decrease (around six-fold) in murine ATPase activity. In the present study with salmon heart, considerable changes in the n-3/n-6PUFA ratio in cardiac phospholipids caused no significant alteration in SR

Table 5. Fatty acid composition of heart phosphatidylethanolamine from salmon fed increasing levels of linoleic acid

Fatty acid	Diet 1	Diet 2 (weight %)	Diet 3
Total saturates	30.3 ± 1.9	31.7 ± 3.0	30.5 ± 1.1
Total monoenes	10.2 ± 0.4‡	9.1 ± 1.7‡§	7.8 ± 0.2§
18:2(n-6)	2.5 ± 0.1‡	5.3 ± 0.9§	7.9 ± 0.9‖
20:2(n-6)	0.2 ± 0.1‡	0.4 ± 0.0§	0.7 ± 0.1‖
20:3(n-6)	0.2 ± 0.1‡	0.5 ± 0.1§	1.1 ± 0.3‖
20:4(n-6)	1.6 ± 0.2‡	1.7 ± 0.2‡	2.5 ± 0.3§
Total (n-6)*	5.1 ± 0.4‡	8.7 ± 1.0§	13.5 ± 1.4‖
20:5(n-3)	3.4 ± 0.1‡	2.9 ± 0.2§	2.0 ± 0.3‖
22:5(n-3)	2.2 ± 0.1‡	2.0 ± 0.4‡§	1.6 ± 0.3§
22:6(n-3)	41.8 ± 1.1	36.9 ± 3.9	37.4 ± 1.3
Total (n-3)†	47.8 ± 1.2	41.9 ± 4.3	42.2 ± 1.6
Total PUFA	52.9 ± 1.0	51.0 ± 3.8	55.6 ± 1.3
(n-3)/(n-6)	9.5 ± 0.8‡	4.6 ± 0.7§	3.2 ± 0.4‖
20:4/20:5	0.5 ± 0.1‡	0.6 ± 0.1‡	1.3 ± 0.3§

Footnotes as described in Table 4.

Table 6. Fatty acid composition of heart phosphatidylserine from salmon fed increasing levels of linoleic acid

Fatty acid	Diet 1	Diet 2 (weight %)	Diet 3
Total saturates	38.1 ± 4.5‡	46.5 ± 3.5§	46.6 ± 1.8§
Total monoenes	10.2 ± 0.7‡	8.5 ± 0.9§	5.7 ± 0.5‖
18:2(n-6)	3.0 ± 0.6‡	5.0 ± 0.7§	6.4 ± 1.1‖
20:2(n-6)	0.3 ± 0.1‡	0.5 ± 0.1§	0.8 ± 0.1‖
20:3(n-6)	0.3 ± 0.1‡	0.6 ± 0.2§	1.2 ± 0.2‖
20:4(n-6)	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
Total (n-6)*	5.3 ± 0.8‡	7.6 ± 0.9§	9.8 ± 1.2‖
20:4(n-3)	0.5 ± 0.1‡	trace§	trace§
20:5(n-3)	1.2 ± 0.1‡	0.9 ± 0.1§	0.5 ± 0.1‖
22:5(n-3)	2.2 ± 0.3‡	1.6 ± 0.2§	1.4 ± 0.2§
22:6(n-3)	34.8 ± 3.9	31.7 ± 2.4	29.9 ± 1.5
Total (n-3)†	38.9 ± 4.1‡	34.3 ± 2.2‡§	31.8 ± 1.7§
Total PUFA	44.2 ± 3.5	41.9 ± 2.6	41.6 ± 1.7
(n-3)/(n-6)	7.6 ± 1.8‡	4.6 ± 0.6§	3.3 ± 0.5‖
20:4/20:5	0.4 ± 0.1‡	0.4 ± 0.1‡	1.2 ± 0.5§

Footnotes as described in Table 4.
Trace = value < 0.05.

Table 7. Fatty acid composition of heart phosphatidylinositol from salmon fed increasing levels of linoleic acid

Fatty acid	Diet 1	Diet 2 (weight %)	Diet 3
Total saturates	35.9 ± 4.5	37.2 ± 1.2	40.3 ± 1.6
Total monoenes	9.6 ± 0.6‡	7.9 ± 0.4§	5.9 ± 0.3‖
18:2(n-6)	2.6 ± 0.2‡	5.3 ± 0.5§	6.1 ± 0.6‖
20:2(n-6)	trace‡	0.3 ± 0.1§	0.3 ± 0.1§
20:3(n-6)	0.5 ± 0.1‡	0.9 ± 0.1§	1.3 ± 0.1‖
20:4(n-6)	12.2 ± 0.9‡	13.3 ± 0.6‡	14.9 ± 0.8§
Total (n-6)*	16.5 ± 1.9‡	20.9 ± 1.2§	23.4 ± 0.5‖
20:4(n-3)	0.5 ± 0.1‡	0.4 ± 0.1‡	trace§
20:5(n-3)	6.6 ± 0.7‡	5.3 ± 0.3§	3.0 ± 0.6‖
22:5(n-3)	1.0 ± 0.4	0.7 ± 0.1	0.6 ± 0.2
22:6(n-3)	25.9 ± 5.0	25.7 ± 1.7	21.8 ± 0.6
Total (n-3)†	34.2 ± 5.8‡	32.2 ± 2.0‡	25.4 ± 0.5§
Total PUFA	50.7 ± 5.6	53.0 ± 2.9	48.8 ± 0.3
(n-3)/(n-6)	2.1 ± 0.4‡	1.6 ± 0.1§	1.1 ± 0.1‖
20:4/20:5	1.9 ± 0.3‡	2.6 ± 0.1‡	5.1 ± 1.2§

Footnotes as described in Table 4.
Trace = values < 0.05.

Table 8. Production of eicosanoids by isolated cardiac-myocytes from salmon fed increasing levels of linoleic acid

Eicosanoid	Diet 1	Diet 2	Diet 3
	(pg/mg protein)		
6-ketoPGF$_{1\alpha}$	363.3 ± 76.5	531.7 ± 71.5	533.9 ± 290.1
TXB$_2$	194.8 ± 35.6*	238.0 ± 42.0*	439.4 ± 71.9†

Values are mean ± SD for three cardiac-myocyte preparations per treatment. Values in the same row with different symbols are significantly different ($P < 0.05$).

Ca^{2+}–Mg^{2+} ATPase activity. The major alteration in the fatty acid composition of murine SR was an increase in DHA, particularly in PC of mice given menhaden oil, whereas the present study showed no significant dietary-induced alteration in phospholipid DHA. Rabbits given menhaden oil showed no incorporation of DHA but some incorporation of EPA into skeletal muscle SR and consequently, no alteration of skeletal muscle SR Ca^{2+}–Mg^{2+} ATPase was observed (Gould *et al.*, 1987). Thus, it appears that DHA may be essential in the control of the SR calcium pump. While the work of Swanson *et al.* (1989) indicates decreased activity of the SR calcium pump in mice fed menhaden oil, a study by Karmazyn *et al.* (1987) observed enhanced uptake of Ca^{2+} in cardiac myocytes from rats fed cod-liver oil. Infante (1987) suggests that low dietary n-3 (or high n-6) PUFA may encourage uncoupling of the SR Ca^{2+}-ATPase, such that ATP hydrolysis may be occurring in the absence of calcium translocation. This possibility was not examined in the present work. The importance of the dietary n-3/n-6PUFA ratio on the microenvironment of the Ca^{2+}–Mg^{2+} ATPase and thus, the control of Ca^{2+}/ATP coupling ratio is an obvious area for further study.

The beneficial effects of fish oil supplements in attenuating a number of atherothrombotic, autoimmune and inflammatory conditions in mammals are, in part, due to the modulation of AA-derived eicosanoid production by EPA and DHA (Weber, 1990). These effects may be due to direct inhibition of cyclooxygenase by EPA and/or DHA (Culp *et al.*, 1979), or by the production of EPA-derived eicosanoids which have much less biopotency compared with their AA-derived homologues (Bruckner *et al.*, 1984). Cardiac-derived prostaglandins are important modulators of myocardial function (Karmazyn and Dhalla, 1983) with prostacyclin (PGI_2) being the primary cyclooxygenase-derived product in the heart (Karmazyn *et al.*, 1987). In this study, the Atlantic salmon fed increased levels of LA had correspondingly increased 20:4/20:5 ratios in their membrane phospholipids which could, in principle, alter the balance and biopotency of eicosanoid production. In the present study, the production of 6-ketoPGF$_{1\alpha}$ the stable metabolite of PGI_2, and TXB_2, the stable metabolite of thromboxane (TXA_2) were both increased in stimulated cardiac myocytes from salmon fed increasing levels of dietary LA. However, the observed increases were only significant with TXB_2 production at the highest level of dietary LA. Thromboxane is an important mediator of platelet aggregation, vascular tone and intracellular calcium concentration (Weber, 1990). Studies investigating skeletal muscle synthesis have shown that PGI_2 is stimulatory whereas PGE_2 is inhibitory to muscle fibre formation (McLennan, 1991). PGE_2 can also stimulate protein catabolism (Barnett and Ellis, 1987). Further studies with salmon cardiomyocytes will address the influence of dietary lipids on the production of these two eicosanoids.

A recent study has shown that alterations in membrane n-3/n-6PUFA ratio in cultured rat ventricular myocytes can influence the activity of enzymes involved in lipid metabolism (Nalbone *et al.*, 1990). Specifically, myocytes cultured in high n-6PUFA-containing media had increased phospholipase A_2 activity and decreased lysophospholipase activity when compared with myocytes grown in high n-3PUFA-containing media. In n-6PUFA enhanced tissues these changes could affect fatty acid turnover and result in enhanced production of eicosanoids. In addition, lyso PC is a known cytolytic agent (Choy *et al.*, 1989) and its production could disrupt membrane integrity.

This present study with Atlantic salmon demonstrates that cardiac membrane fatty acid composition and eicosanoid production can be profoundly altered by increasing dietary LA. These modifications and their possible consequences in terms of cardiac physiology and pathophysiology (Bell *et al.*, 1991) are the subject of continuing research.

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PROSTAGLANDINS LEUKOTRIENES AND ESSENTIAL FATTY ACIDS

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Effects of Increasing Dietary Linoleic Acid on Phospholipid Fatty Acid Composition and Eicosanoid Production in Leucocytes and Gill Cells of Atlantic Salmon (*Salmo salar*)

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ABSTRACT. **Diets containing linoleic acid at 10, 25 and 45% of total dietary fatty acids were fed to three groups of post-smolt Atlantic salmon (*Salmo salar*) for 18 weeks. Incorporation of linoleic acid into membrane phospholipids of leucocytes and gills increased in response to dietary intake. In general, there was an increase in arachidonic acid and a decrease in eicosapentaenoic acid in the individual phospholipids of both cell types in response to increasing dietary linoleic acid. These changes in eicosanoid precursors were reflected in significantly increased plasma concentrations of 6-keto-$PGF_{1\alpha}$ and TXB_2 in salmon given the highest dietary linoleic acid. In whole blood stimulated with the calcium ionophore A23187, LTB_4, 12-HETE and TXB_2 were significantly increased and 12-HEPE significantly decreased in response to increasing dietary linoleic acid. In isolated gill cells stimulated with A23187, 12-HEPE, 12-HETE, 14-HDHE and TXB_2 were all decreased in response to increasing dietary linoleic acid, although the ratio of 12-HEPE/12-HETE was also decreased.**

INTRODUCTION

The availability of polyunsaturated fatty acids (PUFA) for eicosanoid production is dictated by phospholipid fatty acid composition which is, in turn, influenced by dietary PUFA intake (1). In humans consuming 'Western-type' diets high in n-6 PUFA, arachidonic acid (20:4(n-6); AA) is the dominant precursor for eicosanoid production and this dietary regime is probably, at least partly, responsible for the high incidence of atherothrombotic, auto-immune and inflammatory conditions prevalent in Western society (2). Considerable evidence now suggests that many of these conditions can be attenuated by intake of dietary fish oil supplements which are rich in eicosapentaenoic acid (20:5(n-3); EPA) and docosahexaenoic acid (22:6(n-3); DHA) (3).

Fish offer a particularly useful model system for studying the production and interaction of eicosanoids derived from both AA and EPA because their tissues are naturally abundant in n-3 PUFA (4, 5). However, despite their abundance of n-3 PUFA, fish tissues tend to produce considerable amounts of AA-derived prostaglandins (6, 7) and indeed AA would seem to be the preferred substrate for cyclooxygenase in plaice skin and neutrophils and in turbot gills (7–9). Atlantic salmon given diets containing corn oil showed increased AA and decreased EPA in their tissue phospholipids (10). The same species given diets containing sunflower oil had an increased AA:EPA ratio in individual phospholipids and subsequently showed decreased production of EPA-derived lipoxygenase metabolites by leucocytes and gill cells (11).

Like humans, farmed Atlantic salmon are susceptible to stress-induced disease conditions. One particularly serious condition in salmon is known as 'pancreas disease' which is characterised by total loss of the exocrine pancreas (12) and is sometimes accompanied by cardiomyopathy (13). Due to the inclusion of some plant-derived lipids in their dietary regime, farmed salmon contain around 5 times the amount of n-6 PUFA compared to their wild counterparts and it is possible that the subsequent reduction in n-3/n-6 PUFA ratio is responsible for increased AA-derived eicosanoid production in farmed salmon.

In the present study three groups of Atlantic salmon were given diets containing linoleic acid

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(18:2n-6; LA) at 10, 25 and 45% of total dietary fatty acids. Our objectives were:

1. To measure the effects of the different diets on the fatty acid compositions of the phospholipid classes in leucocytes and gill cells. These cells were chosen because of their high potential for synthesising eicosanoids (14, 15).
2. To measure the levels of the antagonistic cyclooxygenase products 6-keto prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and thromboxane B_2 (TXB_2) in blood plasma.
3. To measure the production of TXB_2, leukotriene (LT) B_4 and B_5 in leucocytes stimulated with the calcium ionophore A23187.
4. To measure the 12-lipoxygenase products and the above cyclooxygenase metabolites derived from isolated gill cells stimulated with A23187.

MATERIALS AND METHODS

Animals and diets

300 Atlantic salmon S1 smolts (salmon undergoing seawater transition in 1 year) were distributed randomly into three tanks supplied continuously with non-recirculated seawater. The salmon (mean weight 36 g) were obtained from the S.O.A.F.D. Fish Cultivation Unit, Aultbea, Wester Ross, Scotland, and were subjected to natural photoperiod over the experimental period (May–October). The water temperature varied from 10–14°C over this period. Diets were provided by automatic feeder and were adjusted to provide 2% of biomass per day, 7 days each week. Total biomass was measured every 28 days and the ration adjusted accordingly. The diets were formulated to satisfy the nutritional requirements of salmon (Table 1) (16). The lipid component was formulated to supply linoleic acid at 10, 25 and 45% of dietary fatty acids by blending fish oil, sunflower oil, grapeseed oil and safflower oil. All diets satisfied the minimum requirement of salmonids for n-3 PUFA (*c.* 1% of diet as n-3 PUFA) (17). The fatty acid compositions of the three diets are given in Table 2. Analyses were performed between 18 and 20 weeks after the start of the experiment.

Table 1 Compositions of experimental diets (g/kg)

Ingredient	Diet 1	Diet 2	Diet 3
Fishmeal	650	650	650
Starch (pre-cooked)	150	150	150
Fish oil[1]	78	40	—
Sunflower oil[2]	22	60	—
'Vitelma' oil[3]	—	—	100
Mineral mix[4]	24	24	24
Vitamin mix[4]	10	10	10
α-Cellulose	61.6	61.6	61.6
Choline chloride	4	4	4
Antioxidant mix[4]	0.4	0.4	0.4

[1] 'Fosol', Seven Seas Ltd, Hull, UK.
[2] Tesco Ltd, Cheshunt, UK.
[3] 'Vitelma' is a blend of sunflower, safflower and grapeseed oil produced by Vandemoortele (UK) Ltd, Hounslow, UK.
[4] The composition of these mixes have been described in detail previously (21).

Table 2 Fatty acid compositions of diets (weight %)

Fatty acid	Diet 1	Diet 2	Diet 3
14:0	6.1	4.4	2.5
16:0	14.4	12.7	12.0
18:0	2.8	3.2	4.3
16:1n-7	4.6	3.4	2.3
18:1n-9	13.5	15.8	15.3
18:1n-7	2.1	1.8	1.5
20:1n-9	7.0	5.5	2.9
22:1n-11	9.0	7.0	3.3
24:1	0.8	0.6	0.3
18:2n-6	10.5	24.6	44.7
20:2n-6	0.1	0.1	trace[1]
20:3n-6	trace	trace	trace
20:4n-6	0.6	0.4	0.2
18:3n-3	0.9	0.7	0.5
18:4n-3	2.3	1.6	0.9
20:5n-3	5.7	4.6	2.4
22:5n-3	0.8	0.6	0.2
22:6n-3	7.6	6.2	3.2
Total n-6	12.0	25.8	45.2
Total n-3	18.0	14.1	7.4
n-3/n-6	1.5	0.6	0.2

[1] Trace = value < 0.05%

Leucocyte isolation

Five ml of blood were collected in heparinised syringes from the caudal vein of 6 fish per dietary treatment and diluted with 2 volumes of phosphate buffered saline (PBS). Four ml of the diluted blood were layered onto 6 ml of lymphocyte separation medium (Gibco Ltd, Paisley, Scotland) and centrifuged at 400 × g for 30 min at 4°C. The cells around the interface of the erythrocytes were harvested and washed in PBS. If erythrocyte contamination was greater than 1%, the gradient centrifugation was repeated. The leucocytes obtained from 6 samples per dietary treatment were pooled prior to lipid extraction.

Preparation of isolated gill cells

Fish were killed by a blow to the head, the pericardium cut open and the gills perfused via the bulbous arteriosus with 15 ml of ice-cold modified Hanks' medium to remove blood. The Hanks' medium is a specially formulated calcium-free medium for use with salmonid fish (18). The composition was: 10.29 g/L NaCl, 0.4 g/L KCl, 0.2 g/L $MgSO_4.7H_2O$, 0.06 g/L KH_2PO_4, 0.13 g/L $Na_2HPO_4.12H_2O$, 0.5 g/L $NaHCO_3$, and 2.38 g/L (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphenic acid]) (HEPES). The intact gill arches were dissected out and placed in ice-cold medium. The gill filaments

were dissected from the arches and chopped finely with scissors before addition of 25 ml of medium containing 0.1% (w/v) collagenase (type IV, Sigma Chemical Co, Poole, Dorset). This suspension was incubated for 45 min at room temperature and was constantly stirred using a magnetic stirrer. The suspension was finally filtered through nylon gauze (190 & 100 μm) and the filtered cells collected by centrifugation at 400 × g for 2 min. The cells were washed twice in Hanks' medium and finally resuspended in 1 ml of the same medium containing 1 mM $CaCl_2$, 50 μl was removed for protein determination (19).

Extraction of lipids and fatty acid analysis

Total lipid was extracted from leucocytes, gills and diets by the method of Folch et al (20). The procedures have been described in detail elsewhere (21). All solvents contained butylated hydroxytoluene (BHT) (0.05% w/v) as antioxidant. Total lipid extracts from leucocytes and gills were separated into phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) by thin-layer chromatography (TLC) using chloroform:ethanol:triethylamine:water (35:35:35:6 by volume) as developing solvent, as described by Leray et al (22). The plates were sprayed with 0.1% 2',7'-dichlorofluorescein in 97% methanol containing 0.05% butylated hydroxytoluene (BHT) and the lipid bands visualised under UV light. The phospholipids were scraped from the plates and converted to fatty acid methyl esters by acid-catalysed transmethylation carried out overnight at 50°C according to Christie (23). The analysis of fatty acid methyl esters was performed using a Packard 436 gas chromatograph (Chrompak UK Ltd, London) equipped with a capillary column (50 m × 0.22 mm) coated with free fatty acid phase (S.G.E. Ltd, Milton Keynes, UK). Individual methyl esters were identified by comparison with known standards and by reference to published data (5, 24).

Ionophore challenge and eicosanoid extraction

Glass test-tubes used in the incubations were precoated with 'Sigmacote' prior to use to prevent cell adhesion. Isolated gill cells were suspended in 1 ml of modified Hanks' medium containing 1 mM $CaCl_2$ as described above. One ml of whole blood was mixed with an equal volume of the above medium and both blood and gill cells were incubated in a shaking water bath at 18°C for 10 min. Calcium ionophore A23187 was added in 5 μl of dimethyl sulphoxide at a final concentration of 20 μM and the incubation continued for a further 20 min. The cells were sedimented by centrifugation (12 000 × g, 2 min) and the eicosanoids extracted from the supernatant using C18 ODS 'Sep-Pak' mini-columns (Millipore (UK) Ltd., Watford) by the method of Powell (25).

High performance liquid chromatography

Hydroxy fatty acids and leukotrienes were quantified by reverse phase high performance liquid chromatography (HPLC) using a Spherisorb 5 μm octadecyl silane (ODS) 2 column (25 cm × 4.6 mm, Anachem, Luton, UK). The chromatographic system was equipped with Waters Model M-45 pumps and the effluent was monitored at 280 nm and 235 nm to detect leukotrienes and hydroxy fatty acids, respectively, using a Pye-Unicam LC-UV detector. An isocratic solvent system containing acetonitrile:methanol:water:acetic acid:phosphoric acid:ammonia (40:29:30:0.5:0.3:0.2 by vol) was used. Leukotrienes and monohydroxy fatty acids were eluted isocratically at a flow rate of 1 ml/min as described previously (11). Quantitation was performed with external standards of 12-hydroxy-5,8,10,14,17-eicosapentaenoic acid (12-HEPE), 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), LTB_5 and LTB_4.

Labeling of gill cells with [1-^{14}C]-DHA

Isolated gill cells produced as described above were resuspended in 2 ml Dulbeccos' modification of Eagles' medium (DMEM). After 10 min at 18°C in a shaking water bath, 0.5 μCi radiolabeled DHA (in 5 μl ethanol) was added and the incubation continued for 5 h. The cells were sedimented by centrifugation (400 × g, 5 min) and washed in 5 ml PBS containing 1% Bovine serum albumin (fatty acid free). The cells were resuspended in 2 ml DMEM and incubated in the shaking water bath for 10 min, before addition of A23187, as described previously. Eicosanoids were extracted as described above and dissolved in 0.2 ml prior to HPLC. Eicosanoids were separated by HPLC as described above and the 1 ml fractions collected were counted in a scintillation counter after addition of 5 ml 'Ecoscint A' (BS and S (Scotland) Ltd, Edinburgh, UK).

Determination of 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2

6-Keto-$PGF_{1\alpha}$ and TXB_2, the stable metabolites of prostacyclin (PGI_2) and TXA_2, respectively, were measured by radioimmunoassay using the tritium isotope kits supplied by Amersham (UK) Ltd.

Materials

LTB_4, A23187, 'Sigmacote' and collagenase were from Sigma Chemical Co Ltd, Poole, UK. LTB_5, 12(R, S)-HETE and 12(S)-HEPE were from Cascade Biochem Ltd, Reading, UK. [1-^{14}C]-DHA was obtained from Du Pont (UK) Ltd, Stevenage, UK. TLC plates pre-coated with silica gel 60 were obtained from Merck, Darmstadt, Germany. All solvents were of HPLC grade and were obtained from Rathburn Chemicals Ltd, Walkerburn, UK.

Statistical analysis

Significance of difference ($p < 0.05$) between dietary treatments was determined by analysis of variance. Data which were non-homogeneous were subjected to arc sin square root transformation before analysis. Differences between means were calculated using Duncans' multiple range test (26).

RESULTS

The fatty acid compositions of leucocyte phospholipids are summarised in Tables 3–6. In PC

Table 3 Fatty acid compositions of leucocyte phosphatidylcholine from salmon given increasing levels of dietary linoleic acid

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		weight %	
14:0	3.7	3.3	2.9
16:0	31.7	30.1	31.8
18:0	2.3	2.5	2.9
Total saturates[1]	38.9	36.6	38.3
16:1n-7	3.4	2.8	2.9
18:1n-9	17.9	16.3	16.6
18:1n-7	4.0	3.1	3.2
20:1n-9	0.9	1.0	0.6
24:1	0.3	0.2	0.2
Total monoenes[2]	26.6	23.4	23.6
18:2n-6	3.1	5.5	8.4
20:2n-6	0.4	1.1	1.3
20:3n-6	0.4	1.1	1.8
20:4n-6	2.7	4.2	4.9
Total n-6[3]	7.4	12.8	17.4
20:5n-3	8.1	5.3	4.7
22:5n-3	0.9	0.7	0.7
22:6n-3	11.7	10.6	11.5
Total n-3[4]	21.6	17.3	17.3
Total PUFA	29.0	30.1	34.7
n-3/n-6	2.9	1.4	1.0
20:4/20:5	0.3	0.8	1.0

Results are obtained from pooled leucocyte samples from 6 fish per dietary treatment (see Materials and Methods for details)
[1] Includes 15:0, 17:0, 20:0 and 22:0.
[2] Includes 20:1n-7 and 22:1n-11.
[3] Includes 18:3n-6, 22-4n-6 and 22:5n-6.
[4] Includes 18:3n-3, 18:4n-3 and 20:4n-3.

Table 4 Fatty acid composition of leucocyte phosphatidylethanolamine from salmon given increasing levels of dietary linoleic acid

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		weight %	
14:0	2.0	2.4	1.4
16:0	13.0	11.1	11.3
18:0	5.5	5.9	6.8
Total saturates[1]	21.2	20.3	20.6
16:1n-7	3.0	2.1	1.9
18:1n-9	12.9	11.2	8.8
18:1n-7	3.4	2.5	2.3
20:1n-9	2.2	1.2	0.7
24:1	0.3	0.2	0.2
Total monoenes[2]	22.9	17.6	14.0
18:2n-6	5.6	9.5	12.0
20:2n-6	0.5	1.0	1.2
20:3n-6	0.3	0.9	1.2
20:4n-6	3.4	5.6	6.5
Total n-6[3]	10.2	17.6	21.6
20:5n-3	3.3	2.6	2.0
22:5n-3	0.9	1.0	0.8
22:6n-3	29.2	29.1	29.0
Total n-3[4]	34.3	33.4	32.0
Total PUFA	44.5	51.0	53.6
n-3/n-6	3.4	1.9	1.5
20:4/20:5	1.0	2.2	3.3

Footnotes as described in Table 3.

Table 5 Fatty acid composition of leucocyte phosphatidylserine from salmon given increasing levels of dietary linoleic acid

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		weight %	
14:0	2.1	3.1	3.4
16:0	23.9	22.1	17.1
18:0	17.4	24.6	20.2
Total saturates[1]	44.7	50.9	41.8
16:1n-7	2.2	1.1	2.1
18:1n-9	11.6	9.8	11.1
18:1n-7	2.9	1.0	1.5
20:1n-9	1.8	0.5	2.1
24:1	0.6	0.3	0.4
Total monoenes[2]	19.3	13.0	17.6
18:2n-6	1.5	1.7	4.7
20:2n-6	0.3	0.4	0.7
20:3n-6	0.2	0.5	0.6
20:4n-6	0.8	1.7	2.7
Total n-6[3]	3.5	5.2	9.4
20:5n-3	1.0	0.7	0.4
22:5n-3	1.3	1.8	1.0
22:6n-3	21.8	23.7	21.9
Total n-3[4]	24.4	26.3	23.3
Total PUFA	27.9	31.5	32.7
n-3/n-6	7.0	6.1	2.5
20:4/20:5	0.8	2.4	6.8

Footnotes as described in Table 3.

Table 6 Fatty acid composition of leucocyte phosphatidylinositol from salmon given increasing levels of dietary linoleic acid

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		weight %	
14:0	0.7	3.5	3.7
16:0	11.6	9.5	6.4
18:0	26.9	32.0	35.8
Total saturates[1]	40.3	46.3	47.1
16:1n-7	2.0	2.1	1.0
18:1n-9	12.4	7.1	5.0
18:1n-7	2.6	1.1	0.8
20:1n-9	4.2	0.4	0.1
24:1	0.6	0.1	0.1
Total monoenes[2]	22.4	10.7	6.8
18:2n-6	2.0	1.6	2.7
20:2n-6	0.2	0.5	0.5
20:3n-6	0.2	0.4	0.6
20:4n-6	22.8	32.0	36.9
Total n-6[3]	25.5	34.5	40.7
20:5n-3	0.5	0.5	0.4
22:5n-3	0.3	trace	trace
22:6n-3	2.1	3.3	2.9
Total n-3[4]	3.5	3.8	3.3
Total PUFA	29.0	38.3	44.0
n-3/n-6	0.14	0.11	0.08
20:4/20:5	45.6	64.0	92.3

Footnotes as described in Table 3.

Table 7 Fatty acid composition of gill phosphatidylcholine from salmon given increasing levels of dietary linoleic acid

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		weight %	
14:0	3.9 ± 0.4^a	3.3 ± 0.1^b	2.8 ± 0.4^b
16:0	33.8 ± 2.4	35.5 ± 0.8	35.9 ± 1.3
18:0	2.6 ± 0.2^a	3.0 ± 0.1^b	4.2 ± 0.7^c
Total saturates[1]	41.8 ± 2.6	43.1 ± 0.9	44.1 ± 2.2
16:1n-7	4.2 ± 0.4^a	3.6 ± 0.2^a	2.9 ± 0.1^b
18:1n-9	17.4 ± 1.2	18.5 ± 1.0	17.7 ± 0.7
18:1n-7	2.5 ± 0.2^a	2.3 ± 0.2^{ab}	1.9 ± 0.2^b
20:1n-9	1.1 ± 0.1^a	0.9 ± 0.1^a	0.6 ± 0.1^b
22:1n-11	0.4 ± 0.1^a	0.2 ± 0.1^{ab}	0.1 ± 0.0^b
24:1	0.4 ± 0.1	0.8 ± 0.3	0.5 ± 0.3
Total monoenes[2]	26.1 ± 1.7^a	26.6 ± 1.53	23.7 ± 1.0^b
18:2n-6	2.4 ± 0.2^a	5.0 ± 0.3^b	8.9 ± 0.8^c
20:2n-6	0.3 ± 0.1^a	0.7 ± 0.1^b	1.2 ± 0.2^c
20:3n-6	0.2 ± 0.1^a	0.6 ± 0.1^b	1.5 ± 0.3^c
20:4n-6	1.3 ± 0.2^a	1.5 ± 0.3^a	2.5 ± 0.7^b
Total n-6[3]	4.8 ± 0.6^a	8.3 ± 0.6^b	14.4 ± 1.5^c
20:5n-3	3.7 ± 0.3^a	2.9 ± 0.7^a	1.7 ± 0.2^c
22:5n-3	0.5 ± 0.0	0.4 ± 0.1	0.4 ± 0.1
22:6n-3	15.7 ± 1.7^a	13.6 ± 0.6^b	12.1 ± 0.5^b
Total n-3[4]	20.1 ± 1.7^a	17.1 ± 1.2^b	14.2 ± 0.6^c
Total PUFA	24.9 ± 1.8	25.4 ± 1.8	28.6 ± 2.2
n-3/n-6	4.3 ± 0.6^a	2.1 ± 0.0^b	1.0 ± 0.1^c
20:4/20:5	0.4 ± 0.1^a	0.5 ± 0.1^a	1.5 ± 0.5^b

Results are mean ± SD for 4 fish per treatment.
[1] Includes 15:0, 17:0, 20:0 and 22:0.
[2] Includes 20:1n-7.
[3] Includes 18:3n-6, 22:4n-6 and 22:5n-6.
[4] Includes 18:3n-3, 18:4n-3 and 20:4n-3.

Values in the same row with different superscript letters are significantly different ($p < 0.05$).
SD of 0.0 is less than 0.05.

(Table 3) 18:2n-6, 20:2n-6, 20:3n-6, 20:4n-6 and total n-6 PUFA all increased with increasing dietary intake of LA. EPA and total n-3 PUFA all decreased with increasing dietary LA. Consequently, the n-3/n-6 PUFA ratio decreased and the 20:4/20:5 ratio increased with increasing dietary LA. Similar changes occurred in the fatty acid compositions of leucocyte PE and PS (Tables 4 & 5). The fatty acid composition of leucocyte PI was less affected by dietary LA but AA was increased with increasing LA (Table 6). In general, the saturated and monoenoic fatty acids in leucocyte phospholipids reflected dietary intake.

The fatty acid compositions of gill phospholipids are shown in Tables 7–10. In PC (Table 7) 18:2n-6, 20:2n-6, 20:3n-6 and total n-6 PUFA were all increased significantly with increasing dietary LA, but AA and the 20:4/20:5 ratio were only increased significantly in the fish given the highest dietary LA. EPA, total n-3 PUFA and n-3/n-6 PUFA ratio were significantly reduced with increasing dietary LA while DHA was significantly reduced in fish given 25 and 45% LA. Very similar changes occurred in gill PE fatty acid composition (Table 8) but EPA and total n-3 PUFA were significantly reduced only in fish given the highest dietary LA, while DHA was unaffected. Gill PS fatty acid composition (Table 9) showed changes similar to those in PE except that LA, 20:2n-6 and 20:3n-6 were only significantly increased in fish given the highest dietary level of LA. The n-3/n-6 PUFA ratio was significantly decreased only in fish given the highest dietary LA. In gill PI fatty acid composition (Table 10) 18:2n-6, 20:2n-6, 20:3n-6, total n-6 PUFA and the 20:4/20:5 ratio were all increased significantly with increasing dietary LA, while total PUFA were increased significantly only in fish given the highest LA. EPA, total n-3 PUFA and n-3/n-6 PUFA ratio were all significantly reduced with increasing dietary LA, while 22:5n-3 and DHA were significantly reduced in fish given 25 and 45% LA. As with leucocytes, the saturated and monoenoic fatty acid composition of gill phospholipids reflected dietary intake.

The concentrations of plasma 6-keto-$PGF_{1\alpha}$ and TXB_2 are shown in Table 11. Both cyclooxygenase products were significantly increased in plasma of salmon given the highest dietary LA. However, while 6-keto-$PGF_{1\alpha}$ was increased around 1.5-fold, TXB_2 was increased over 3.5-fold. The production of eicosanoids by whole blood stimulated with the calcium ionophore A23187 is shown in Table 12.

Table 8 Fatty acid composition of gill phosphatidylethanolamine from salmon given increasing levels of dietary linoleic acid

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		weight %	
14:0	1.1 ± 0.4	1.2 ± 0.4	0.7 ± 0.3
16:0	15.2 ± 1.0	13.6 ± 1.3	15.2 ± 1.6
18:0	6.1 ± 0.2[a]	7.0 ± 0.4[b]	8.3 ± 0.7[c]
Total saturates[1]	25.7 ± 0.8	25.4 ± 1.9	27.3 ± 2.0
16:1n-7	1.7 ± 0.2	1.5 ± 0.4	1.8 ± 0.6
18:1n-9	8.0 ± 0.6	8.0 ± 0.4	7.1 ± 0.5
18:1n-7	2.3 ± 0.2[a]	2.1 ± 0.2[a]	1.4 ± 0.2[b]
20:1n-9	1.5 ± 0.2[a]	1.1 ± 0.1[b]	0.8 ± 0.1[c]
24:1	0.3 ± 0.1	0.3 ± 0.3	0.2 ± 0.1
Total monoenes[2]	14.2 ± 0.9[a]	13.2 ± 0.8[a]	10.8 ± 0.6[b]
18:2n-6	2.8 ± 0.3[a]	5.9 ± 0.6[b]	8.4 ± 0.6[c]
20:2n-6	0.4 ± 0.1[a]	0.8 ± 1.2[b]	1.2 ± 0.2[c]
20:3n-6	0.2 ± 0.1[a]	0.6 ± 0.1[b]	1.2 ± 0.2[c]
20:4n-6	2.6 ± 0.5[a]	2.9 ± 0.4[a]	5.1 ± 1.3[b]
Total n-6[3]	6.6 ± 1.0[a]	10.9 ± 1.1[b]	16.7 ± 1.7[c]
20:5n-3	3.2 ± 0.6[a]	2.9 ± 0.4[a]	2.0 ± 0.3[b]
22:5n-3	0.8 ± 0.1	0.8 ± 0.1	0.6 ± 0.1
22:6n-3	36.2 ± 1.5	32.6 ± 2.9	31.6 ± 2.7
Total n-3[4]	40.4 ± 1.2[a]	36.3 ± 2.7[a]	34.2 ± 3.1[b]
Total PUFA	47.0 ± 1.3	47.2 ± 2.3	51.5 ± 3.3
n-3/n-6	6.2 ± 1.1[a]	3.4 ± 0.5[b]	2.0 ± 0.2[c]
20:4/20:5	0.8 ± 0.0[a]	1.0 ± 0.2[a]	2.7 ± 1.0[b]

Footnotes as described in Table 7 except; 3 Includes 20:1n-7 and 22:1n-11.

Table 9 Fatty acid composition of gill phosphatidylserine from salmon given increasing levels of dietary linoleic acid

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		weight%	
14:0	0.4 ± 0.1[a]	0.3 ± 0.2[ab]	0.2 ± 0.1[b]
16:0	10.7 ± 0.4	10.2 ± 1.1	10.2 ± 0.4
18:0	20.4 ± 0.7[a]	21.6 ± 2.8[ab]	23.9 ± 0.7[b]
Total saturates[1]	32.8 ± 1.5[a]	33.6 ± 2.8[ab]	36.2 ± 0.8[b]
16:1n-7	1.6 ± 0.2[a]	1.4 ± 0.4[a]	0.8 ± 0.1[b]
18:1n-9	7.3 ± 0.3[a]	6.4 ± 0.6[a]	5.2 ± 0.2[b]
18:1n-7	1.3 ± 0.1[a]	1.1 ± 0.1[ab]	1.0 ± 0.1[b]
20:1n-9	1.9 ± 0.4[a]	1.2 ± 0.1[b]	0.9 ± 0.2[b]
24:1	1.0 ± 0.2[a]	0.7 ± 0.1[ab]	0.5 ± 0.1[b]
Total monoenes[2]	14.2 ± 1.5[a]	11.7 ± 1.0[b]	9.0 ± 0.4[c]
18:2n-6	1.9 ± 0.2[a]	2.3 ± 0.3[ab]	2.9 ± 0.4[b]
20:2n-6	0.4 ± 0.1[a]	0.7 ± 0.2[a]	1.1 ± 0.1[b]
20:3n-6	0.5 ± 0.1[a]	0.7 ± 0.2[ab]	1.1 ± 0.2[b]
20:4n-6	0.9 ± 0.1[a]	1.5 ± 0.6[ab]	1.6 ± 0.2[b]
Total n-6[3]	5.4 ± 0.2[a]	6.3 ± 0.7[a]	8.1 ± 0.6[b]
20:5n-3	0.9 ± 0.1[a]	0.9 ± 0.1[a]	0.6 ± 0.1[b]
22:5n-3	1.3 ± 0.1	1.3 ± 0.2	1.1 ± 0.1[b]
22:6n-3	33.5 ± 1.1	35.0 ± 3.3	32.1 ± 0.3
Total n-3[4]	36.6 ± 1.3	37.6 ± 3.5	33.8 ± 0.1
Total PUFA	42.0 ± 1.5	43.9 ± 3.8	41.9 ± 0.6
n-3/n-6	6.7 ± 0.2[a]	6.0 ± 0.6[a]	4.2 ± 0.4[b]
20:4/20:5	6.7 ± 0.2[a]	1.6 ± 0.5[a]	2.8 ± 0.4[b]

Footnotes as described in Table 8.

Table 10 Fatty acid composition of gill phosphatidylinositol from salmon given increasing levels of dietary linoleic acid

Fatty acid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		weight %	
14:0	0.7 ± 0.2	0.7 ± 0.2	0.6 ± 0.2
16:0	10.9 ± 0.5[a]	14.7 ± 0.4[b]	14.2 ± 0.6[b]
18:0	22.6 ± 0.9[a]	21.2 ± 2.4[ab]	20.6 ± 0.9[b]
Total saturates[1]	35.6 ± 1.0	38.5 ± 2.4	36.4 ± 0.7
16:1n-7	2.5 ± 0.2[a]	2.6 ± 0.5[a]	3.7 ± 0.3[b]
18:1n-9	10.0 ± 0.4[a]	11.5 ± 0.7[b]	11.2 ± 0.4[b]
18:1n-7	1/7 ± 0.1[a]	3.1 ± 0.3[b]	2.1 ± 0.1[c]
20:1n-9	1.5 ± 0.2[a]	1.8 ± 0.3[b]	1.0 ± 0.1[b]
24:1	0.9 ± 0.1	0.8 ± 0.2	0.8 ± 0.2
Total monoenes[2]	16.0 ± 0.7[a]	19.7 ± 1.2[b]	18.8 ± 0.2[b]
18:2n-6	2.2 ± 0.2[a]	4.7 ± 0.5[b]	6.8 ± 0.3[c]
20:2n-6	0.3 ± 0.1[a]	0.5 ± 0.1[a]	1.0 ± 0.1[b]
20:3n-6	0.5 ± 0.1[a]	1.1 ± 0.1[b]	1.5 ± 0.1[c]
20:4n-6	8.2 ± 0.3	8.0 ± 0.8	9.0 ± 0.3
Total n-6[1]	12.5 ± 0.3[a]	17.5 ± 0.8[b]	20.3 ± 0.7[c]
20:5n-3	2.7 ± 0.2[a]	2.0 ± 0.2[b]	1.2 ± 0.2[c]
22:5n-3	1.2 ± 0.2[a]	0.7 ± 0.1[b]	0.5 ± 0.1[b]
22:6n-3	17.2 ± 0.4[a]	14.5 ± 0.6[b]	14.6 ± 0.3[b]
Total n-3[2]	22.2 ± 0.1[a]	18.2 ± 0.4[b]	17.2 ± 0.4[c]
Total PUFA	34.7 ± 0.2[a]	35.7 ± 0.4[a]	37.5 ± 0.9[b]
n-3/n-6	1.8 ± 0.1[a]	1.0 ± 0.1[b]	0.8 ± 0.1[b]
20:4/20:5	3.0 ± 0.2[a]	4.1 ± 0.3[b]	7.9 ± 1.1[c]

Footnotes as described in Table 8.

Table 11 Plasma 6-keto-$PGF_{1\alpha}$ and TXB_2 concentrations in salmon given increasing dietary levels of linoleic acid

Eicosanoid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		pg/ml plasma	
6-keto-$PGF_{1\alpha}$	1103 ± 84[a]	1005 ± 177[a]	1664 ± 323[b]
TXB_2	402 ± 130[a]	293 ± 54[a]	1418 ± 1083[b]

Values are mean ± SD for 4 fish per treatment.
Values in the same row with different superscript letters are significantly different.

LTB_4, 12-HETE, TXB_2 and 12-HETE/12-HEPE ratio were all significantly increased, whereas 12-HEPE was significantly reduced in fish given the highest dietary LA. While LTB_5 was not significantly affected, the ratio of LTB_4/LTB_5 was significantly increased in fish given the highest dietary LA.

The production of eicosanoids by isolated gill cells stimulated with A23187 is shown in Table 13. The production of both 12-HEPE and 12-HETE was significantly reduced with increasing dietary linoleic acid although the ratio 12-HETE/12-HEPE indicated that the EPA-derived species was more affected. The production of a DHA-derived 12-lipoxygenase product, eluting immediately before 12-HETE on HPLC, was confirmed by labeling gill

Table 12 Eicosanoid production by whole blood of salmon given increasing dietary linoleic acid, after stimulation with calcium ionophore A23187

Eicosanoid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		ng/ml whole blood	
LTB_4	54.8 ± 21.8[a]	75.3 ± 15.0[ab]	101.4 ± 27.3[b]
LTB_5	38.3 ± 10.2	28.7 ± 8.3	29.2 ± 2.2
LTB_4/LTB_5	1.44 ± 0.60[a]	2.85 ± 0.99[ab]	3.53 ± 1.17[b]
12-HETE	55.6 ± 7.1[a]	53.3 ± 14.1[a]	92.7 ± 8.8[b]
12-HEPE	40.5 ± 6.8[a]	24.7 ± 3.9[b]	20.8 ± 3.4[b]
12-HETE/12-HEPE	1.69 ± 0.39[a]	2.20 ± 0.60[ab]	3.81 ± 1.51[b]
TXB_2	36.4 ± 8.7[a]	53.1 ± 19.9[a]	136.5 ± 41.1[b]

Footnotes as described in Table 11.

Table 13 Eicosanoid production by isolated gill cells from salmon given diets with increasing linoleic acid, after stimulation with calcium-ionophore A23187

Eicosanoid	Diet 1 (10% LA)	Diet 2 (25% LA)	Diet 3 (45% LA)
		ng/mg protein	
12-HEPE	48.8 ± 11.3[a]	22.1 ± 5.9[b]	5.9 ± 2.1[c]
12-HETE	17.7 ± 4.9[a]	13.1 ± 2.5[ab]	6.9 ± 3.8[b]
12-HETE/12-HEPE	0.33 ± 0.04[a]	0.64 ± 0.16[b]	0.98 ± 0.15[c]
14-HDHE	43.1 ± 15.4[a]	19.7 ± 4.4[b]	10.8 ± 4.4[c]
6-keto-$PGF_{1\alpha}$	0.23 ± 0.06[a]	ND	0.14 ± 0.06[b]
TXB_2	0.30 ± 0.14	ND	0.30 ± 0.12

Footnotes as described in Table 11.
ND = not determined.

cells with [1-^{14}C]-DHA. The peak detected by absorbance at 235 nm co-eluted with the maximum radioactive peak when eicosanoids derived from gill cells labeled with [1-^{14}C]-DHA were analysed by HPLC. We presume it is the 12-lipoxygenase product of DHA, 14-hydroxy-4,7,10,13,16,19-docosahexaenoic acid (14-HDHE). Production of the putative 14-HDHE was also decreased with increasing dietary linoleic acid. Production of TXB_2 was not affected by dietary linoleic acid but the amount of 6-keto-$PGF_{1\alpha}$ was significantly reduced.

DISCUSSION

The increase in dietary LA is reflected by increased incorporation of this fatty acid into the membrane phospholipids of leucocytes and gill cells. The concomitant incorporation of increased levels of 20-carbon n-6 PUFA in these phospholipids confirms the presence of active Δ-6- and Δ-5-desaturation and elongation of C18 PUFA in salmon. 20:3n-6 is increased by up to 4-fold in leucocyte and 7-fold in gill phospholipids which is considerably greater than the rise in AA. The accumulation of 20:3n-6 suggests that Δ-5-desaturase is rate limiting in Atlantic salmon which is in agreement with a previous study in isolated hepatocytes of rainbow trout (27). While increasing dietary LA caused a moderate increase in phospholipid AA, it simultaneously resulted in decreased EPA and thereby a substantially increased 20:4/20:5 eicosanoid precursor ratio. However, while the diets contained increasing levels of LA they also contained decreasing levels of EPA. Therefore, the observed increase in phospholipid AA as a result of increasing dietary LA may not purely reflect increased desaturation and elongation of LA but may also result from decreased dietary EPA. In a previous study (10), the sum of AA and EPA in liver and muscle polar lipids was maintained at a constant level despite dietary fluctuation and thus it appeared that these two fatty acids were interchangeable. Therefore, although fish will preferentially incorporate EPA into their tissue phospholipids, they can substitute AA when dietary EPA, which may be specifically required for production of DHA, is reduced.

In the present study we used whole blood, rather than isolated leucocytes, to study eicosanoid metabolism. This method was chosen because it closely resembles the in vivo situation where interaction between cell types can profoundly influence the overall eicosanoid spectrum produced (28). Studies

with a related salmonid species, the rainbow trout, suggest that erythrocytes are not capable of eicosanoid synthesis (29). The changes in leucocyte membrane fatty acid composition in the present study are reflected in the nature of the eicosanoids generated on stimulation with A23187. Increasing dietary LA results in increased production of AA-derived eicosanoids and increased ratios of LTB_4/LTB_5 and 12-HETE/12-HEPE. LTB_4 is a particularly powerful chemotactic agent for neutrophils and it is also active in stimulating cellular uptake of calcium (30). While LTB_5 has similar activity it is around 30 times less potent (31). TXA_2 is a potent agonist for platelet aggregation, vasoconstriction and increasing intracellular calcium (32). The production of these highly bioactive species, which is enhanced in salmon given increased levels of LA, could clearly result in altered immune function, increased inflammatory activity and other undesirable pathophysiological effects.

A previous study using gill homogenates from rainbow trout demonstrated a 12-lipoxygenase activity capable of converting exogenous radiolabeled AA and EPA to their corresponding 12-hydroxy metabolites (15). The same authors, again using gill homogenates, demonstrated that AA was the major fatty acid released from endogenous PUFA and that 12-HETE was the predominant metabolite (33), and they postulated that the precursors for the lipoxygenase metabolites were derived from AA-rich PI. In a previous study using salmon given diets containing fish oil (11) and in the present study with salmon given 10% LA, the major lipoxygenase metabolite produced by intact, isolated gill cells was EPA-derived 12-HEPE. Only in salmon given the highest level of dietary LA did the ratio of 12-HETE/12-HEPE increase towards unity. The ratio of the two 12-lipoxygenase products in gill cells is most closely related to the ratio of AA:EPA in gill PC and least closely to the corresponding ratio in PI. This suggests that the former phospholipid is much more likely than the latter to be the source of precursors for the gill lipoxygenase. A similar relationship has also been observed in mammalian platelets (34) and cultured umbilical cells (35). However, the present studies with whole blood show that the average ratios for 12-HETE/12-HEPE and LTB_4/LTB_5 most closely approach the AA/EPA ratio in PE.

Previous studies showed that human platelets could synthesise 14-HDHE from exogenous [1-^{14}C]-DHA (36) but rat platelets and guinea-pig epidermis did not produce DHA-derived lipoxygenase metabolites from endogenous DHA (37, 38). In the present study we demonstrated that DHA released after stimulation of gill cells with A23187 was a substrate for 12-lipoxygenase. While the production of 14-HDHE, from endogenous DHA, has been observed in homogenates of trout gill (33), this is the first evidence that 12-lipoxygenase products of DHA are generated by intact gill cells.

While increasing dietary LA caused a reduction in the ratio of EPA:AA-derived lipoxygenase products in gills, it also caused a dramatic reduction in total lipoxygenase metabolites produced from AA, EPA and DHA. Similarly, there was a decrease in 6-keto-$PGF_{1\alpha}$ but no significant decrease in TXB_2 produced by gill cells in fish given the highest dietary LA. It is difficult to account for the marked decrease in eicosanoid products in gills in terms of altered levels of their substrate fatty acids esterified in phospholipids, especially in the cases of AA and DHA. It may be notable, however, that the amount of 20:3n-6 was increased up to 7-fold in fish given the highest dietary LA and this fatty acid is known to inhibit production of AA-derived lipoxygenase metabolites (38). PGE_1, which is derived from 20:3n-6, can cause increased synthesis of cAMP, a potent inhibitor of AA mobilisation (39). Recent studies with rats have shown that alteration of dietary n-3/n-6 PUFA ratios can affect the activity of enzymes involved in lipid metabolism. Thus, the activity of phospholipase A_2 was found to be either increased (40) or decreased (41), depending on the tissue, when dietary n-3/n-6 PUFA was reduced. A decrease in phospholipase activity in gills of salmon given high dietary LA, which would result in less available substrate for lipoxygenase and cyclooxygenase, could explain the diminished eicosanoid production in gills observed in the present study. While the role of 12-lipoxygenase metabolites is, at present, not clearly understood there is evidence that they are involved in the regulation of ion channels (42, 43). Gills are fundamentally involved in osmoregulation and any compromise in the control of ionic fluxes could clearly have very serious implacations for the physiological integrity of the fish.

This study has demonstrated that membrane phospholipid fatty acid compositions in gills and leucocytes of salmon can be altered by giving diets with increasing levels of dietary LA. The resulting changes in eicosanoid precursors are generally reflected in an increase in AA-derived eicosanoids which could have important consequences for the occurence of stress-induced conditions in these fish.

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The incorporation and metabolism of polyunsaturated fatty acids in phospholipids of cultured cells from chum salmon (*Oncorhynchus keta*)

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Abstract

The incorporation and metabolism of (n – 3) and (n – 6) polyunsaturated fatty acids were studied in a cell line derived from chum salmon heart (CHH-1). Supplementing media with 25 μM fatty acid considerably altered the cellular fatty acid composition but did not affect the lipid class composition or cause the appearance of cytoplasmic lipid droplets. CHH-1 cells exhibited considerable Δ-6-desaturase activity but showed no preference between (n – 3) and (n – 6)PUFA substrates. CHH-1 cells also possess Δ-5-desaturase activity which showed preference towards (n – 3)PUFA, but Δ-4-desaturase activity was totally absent. Elongation of 20-carbon PUFA was especially active in CHH-1 cells with 22-carbon PUFA being specifically incorporated into PE and PS lipid classes. The fatty acid composition of PI indicated specific incorporation of 20-carbon PUFA into this lipid class. Supplementation with 22:6(n – 3) generated fatty acid compositions more closely resembling those of intact salmonid hearts. Substantial chain shortening of 22:6(n – 3) to 20:5(n – 3) occurred.

Introduction

Fish tissues are characterized by high levels of polyunsaturated fatty acids (PUFA) which are predominantly of the (n – 3) series as opposed to the (n – 6) series PUFA which predominate in terrestrial animals (Padley *et al.* 1986; Henderson and Tocher 1987). In general, freshwater fish can metabolize linolenic acid, 18:3(n – 3), by Δ-6-desaturation to 18:4(n – 3) and after subsequent elongation and Δ-5- and Δ-4-desaturation, produce docosahexaenoic acid, 22:6(n – 3) (Henderson and Tocher 1987). Marine fish, such as the turbot (*Scophthalmus maximus*), apparently lack Δ-5-desaturase and require the long chain PUFA eicosapentaenoic acid, 20:5(n – 3), and 22:6(n – 3) to be supplied by the diet (Owen *et al.* 1975). These metabolic pathways have recently been confirmed in cultured cells from turbot and rainbow trout (*Oncorhynchus mykiss*) (Tocher 1990; Tocher and MacKinlay 1990). In an anadramous species, Atlantic salmon (*Salmo salar*), the ability to elongate and desaturate 18:2(n – 6) was apparently lost during smoltification (Ackman and Takeuchi 1986) but

Abbreviations: BHT, butylated hydroxytoluene; BSA, bovine serum albumin; CL, cardiolipin; FCS, fetal calf serum; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SM, sphingomyelin.

was regained in post-smolts (Bell *et al.* 1989).

In cultured cells, the only added lipid tends to be derived from the serum supplement which is usually of mammalian origin. This results in cultured cells having a fatty acid composition which largely reflects that of the serum supplement (Spector *et al.* 1981). Fetal calf serum (FCS) is normally the serum of choice but fish cells cultured in FCS are generally deficient in (n – 3)PUFA and usually contain elevated concentrations of (n – 6)PUFA (Tocher *et al.* 1988).

In a recent study with Atlantic salmon, we observed the development of a severe cardiomyopathy in fish given diets containing sunflower oil (high in 18:2(n – 6)) whereas no lesion was seen in fish given diets containing fish oil (Bell *et al.* 1991). A number of other cardiac abnormalities including epicarditis and haemopericarditis have also been observed in farmed Atlantic salmon (Ferguson *et al.* 1986; Raynard and Houghton 1991).

Feeding diets with varying levels of (n – 3) and (n – 6)PUFA to rats resulted in modification of cardiac membrane lipids (Swanson and Kinsella 1986) and subsequent alteration of membrane bound enzyme activities (Leonardi *et al.* 1987; Swanson *et al.* 1989). In the present study we investigated the incorporation and metabolism of (n – 3) and (n – 6)PUFA in an established cell line (CHH-1) from chum salmon (*Oncorhynchus keta*) (Lannan *et al.* 1984). These cells had been derived from primary cultures of heart cells from juvenile salmon prepared as described by Wolf and Quimby (1976). Our aim was to determine the pathways of desaturation and elongation present in these cells and to observe whether there was preferential metabolism of (n – 3)PUFA compared to (n – 6) PUFA.

Materials and methods

Materials

Eagle's Minimum Essential Medium (EMEM), trypsin-EDTA and fetal calf serum (FCS) were obtained from Northumbria Biologicals Ltd., Cramlington, U.K. Tryptose phosphate broth, 10× concentrated non-essential amino acids, sodium bicarbonate, antibiotics and HCl were obtained from Flow Laboratories, Rickmansworth, U.K. All PUFA (approx. 99% pure), fatty acid-free bovine serum albumin (BSA), butylated hydroxy toluene (BHT), and Trizma were obtained from Sigma Chemical Co. Ltd., Poole, U.K. All solvents were HPLC grade and were obtained from Rathburn Chemicals, Walkerburn, U.K.

Cells and medium

The chum salmon heart cell line (CHH-1) was obtained from Dr. D. Smail, S.O.A.F.D. Marine Laboratory, Aberdeen, U.K., and was maintained in EMEM (with Earle's salts) containing 1% tryptose phosphate broth, 4 mM Tris-HCl buffer (Trizma, pH 7.4), 1% non-essential amino acids, 0.275% sodium bicarbonate, 2 mM HCl, antibiotics (50 I.U.ml^{-1} penicillin and 50 μg.ml^{-1} streptomycin) and either 10% or 1% FCS.

PUFA supplements

PUFA supplements were added to the CHH-1 cultures as BSA complexes prepared largely by the method of Spector and Hoak (1969). To reduce oxidation all procedures were carried out at room temperature under a stream of nitrogen. The PUFA-BSA complexes were filter sterilized through 0.2 μm filters (Flowpore, Flow Laboratories) prior to use. The fatty acid concentrations were assayed after addition of an internal standard (17:0) to an aliquot of the complex. The lipids were then extracted, transmethylated and quantitated by gas-liquid chromatography. PUFA concentrations were in the range 1.5–2.0 mM (BSA = 0.25 mM) giving a PUFA:BSA ratio of 6–8. The PUFA:BSA mixtures were stored under nitrogen at −20°C in darkened vials.

Incubation conditions

For routine cell cultivation the cultures were grown in sealed 75 cm^2 flasks (Northumbria Biological

Ltd.) in 15 ml medium containing 10% or 2% FCS. Cells were harvested for analysis or subculture within 24h of achieving confluence (usually after 5–7 days). Cells incubated with PUFA were subcultured into 225 cm^2 flasks with 50 ml medium containing 1% FCS and an appropriate volume of PUFA:BSA complex to give a final fatty acid concentration of 25 μM. FCS contains approximately 0.37% lipid of which around 50% is fatty acid. The lipid class composition and fatty acid composition of FCS has been given in detail previously (Tocher *et al.* 1988). Previous studies with cultured fish cells (Tocher *et al.* 1988, 1989) indicated that this fatty acid concentration resulted in considerable incorporation and metabolism into phospholipids without causing increased deposition of neutral lipid. Control flasks received the same volume of fatty acid free BSA solution. All incubations were performed at 22°C.

Cell harvesting and lipid extraction

Cells were harvested within 24h of reaching confluence. The media were removed by aspiration and the cells were washed with 20 ml phosphate-buffered saline before addition of 3 ml trypsin-EDTA (0.05% and 0.02% respectively). Once the cells became dissociated 6 ml of basal medium were added to inhibit trypsin activity. Cells from two 225 cm^2 flasks were pooled and centrifuged at 600 × g for 10 min at 4°C, the supernatant poured off and the cell pellet washed twice with 25 ml of phosphate-buffered saline to ensure removal of serum. Total lipid was extracted from the cells largely by the method of Folch *et al.* (1957). Cells were homogenised in 5 ml of ice-cold chloroform: methanol (2:1, v/v), 1 ml of 0.88% KCl added, followed by mixing on a vortex mixer. The phases were separated by centrifugation, the chloroform layer removed and dried under nitrogen and the residue dissolved in chloroform:methanol (2:1, v/v) containing 0.05% BHT at a final concentration of 50 mg/ml.

Lipid class analysis

Approximately 10 μg of total lipid were applied to a 10 × 10 cm high-performance thin-layer chromatography (HPTLC) plate that had been pre-run in hexane:diethyl ether (1:1, v/v) and activated at 110°C for 30 min. The plates were developed to 6 cm in methylacetate:isopropanol:chloroform:methanol:0.25% aqueous KCl (25:25:25:10:9 by volume) to separate phospholipid classes with neutral lipids running at the solvent front (Vitiello and Zanetta 1978). After drying, the plates were developed fully in hexane:diethyl ether:acetic acid (85:15:1.5, v/v/v) to separate neutral lipids and cholesterol. Lipid classes were visualised by charring at 160°C for 15 min after spraying with 3% copper acetate (w/v) in 8% phosphoric acid (v/v) and identified by comparison with commercially available standards. Lipid classes were quantified by scanning densitometry using a Shimadzu CS-930 dual wavelength TLC scanner and a DR-2 recording integrator. Data are means of triplicate analyses; standard deviations are omitted for clarity but were generally less than 5% of the mean.

Lipid class separation and fatty acid analysis

Total lipid (approx. 1 mg) was applied in a 1 cm streak to a 10 × 10 cm HPTLC plate and separated into phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) fractions as decribed by Vitiello and Zanetta (1978). The plates were sprayed with 0.1% 2′,7′-dichlorofluorescein in 97% methanol containing 0.05% BHT, and the lipid bands were visualised under UV light. The lipid bands were scraped from the plate and acid-catalysed transmethylation was performed overnight at 50°C by the method of Christie (1982). The fatty acid methyl esters were separated and quantified by gas-liquid chromatography (Carlo Erba GC 6000 Vega series 2, Fisons Ltd., Crawley, U.K.) using a capillary column (CP-Wax 51, 50 m × 0.32 mm, Chrompack (U.K.) Ltd., London) with on-column injection using hydrogen as carrier gas and with a two-stage thermal gradient from 50°C to 225°C.

Table 1. Lipid class composition of CHH-1 cells cultured in 10% fetal calf serum (FCS) or 1% FCS supplemented with various fatty acids. Results are means of triplicate experiments

	Lipid Supplement							
Lipid	10% FCS	18:2 n-6	18:3 n-3	18:3 n-6	20:3 n-6	20:4 n-6	20:5 n-3	22:6 n-3
%Phospholipids	69.0	63.8	57.1	59.7	66.4	69.5	64.8	60.2
%Neutral lipids	29.6	34.6	40.4	38.6	33.6	30.5	34.9	39.8
Phospholipid classes: (% total lipid)								
SM	3.3	3.5	3.5	3.6	3.9	3.9	3.7	3.6
PC	23.0	22.9	20.1	22.1	28.8	27.4	23.6	24.0
PE	16.2	17.6	16.1	16.9	18.1	19.9	18.6	16.7
PS	9.6	6.6	6.2	6.5	4.7	7.1	7.3	5.7
PI	12.3	8.9	7.4	7.2	6.8	7.8	7.8	6.3
PA/CL	4.6	4.4	3.9	3.7	4.3	3.6	4.0	4.0
Neutral classes: (% total lipid)								
Triacylglycerol	5.9	8.8	12.3	11.7	12.6	9.0	6.4	16.3
Free Fatty Acids	0.8	1.3	3.7	3.3	5.0	5.4	6.3	7.6
Diacylglycerol	0.3	1.0	3.2	2.7	1.9	3.3	3.7	3.0
Cholesterol	22.5	23.6	21.3	21.0	14.1	12.8	18.7	13.1

– = not detected.

Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman 1980; Bell *et al.* 1983).

Statistical analysis

Significance of difference ($p < 0.05$) between data in Table 2 and data in Tables 3–9 was determined by Student's t-test.

Results

The lipid class compositions of CHH-1 cells grown in 10% FCS or 1% FCS plus various fatty acids at 25 μM are shown in Table 1. In all cases, PC was the predominant phospholipid closely followed by PE which together accounted for approximately 40% of the total lipid. PS and PI were present in approximately equal amounts with PI always the dominant species followed by PA/CL and SM. Some accumulation of neutral lipid was observed in cells supplemented with 18:3(n-3) and 22:6(n-3) but microscopic examination of the cells indicated minimal presence of lipid droplets in all of the experimental treatments. Cells grew well and attained confluence in 5–7 days with all fatty acid supplements except 20:3(n-6). Cells supplemented with 20:3(n-6) did not attain confluence, after 4–5 days they appeared pyknotic and after 6–7 days they began to detach from the flask.

The fatty acid compositions of phospholipid classes of cells cultured in 10% FCS are shown in Table 2. The (n-9) series PUFA are dominant in all phospholipid classes, except PI, where (n-6) species predominate. 18:2(n-9) is the predominant (n-9)PUFA in all phospholipid classes except PI where 20:2(n-9) predominates. 20:3(n-9), which could not be separated from 20:2(n-6) in this chromatographic system, did not accumulate in CHH-1 cells. The (n-6)PUFA were generally more abundant than (n-3)PUFA, except in PS, and the major (n-6) species were 20:4, 18:2 and 20:2. The major (n-3)PUFA were 22:5 and 22:6. The greatest amount of PUFA were found in PI with the least being in PC, but in all classes the monoenes, mostly 18:1(n-9), and the saturates, mostly 16:0

Table 2. Fatty acid composition of phospholipid classes of CHH-1 cells cultured in medium containing 10% fetal calf serum

Fatty acid	Phospholipid class			
	PC	PS	PI	PE
14:0	1.5 ± 0.1	0.8 ± 0.1	0.4 ± 0.0	0.3 ± 0.0
16:0	18.8 ± 0.2	9.8 ± 0.1	4.4 ± 0.1	5.0 ± 0.2
18:0	6.5 ± 0.1	26.4 ± 1.4	19.2 ± 1.7	5.1 ± 0.3
Total saturates[a]	27.6 ± 0.3	37.7 ± 1.4	25.0 ± 1.5	10.8 ± 0.4
16:1n-9	5.2 ± 0.2	3.1 ± 0.1	0.8 ± 0.1	3.3 ± 0.0
16:1n-7	4.9 ± 0.0	2.8 ± 0.1	1.1 ± 0.1	3.2 ± 0.2
18:1n-9	44.2 ± 1.0	25.1 ± 0.3	25.8 ± 0.9	36.2 ± 0.4
18:1n-7	3.1 ± 0.1	1.7 ± 0.1	2.2 ± 0.5	2.9 ± 0.1
20:1n-9	0.8 ± 0.4	1.0 ± 0.1	0.4 ± 0.0	0.5 ± 0.4
24:1	0.7 ± 0.2	0.9 ± 0.4	1.0 ± 0.5	0.5 ± 0.1
Total monoenes	58.9 ± 0.5	34.6 ± 0.6	31.3 ± 0.9	46.6 ± 0.4
18:2n-9	4.3 ± 0.2	5.6 ± 0.0	2.6 ± 0.3	5.3 ± 0.1
20:2n-9	3.8 ± 0.1	4.6 ± 0.1	11.1 ± 0.4	4.3 ± 0.1
Total n-9	8.1 ± 0.3	10.2 ± 0.2	13.7 ± 0.7	9.6 ± 0.0
18:2n-6	0.6 ± 0.1	1.0 ± 0.1	0.7 ± 0.0	1.0 ± 0.1
18:3n-6	0.2 ± 0.1	0.7 ± 0.4	0.5 ± 0.3	0.1 ± 0.1
20:2n-6[b]	0.3 ± 0.0	0.3 ± 0.0	3.6 ± 0.1	1.5 ± 0.1
20:3n-6	0.3 ± 0.0	0.6 ± 0.2	2.1 ± 0.2	0.5 ± 0.0
20:4n-6	0.4 ± 0.1	0.6 ± 0.1	7.8 ± 1.0	4.5 ± 0.2
22:2n-6	-	0.4 ± 0.0	0.5 ± 0.2	0.3 ± 0.1
22:4n-6	-	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.0
22:5n-6	0.4 ± 0.3	0.7 ± 0.1	0.4 ± 0.0	0.4 ± 0.2
Total n-6	2.2 ± 0.4	4.9 ± 0.3	16.1 ± 1.5	8.7 ± 0.2
18:3n-3	t	0.1 ± 0.0	0.1 ± 0.0	t
18:4n-3	t	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
20:5n-3	0.1 ± 0.0	0.1 ± 0.0	0.8 ± 0.3	1.4 ± 0.0
22:5n-3	0.3 ± 0.0	3.6 ± 0.5	4.2 ± 0.7	2.8 ± 0.0
22:6n-3	0.2 ± 0.0	2.2 ± 0.3	3.7 ± 0.8	4.2 ± 0.2
Total n-3	0.6 ± 0.0	6.2 ± 0.7	9.0 ± 1.2	8.5 ± 0.2
Total PUFA	10.9 ± 0.6	21.3 ± 0.6	38.8 ± 0.5	26.8 ± 0.4
n-3/n-6	0.3 ± 0.1	1.4 ± 0.4	0.6 ± 0.2	1.0 ± 0.1
Total dimethyl acetals	0.4 ± 0.0	-	-	13.3 ± 0.7
Total unidentified	2.2 ± 0.2	6.4 ± 0.2	4.9 ± 2.0	2.5 ± 0.3

Data are shown as means ± SD of triplicate experiments; results are expressed as % by weight; [a] includes 15:0, 17:0 and 20:0; [b] includes 20:3n-9; t = trace value less than 0.1%; SD < 0.05 are tabulated as 0.0.

and 18:0, formed the main bulk of phospholipid fatty acids.

The data in the following tables (Tables 3–9) are dealt with in comparison to data in Table 2. The (n-6)PUFA composition of phospholipid classes from CHH-1 cells supplemented with 18:2(n-6) is shown in Table 3, where the total (n-6)PUFA content of all phospholipid classes is significantly increased. Apart from 18:2(n-6), there were significant increases in the Δ-6-desaturation product 18:3(n-6) and the elongation product 22:3(n-6) in PC and PE. 20:2(n-6) and 20:3(n-6) were significantly increased in all phospholipid classes. 20:3(n-6) showed the greatest increase, particularly in PI. The highest percentage of 18:2(n-6) was observed in PE.

Table 3. (n – 6)PUFA composition of phospholipid classes from CHH-1 cells cultured in medium supplemented with 18:2(n – 6)

n – 6PUFA	PC	PS	PI	PE
18:2	23.8 ± 1.5*	19.7 ± 1.5*	17.2 ± 1.1*	34.4 ± 0.2*
18:3	0.8 ± 0.1*	1.0 ± 0.1	0.7 ± 0.1	0.9 ± 0.0*
20:2	0.8 ± 0.1*	1.4 ± 0.3*	5.1 ± 0.2*	1.9 ± 0.1*
20:3	1.2 ± 0.2*	3.7 ± 0.5*	11.8 ± 0.7*	1.8 ± 0.1*
20:4	0.2 ± 0.1	0.5 ± 0.2	5.2 ± 0.8*	1.3 ± 0.2*
22:2	0.2 ± 0.0*	0.4 ± 0.1	0.4 ± 0.1	0.6 ± 0.2
22:3	0.1 ± 0.0*	0.6 ± 0.2	0.2 ± 0.2	0.3 ± 0.1*
22:4	t	0.2 ± 0.0*	0.1 ± 0.1*	0.2 ± 0.0
22:5	0.2 ± 0.1	0.2 ± 0.1*	0.2 ± 0.1*	t
Total	27.3 ± 1.8*	27.7 ± 1.0*	40.9 ± 2.6*	41.4 ± 0.4*

Data are shown as means ± SD of triplicate experiments; results are expressed as a % of total fatty acids; t = trace value less than 0.1%; SD < 0.05 are tabulated as 0.0; * Values are significantly different ($p < 0.05$) compared to cells cultured in 10% FCS.

Table 4. (n – 3)PUFA composition of phospholipid classes from CHH-1 cells cultured in medium supplemented with 18:3(n – 3)

n – 3PUFA	PC	PS	PI	PE
18:3	24.9 ± 2.3*	21.0 ± 0.8*	12.5 ± 1.0*	30.1 ± 0.4*
18:4	1.4 ± 0.1*	1.4 ± 0.1*	0.7 ± 0.0*	1.3 ± 0.1*
20:3	2.0 ± 0.5*	1.8 ± 0.3*	2.4 ± 0.2*	2.8 ± 0.6*
20:4	3.2 ± 0.5*	4.2 ± 0.2*	9.4 ± 0.1*	5.3 ± 0.8*
20:5	0.3 ± 0.1*	0.5 ± 0.1*	4.5 ± 0.8*	2.6 ± 0.0*
22:5	0.2 ± 0.0	1.8 ± 0.3*	1.2 ± 0.3*	1.4 ± 0.1*
22:6	0.1 ± 0.0	1.3 ± 0.2*	0.7 ± 0.3*	1.2 ± 0.1*
Total	32.1 ± 3.4*	32.0 ± 0.9*	31.0 ± 1.3*	44.7 ± 1.9*

Data presented as described in Table 3.

The (n – 3)PUFA composition of phospholipid classes from CHH-1 cells supplemented with 18:3(n – 3) is shown in Table 4. Supplementing the media with 25 μM 18:3(n – 3) significantly increased the total (n – 3)PUFA content of all phospholipid classes with the highest levels again occurring in PE. The Δ-6-desaturase product, 18:4(n – 3) was significantly increased in all phospholipid classes as were the elongation products 20:3(n – 3) and 20:4(n – 3), with the latter being the major metabolite of 18:3(n – 3). The Δ-5-desaturation product 20:5(n – 3) was significantly increased in all phospholipid classes with the biggest increase occurring in PI. The elongation and desaturation products of 20:5(n – 3) were significantly decreased in PS, PI and PE.

The (n – 6)PUFA composition of phospholipid classes from CHH-1 cells supplemented with 18:3(n – 6) is shown in Table 5. The increase in total (n – 6)PUFA resulting from 18:3(n – 6) supplementation was generally greater than that for 18:2 (n – 6), with the exception of PE. PC showed the highest incorporation of 18:3(n – 6) and its metabolites including a significant increase in the Δ-5-desaturase product, 20:4(n – 6). The major metabolite was the elongation product, 20:3(n – 6) which showed greatest incorporation into PI but was also significantly increased in all phospholipids. The incorporation of the elongation product 22:3(n – 6) was significantly increased in all phospholipid classes, especially PE.

The (n – 6)PUFA composition of phospholipid

Table 5. (n – 6)PUFA composition of phospholipid classes from CHH-1 cells cultured in medium supplemented with 18:3(n – 6)

n – 6PUFA	PC	PS	PI	PE
18:2	0.4 ± 0.1	0.5 ± 0.0*	0.5 ± 0.1*	0.5 ± 0.1*
18:3	27.4 ± 0.3*	22.9 ± 0.1*	12.7 ± 0.9*	18.0 ± 2.4*
20:2	0.7 ± 0.0*	0.3 ± 0.0	3.6 ± 0.4	1.4 ± 0.3
20:3	14.3 ± 0.4*	6.1 ± 0.0*	16.3 ± 0.2*	10.4 ± 1.0*
20:4	1.3 ± 0.0*	0.8 ± 0.2	6.5 ± 0.4	2.9 ± 0.4*
22:2	0.2 ± 0.0*	0.4 ± 0.1	0.5 ± 0.1	0.6 ± 0.1*
22:3	1.9 ± 0.4*	0.9 ± 0.0*	1.8 ± 0.2*	3.1 ± 0.5*
22:4	0.2 ± 0.0	0.5 ± 0.0	0.4 ± 0.1	0.6 ± 0.1*
Total	46.4 ± 0.2*	32.4 ± 0.4*	42.3 ± 1.9*	37.5 ± 4.5*

Data presented as described in Table 3.

Table 6. (n – 6)PUFA composition of phospholipid classes from CHH-1 cells cultured in medium supplemented with 20:3(n – 6)

n – 6PUFA	PC	PS	PI	PE
18:2	0.4 ± 0.0*	0.7 ± 0.1*	0.4 ± 0.0*	0.4 ± 0.1*
18:3	2.7 ± 0.1*	2.0 ± 0.1*	1.4 ± 0.1*	1.2 ± 0.1*
20:2	1.0 ± 0.1*	0.7 ± 0.1*	5.6 ± 0.4*	2.0 ± 0.1*
20:3	30.5 ± 1.9*	19.2 ± 1.1*	26.3 ± 0.4*	22.3 ± 0.5*
20:4	2.0 ± 0.3*	0.8 ± 0.2	7.8 ± 1.3	3.0 ± 0.2*
22:2	0.2 ± 0.0*	0.6 ± 0.1*	0.4 ± 0.1	0.7 ± 0.2*
22:3	4.5 ± 0.3*	4.6 ± 0.3*	3.4 ± 0.2*	9.7 ± 1.1*
22:4	0.3 ± 0.1*	0.7 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
Total	41.6 ± 1.3*	29.3 ± 1.7*	45.7 ± 1.4*	39.8 ± 1.1*

Data presented as described in Table 3.

classes from CHH-1 cells supplemented with 20:3(n – 6) is shown in Table 6. The increase in total (n – 6)PUFA was of a similar magnitude to that achieved with 18:3(n – 6) with the highest incorporation into PC. PC showed a significant increase in the Δ-5-desaturase product 20:4(n – 6) but the same fatty acid was significantly reduced in PE. All classes had significantly increased levels of the elongation product 22:3(n – 6). All phospholipid classes showed significantly increased 18:3(n – 6) demonstrating the presence of chain shortening activity.

The (n – 6)PUFA composition of phospholipid classes from CHH-1 cells supplemented with 20:4 (n – 6) is shown in Table 7. Supplemented 20:4 (n – 6) generated total (n – 6)PUFA levels in all phospholipid classes similar to 20:3(n – 6) but was preferentially incorporated into PI. Chain elongation to 22:4(n – 6) was extensive with maximum incorporation into PS. No Δ-4-desaturation and elongation to 22:5(n – 6) was observed.

The (n – 3)PUFA composition of phospholipid classes from CHH-1 cells supplemented with 20:5(n – 3) is shown in Table 8. Incorporation of 20:5(n – 3) was greatest into PI although, as a result of extensive elongation to 22:5(n – 3), the highest levels of total (n – 3)PUFA were in PE. The incorporation of 22:5(n – 3) was greatest in PE and PS where it comprised 70 and 91% of the total (n – 3)PUFA respectively. There was no evidence of Δ-4-desaturation and elongation to 22:6(n – 3).

The (n – 3)PUFA composition of phospholipid classes from CHH-1 cells supplemented with 22:6(n – 3) is shown in Table 9. Supplementation with 22:6(n – 3) produced levels of total (n – 3)

Table 7. (n – 6)PUFA composition of phospholipid classes from CHH-1 cells cultured in medium supplemented with 20:4(n – 6)

n – 6PUFA	PC	PS	PI	PE
18:2	0.7 ± 0.3	1.0 ± 0.4	0.6 ± 0.0	0.4 ± 0.0*
18:3	0.7 ± 0.1*	0.8 ± 0.3	0.8 ± 0.3	0.5 ± 0.1*
20:2	0.4 ± 0.1	t	1.7 ± 0.4*	0.7 ± 0.1*
20:3	1.2 ± 0.2*	0.4 ± 0.0	0.8 ± 0.1*	0.9 ± 0.3*
20:4	12.5 ± 0.3*	2.2 ± 0.5*	23.6 ± 1.6*	13.3 ± 0.1*
22:2	0.2 ± 0.0*	0.2 ± 0.0*	0.2 ± 0.1	0.2 ± 0.0
22:3	0.2 ± 0.1*	0.2 ± 0.0*	0.2 ± 0.0*	0.2 ± 0.0*
22:4	21.5 ± 1.2*	26.8 ± 2.4*	14.7 ± 0.8*	22.7 ± 1.8*
22:5	0.1 ± 0.0	0.1 ± 0.1*	t*	0.3 ± 0.1
Total	37.5 ± 1.4*	35.3 ± 2.8*	42.6 ± 1.8*	39.2 ± 1.9*

Data presented as described in Table 3.

Table 8. (n – 3)PUFA composition of phospholipid classes from CHH-1 cells cultured in medium supplemented with 20:5(n – 3)

n – 3PUFA	PC	PS	PI	PE
18:3	t	t	–	–
18:4	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	–
20:3	t	t	0.3 ± 0.1*	t
20:4	0.5 ± 0.1*	0.4 ± 0.1*	0.3 ± 0.1*	0.3 ± 0.0*
20:5	11.0 ± 0.3*	1.6 ± 0.3*	14.8 ± 1.1*	9.7 ± 0.1*
22:5	26.3 ± 0.3*	34.6 ± 0.2*	23.4 ± 1.0*	30.7 ± 0.7*
22:6	0.5 ± 0.0*	1.3 ± 0.1*	1.0 ± 0.1*	2.9 ± 0.1*
Total	38.4 ± 0.6*	38.0 ± 0.2*	39.9 ± 0.3*	43.6 ± 0.7*

Data presented as described in Table 3.

Table 9. (n – 3)PUFA composition of phospholipid classes from CHH-1 cells cultured in medium supplemented with 22:6(n – 3)

n – 3PUFA	PC	PS	PI	PE
18:3	–	–	0.1 ± 0.0	–
18:4	–	–	–	–
20:3	–	*0.3 ± 0.1	*0.3 ± 0.1	*0.3 ± 0.1
20:4	*0.1 ± 0.0	t	t	*0.1 ± 0.0
20:5	*5.2 ± 1.0	*1.2 ± 0.0	*3.8 ± 0.2	*2.0 ± 0.2
22:5	*1.9 ± 0.2	*1.8 ± 0.0	*1.8 ± 0.0	*1.1 ± 0.1
22:6	*27.2 ± 5.4	*37.2 ± 2.6	*33.3 ± 1.1	*44.0 ± 1.5
Total	*34.4 ± 6.5	*40.5 ± 2.5	*40.2 ± 1.5	*47.5 ± 1.0

Data presented as described in Table 3.

PUFA similar to those with 20:5(n – 3). Incorporation of 22:6(n – 3) was particularly high in PE and PS (93 and 92% of total (n – 3)PUFA respectively) while increased 20:5(n – 3) in all phospholipid classes suggested that significant retroconversion of 22:6(n – 3) was occurring.

Discussion

A concentration of 25 μM fatty acid was chosen since supplementation at this level produced considerable alterations in PUFA content, without significantly altering lipid class composition or inducing intracellular lipid droplet formation in a number of previous studies utilising cultured cells (Stubbs and Smith 1984; Tocher 1990; Tocher and MacKinlay 1990). All CHH-1 cells grew well at this concentration of fatty acid supplementation except those grown in 20:3(n – 6) which did not achieve confluence and began to detach from the flask after 6–7 days. The experiment was repeated twice, using the same fatty acid concentration, with two different sources of 20:3(n – 6) and the same result was recorded. While the exact cause of the growth inhibition remains unclear, it seems unlikely that it was due to the presence of toxic oxidation products since measurement of TBA-reactive material in the PUFA-BSA complexes indicated greatest levels of oxidation products in the 20:5(n – 3) and 22:6 (n – 3) supplements which demonstrated no growth inhibition (Results not shown). In a recent study with Atlantic salmon, we observed the development of pathophysiological lesions in heart of fish given diets containing elevated 18:2(n – 6) which caused a decreased (n – 3)/(n – 6)PUFA ratio in cardiac tissue (Bell *et al.* 1991). While CHH-1 cells supplemented with 20:3(n – 6) also had a considerably reduced (n – 3)/(n – 6)PUFA ratio it is unlikely that this was solely to blame for the growth inhibition since supplementation with other (n – 6)PUFA at the same concentration did not affect growth.

The long term culture of fish cells in FCS is known to cause a deficiency in essential fatty acids of the (n – 3) series (Tocher *et al.* 1988). Although the presence of considerable amounts of (n – 9)PUFA indicates that CHH-1 cells grown in 10% FCS were deficient in (n – 3)PUFA, the distribution of fatty acids between the phospholipid classes show many of the features occurring in whole fish. For example, PC contains high levels of 16:0 and 18:1(n – 9), PS and PI high 18:0 and PI high 20:4(n – 6). With all fatty acid supplements PI tended to accumulate 20-carbon PUFA which is consistent with a role as a precursor pool for eicosanoid synthesis as postulated previously (Bell *et al.* 1983). However, the extent of the (n – 3)PUFA deficiency is apparent when comparing the CHH-1 cells grown in 10% FCS with cardiac tissue from Atlantic salmon (Bell *et al.* 1991). While CHH-1 PE contained the highest levels of 20:5(n – 3) and 22:6(n – 3) in any phospholipid class (1.4 and 4.2% respectively) the same fatty acids comprise 4 and 42% of the total in salmon heart.

The increase in 18:3(n – 6) and 20:3(n – 6) on supplementation with 18:2(n – 6) and the increase in 18:4(n – 3) and 20:4(n – 3) on supplementation with 18:3(n – 3) clearly indicates the presence of Δ-6-desaturase activity in CHH-1 cells. The relative percentages of the Δ-6-desaturase products of 18:2(n – 6) and 18:3(n – 3) indicated that there was no specificity for either substrate. This result is similar to that observed by Tocher *et al.* (1989) with cells derived from rainbow trout gonad (RTG-2) and turbot fin (TF), but differed from Atlantic salmon cells (AS) which showed a preference towards the (n – 3) substrate (Tocher and Dick 1990). However, the Δ-5-desaturase in CHH-1 cells showed a preference towards the (n – 3) substrate since supplementation with 18:3(n – 3) resulted in increased 20:5(n – 3) incorporation while 18:2 (n – 6) supplementation resulted in minimal 20:4(n – 6) production. This may not reflect a specific adaptation of fish cells since a similar substrate preference has also been observed in mammals (Stubbs and Smith 1984) and may be enhanced by the (n – 3)PUFA deficiency which exists in cells routinely cultured in FCS. In general, however, the accumulation of 20:3(n – 6) and 20:4(n – 3) on supplementation with 18:2(n – 6) and 18:3(n – 3) is compatible with a low Δ-5-desaturase activity in CHH-1 cells which is considerably less than the activity in both RTG-2 and AS cell lines (Tocher 1990; Tocher and Dick 1990).

CHH-1 cells like other salmonid derived cell lines appear to possess both Δ-6- and Δ-5-desaturase activities, while Δ-4-desaturase is apparently absent. Whether the full desaturase complement was lost as a result of continuous culture or whether the original cells were never capable of Δ-4-desaturation is not known. However, a number of cell lines are known to lose their ability to express desaturase ac-

tivities after long term culture (Maeda *et al.* 1978; Robert *et al.* 1978).

The considerable accumulation of 22C PUFA in comparison to other fish cell lines studied (Tocher 1990; Tocher and MacKinlay 1990; Tocher and Dick 1990) suggests appreciable elongase activity is present in CHH-1 cells. The large concentrations of elongation products arising from supplementation with both 20:4(n – 6) and 20:5(n – 3) may suggest some functional role for long-chain PUFA in these cells. 22:6(n – 3) is a major component of both rat cardiac phospholipids (Swanson and Kinsella 1986) and Atlantic salmon cardiac phospholipids (Bell *et al.* 1991). The concentration of 22:6(n – 3) in membrane phospholipids of cardiomyocytes can profoundly influence the activities in a number of membrane associated enzymes including Ca^{2+}-Mg^{2+} ATPase and phospholipase A (Swanson *et al.* 1989; Nalbone *et al.* 1990). Culture of CHH-1 cells in medium supplemented with 1% FCS and 25 μM 22:6(n – 3) resulted in phospholipid fatty acid compositions similar to those in Atlantic salmon heart (Bell *et al.* 1991). The ability to return these cells to a lipid composition approaching that of the salmonid heart makes them a useful model system to study the effects of membrane fatty acid composition on a number of biochemical and physiological functions.

The ability of CHH-1 cells to extensively elongate both 20:4(n – 6) and 20:5(n – 3) is contrary to the situation in rat heart and isolated cardiomyocytes which are unable to perform elongation (Mohammed *et al.* 1990) or desaturation reactions (Hagve and Sprecher 1989). Future studies in this laboratory using isolated and primary cultures of salmonid cardiomyocytes would hope to elucidate whether the high levels of 22:6(n – 3) in heart are a result of the synthetic capacity of the cardiomyocytes themselves, or are a result of hepatic modulation of dietary fatty acids followed by release, and uptake by extrahepatic tissues.

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PROSTAGLANDINS LEUKOTRIENES AND ESSENTIAL FATTY ACIDS

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Dietary Sunflower, Linseed and Fish Oils Affect Phospholipid Fatty Acid Composition, Development of Cardiac Lesions, Phospholipase Activity and Eicosanoid Production in Atlantic Salmon (*Salmo salar*)

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ABSTRACT. **Atlantic salmon (Salmo salar) post-smolts were fed practical-type diets in which the lipid was supplied either as fish oil (FO), sunflower oil (SFO) or linseed oil (LO) for 12 weeks. In general, the heart phospholipids from SFO-fed fish had increased 18:2n-6, 20:2n-6, 20:3n-6 and 20:4n-6 but decreased 20:5n-3 compared to both other dietary treatments. This was reflected in a decreased n-3/n-6 polyunsaturated fatty acid (PUFA) ratio and an increased 20:4n-6/20:5n-3 or eicosanoid precursor ratio in SFO-fed fish. While heart phospholipids of fish fed LO had increased levels of 18:2n-6, 20:2n-6 and 20:3n-6 compared to fish fed FO, 20:4n-6 levels were reduced, although only significantly in phosphatidylcholine (PC). Dietary-induced changes in phospholipid fatty acid compositions of blood leucocytes were similar to those in heart, although fish fed LO had increased 20:5n-3 compared to fish fed FO. Thromboxane B_2 (TXB_2) produced by stimulated blood cells was reduced in fish fed LO compared to those fed SFO. Prostaglandin E_2 (PGE_2) production was reduced in LO-fed fish compared to both other dietary treatments. Fish fed LO had reduced PC in heart membranes compared to the other two dietary treatments, resulting in a ratio of PC:PE (phosphatidylethanolamine) less than unity. Fish fed SFO developed a marked cardiac histopathology which, while present in FO-fed fish albeit in a less severe form, was virtually absent in fish fed LO. Fish fed SFO had increased heart phospholipase A activity compared to those given either FO or LO.**

INTRODUCTION

Both essential fatty acids, linoleic acid [18:2n-6] and α-linolenic acid [18:3n-3] are metabolised by the same enzyme systems of sequential desaturation and elongation which result in the production of long chain polyunsaturated fatty acids (PUFA) of the n-6 and n-3 series (1). Most freshwater fish are capable of Δ-6-desaturation of 18:3n-3 to 18:4n-3 followed by elongation and Δ-5-desaturation to eicosapentaenoic acid [EPA, 20:5n-3] which finally undergoes elongation and Δ-4-desaturation to give docosahexaenoic acid [DHA, 22:6n-3] (2). Atlantic salmon (Salmo salar) are probably capable of metabolising 18:2n-6 similarly, since feeding diets containing corn oil resulted in increased arachidonic acid [AA, 20:4n-6] in membrane lipids (3). An important consideration in such experiments is that one type of fatty acid can influence the metabolism of the other (4). Thus, an excess of n-6PUFA may inhibit metabolism of 18:3n-3 although, in general, the n-3PUFA are more efficient in inhibiting the metabolism of n-6PUFA than vice versa (5). AA can be metabolised by both cyclooxygenase and lipoxygenase to yield a wide range of highly bioactive eicosanoids (6). EPA can also give rise to eicosanoids although their biopotency is usually considerably less than that of the AA homologues (7). In addition, both EPA and DHA, the major PUFA in fish tissues (2) and 18:3n-3 can directly inhibit cyclooxygenase activity, thereby reducing the production of AA-derived eicosanoids (8, 9). Evidence suggests that over production of AA-derived eicosanoids may be responsible for a number of pathophysiological conditions, including atherothrombotic and chronic inflammatory disorders in humans (10).

Numerous mammalian studies have established that the PUFA composition of cardiac membrane phospholipids can be directly influenced by dietary fat intake. In a previous study with Atlantic salmon, the phospholipid fatty acid composition of heart tissue was consider-

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ably altered by feeding diets high in 18:2n-6 (11). These fish also developed a severe cardiomyopathy which caused extensive thinning of the ventricular muscle and active necrosis in both the atrium and ventricle. Salmon given high levels of dietary 18:2n-6 showed increased levels of AA-derived eicosanoids in plasma and from stimulated blood leucocytes, compared to fish given low 18:2n-6 (12). Prostaglandins (PGs) are important mediators of myocardial physiology (13) and are also involved in controlling muscle protein synthesis (14).

In addition to their role in supplying precursors for eicosanoid metabolism, PUFA are vital components of membrane structure and as such can influence a number of membrane associated functions. In particular, membrane phospholipid fatty acid composition can directly affect the activities of membrane-bound enzymes and receptors. For example, in rat ventricular myocytes the activities of both phospholipase A and lysophospholipase were affected by altering the membrane n-3/n-6PUFA ratio (15).

The object of the present study was to explore essential fatty acid functions in cardiac physiology by feeding salmon diets in which the lipids were supplied either by sunflower (SFO), linseed (LO) or fish oil (FO). This was done by measuring phospholipid fatty acid compositions and PG production in both heart and blood leucocytes, and assessing the occurrence of cardiac lesions and measuring cardiac phospholipase A activity.

MATERIALS AND METHODS

Animals and diets

330 Atlantic salmon S1 smolts (initial mean weight ca. 85 g) were obtained from the SOAFD Fish Cultivation Unit (Aultbea, Wester Ross, Scotland). The salmon were randomly distributed into three tanks of 2000 L capacity each, which were supplied with seawater at a rate of 26 L/min. The tanks were subjected to natural photoperiod and the water temperature over the experimental period (August–November) was 15–10°C. The diets were supplied by automatic feeders which were activated for a few sec every 15 min during daylight h and adjusted initially to provide 28 g/kg biomass each day. When the average weight of the fish reached 120 g the ration was reduced to 20 g/kg each day. Fish were bulk weighed every 28 days and the ration adjusted accordingly. The experiment was conducted in accordance with the British Home Office guidelines regarding research on experimental animals.

The composition of the basal diet has been described in detail previously (11). The diet contained fishmeal (650 g/kg) (LT94, Ewos Ltd, Westfield, Lothian, Scotland), pre-cooked starch (150 g/kg), vitamin mix (10 g/kg), mineral mix (24 g/kg), α-cellulose (65.5 g/kg) and choline chloride (4 g/kg). The lipid component (100 g/kg) was either FO (Fosol, Seven Seas Ltd, Hull, UK), SFO (Tesco, Cheshunt, UK), or LO (ICN Biomedical Ltd, High Wycombe, UK). An antioxidant mix (0.4 g/kg diet) was added to the oil before mixing with the other ingredients (11). The diets were formulated to satisfy the nutritional requirements of salmon (16) and contained 47% protein and 16% lipid. The fatty acid compositions of the three diets are given in Table 1.

Sampling procedure

Samples of heart for lipid analysis and histology were collected after 12 weeks of the dietary treatment. Fish were killed by a blow to the head followed by decapitation and, after weighing, the heart was dissected, weighed and frozen in liquid N2. Samples were stored at –80°C prior to lipid analysis. Isolation of leucocytes and cardiomyocytes, and phospholipase A measurements were performed between 12–16 weeks of the dietary treatment.

Lipid extraction and analysis

Total lipid was extracted from hearts, leucocytes and diets by the method of Folch et al (17). The details of extraction, separation of phospholipid classes and preparation of fatty acid methyl esters have been described previously (11). The fatty acid methyl esters were separated and quantified by gas-liquid chromatography (Carlo Erba Vega 6000, Fisons Ltd, Crawley, UK) using a 50 m × 0.32 mm capillary column (CP-Wax 51, Chrompak Ltd, London, UK). Hydrogen was used as carrier gas and temperature programming was from 50°C at 35°C/min to 150°C and then to 225°C at 2.5°C/min. Individual methyl esters were identified by comparison with known standards and by reference to published data (18).

The quantification of lipid class composition in heart was performed using high-performance thin-layer chro-

Table 1 Fatty acid composition of diets

Fatty acid	FO diet	SFO diet	LO diet
		mol%	
14:0	7.4	2.0	2.0
16:0	14.8	9.5	9.1
18:0	2.2	3.9	3.2
16:1(n-7)	5.1	2.0	2.0
18:1(n-9)	8.9	16.2	15.9
18:1(n-7)	1.8	0.9	1.1
20:1(n-9)	8.8	2.6	2.6
22:1(n-11)	12.7	3.7	3.4
24:1	0.7	0.4	0.4
18:2(n-6)	1.3	37.0	11.2
18:3(n-6)	0.2	0.1	t
20:2(n-6)	0.3	0.1	0.1
20:4(n-6)	0.5	0.4	0.4
18:3(n-3)	1.2	0.5	29.4
18:4(n-3)	2.9	0.9	0.8
20:4(n-3)	0.6	0.2	0.2
20:5(n-30	5.6	3.1	2.9
22:5(n-3)	0.7	0.4	0.4
22:6(n-3)	6.9	3.9	3.6
Total (n-6)	2.3	37.6	11.7
Total (n-3)	17.9	9.0	37.3
(n-3)/(n-6)	7.8	0.2	3.2

t = trace value < 0.05.

matography (HPTLC). 10 µg total lipid were applied to a 10 × 10 cm HPTLC plate that had been pre-run in hexane:diethyl ether (1:1 v/v) and activated at 110°C for 30 min. The plates were developed to 6 cm in methyl acetate: isopropanol: chloroform: methanol: 0.25% (w/v) aqueous KCl (25:25:25:10:9 by volume) to separate phospholipid classes with neutral lipids running at the solvent front (19). After drying, the plates were developed fully in hexane:diethyl ether:acetic acid (80:20:2, v/v/v) to separate neutral lipids and cholesterol. Lipid classes were visualised by charring at 160°C for 15 min after spraying with 3% copper acetate (w/v) in 8% (v/v) phosphoric acid and identified by comparison with commercially available standards. Lipid classes were quantified by scanning densitometry using a Shimadzu CS-9000 dual wavelength TLC scanner and a DR-13 recording integrator.

Leucocyte isolation

Blood collected from 6 fish per dietary treatment was diluted with 3 volumes of Hanks' balanced salt solution containing 20 i.u./ml heparin. 4 ml of diluted blood was layered on to 5 ml of lymphocyte separation medium and centrifuged at 400 × g for 45 min at 4°C. The leucocytes located around the interface of the separation medium and the salt solution were harvested and washed twice in phosphate buffered saline. If erythrocyte contamination was greater than 2% the gradient separation was repeated. The leucocytes obtained from 6 fish were pooled prior to lipid extraction.

Cardiac myocyte isolation

The enzymatic isolation procedure was based on the method of Powell et al (20). Hearts from 2 fish were cannulated via the bulbous arteriosus and perfused with modified Hanks' medium (concentrations in g/L; NaCl, 10.29; KCl, 0.4; $MgSO_4.7H_2O$, 0.2; KH_2PO_4, 0.06; $Na_2HPO_4.12H_2O$, 0.13; $NaHCO_3$, 0.5; HEPES, 2.38) which was gassed continuously with 95% O_2, 5% CO_2. After 10 min the system was changed to recirculation and the medium changed to modified Hanks' plus 0.6% collagenase (w/v). After a further 45 min the hearts were removed and chopped finely in perfusion medium. This material was filtered through 190 µm nylon gauze and the myocytes collected by centrifugation (100 × g, 2 min). The myocytes were washed twice in collagenase-free Hanks' medium and finally resuspended in 1.0 ml of the same medium containing 2 mM $CaCl_2$. The suspensions routinely contained 55–85% of the total cells present as rod-shaped cardiac myocytes. 100 µl of suspension was retained for protein determination.

Challenge with calcium ionophore and eicosanoid extraction

1 ml of whole blood was mixed with an equal volume of Hanks' medium containing 2 mM $CaCl_2$. Both diluted blood (2 ml) and suspended cardiac myocytes (2 ml) were incubated in a shaking water bath at 18°C for 10 min. Calcium ionophore A23187 was added in 2 µl of dimethyl sulphoxide at a final concentration of 10 µM and the incubation continued for a further 20 min. The cells were then sedimented by centrifugation (12 000 × g, 2 min) and the PGs extracted from the supernatant using C18 'Sep-Pak' mini-columns (Millipore (UK) Ltd, Watford) as described by Powell (21).

Measurement of PGE_2 and thromboxane B_2 (TXB_2)

PGE_2 and TXB_2, the stable metabolite of TXA_2, were measured by enzyme immunoassay using kits supplied by Cascade Biochemicals Ltd, Reading, UK.

Determination of phospholipase A activity

Phospholipase A activity was assayed with endogenous substrate largely as described by Szymanska et al (22). One heart was homogenised in 2 ml of 50 mM Tris-HCl (pH8.5) containing 200 mM KCl and 5 mM $CaCl_2$. 1 ml was removed and the lipid extracted immediately by the method of Folch et al (17), 100 µl was removed for protein determination and 1 ml was placed in a shaking water bath at 18°C. After 2 h the reaction was terminated by addition of 5 ml of chloroform:methanol (2:1 v/v) containing 0.05% (w/v) butylated hydroxytoluene (BHT) and the lipids extracted as described above. Free fatty acids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in 0 and 2 h samples were separated by HPTLC and quantified as described in the extraction of lipids section.

Histology

Samples of heart (approximately 50% of the ventricle) were fixed in 20% buffered formol saline at the time of the dissection within 2 min of killing the fish, embedded in paraffin wax and 5 µm sections were stained with haematoxylin and eosin. Pathological assessment was carried out on coded, randomised slides to eliminate bias in interpretation.

Materials

TLC plates (20 cm × 20 cm × 0.25 mm) and HPTLC plates (10 cm × 10 cm × 0.25 mm), pre-coated with silica gel 60 were obtained from Merck (Darmstadt, Germany). All solvents were of HPLC grade and were obtained from Rathburn Chemicals Ltd (Walkerburn, UK). A23187 (Free acid) and collagenase (type IV) were obtained from Sigma Chemical Co. Ltd (Poole, UK). Lymphocyte separation medium, Hanks' balanced salt solution and phosphate buffered saline were obtained from Flow Ltd (Rickmansworth, UK).

Statistical analysis

Significance of difference ($P < 0.05$) between dietary

treatments was determined by analysis of variance (ANOVA). Analyses were performed using a Statgraphics (system 3.0) computer package. Data which were identified as non-homogeneous (using Bartletts' test) were subjected to either arcsine square root or log transformation before analysis. Differences between means were determined by Tukeys' test.

RESULTS

No growth differences were observed over the 12 week experimental period and mortalities were low in all dietary treatments. There was no significant effect of dietary treatment on cardiosomatic index or heart total lipid content and no gross pathologies were evident. The only cardiac histopathological lesion evident was focal degeneration of the myocardium of the spongy layer of the ventricle with associated leucocyte accumulation (Figure). For assessment purposes the ventricle muscle tissue of each fish was categorised into one of five groups ranging from no detectable degeneration through to severe lesion (Table 2). Similar lesions were also evident in the muscle of the auricle. The greatest incidence and severity of lesion was in the SFO-fed fish although some evidence of tissue damage was also apparent in FO-fed fish. Feeding LO appeared to supress lesion occurrence to a greater extent than FO.

The activities of cardiac phospholipase A, measured with endogenous substrate, are shown in Table 3. Results are calculated either as accumulation of free fatty acid or hydrolysis of PC and PE/h per mg protein. In both cases the phospholipase activity in fish given dietary SFO was significantly increased compared to those given either FO or LO.

Table 2 Severity of heart histopathology in salmon fed diets containing FO, SFO or LO

Lesion severity[a]	FO diet	SFO diet	LO diet
		Number of fish	
0	7	6	11
1	2	0	0
2	2	3	1
3	0	2	0
4	0	0	0
Total sample	11	11	12

[a]Lesion severity categories; 0 = no changes evident. 1 = focal increase of endocardial cellularity without evidence of myocardial damage. 2 = focal lymphocyte accumulation with myocardial degeneration, 1–3 lesions per ventrical section. 3 = as 2 but with 4–10 lesions. 4 = as 2 with 10 + lesions.

Table 3 Phospholipase A activities in hearts of salmon fed diets containing FO, SFO or LO

Phospholipase A activity	FO diet	SFO diet	LO diet
nmol fatty acid liberated/h per mg protein	53.1 ± 4.9^{b}	96.4 ± 6.9^{a}	59.9 ± 13.4^{b}
nmol PC and PE hydrolysed/h per mg protein	11.2 ± 1.4^{b}	26.3 ± 7.0^{a}	13.9 ± 3.4^{b}

Values are means ± SD for 4 fish per treatment.
Values in the same row with different superscript letters are significantly different ($P < 0.05$).

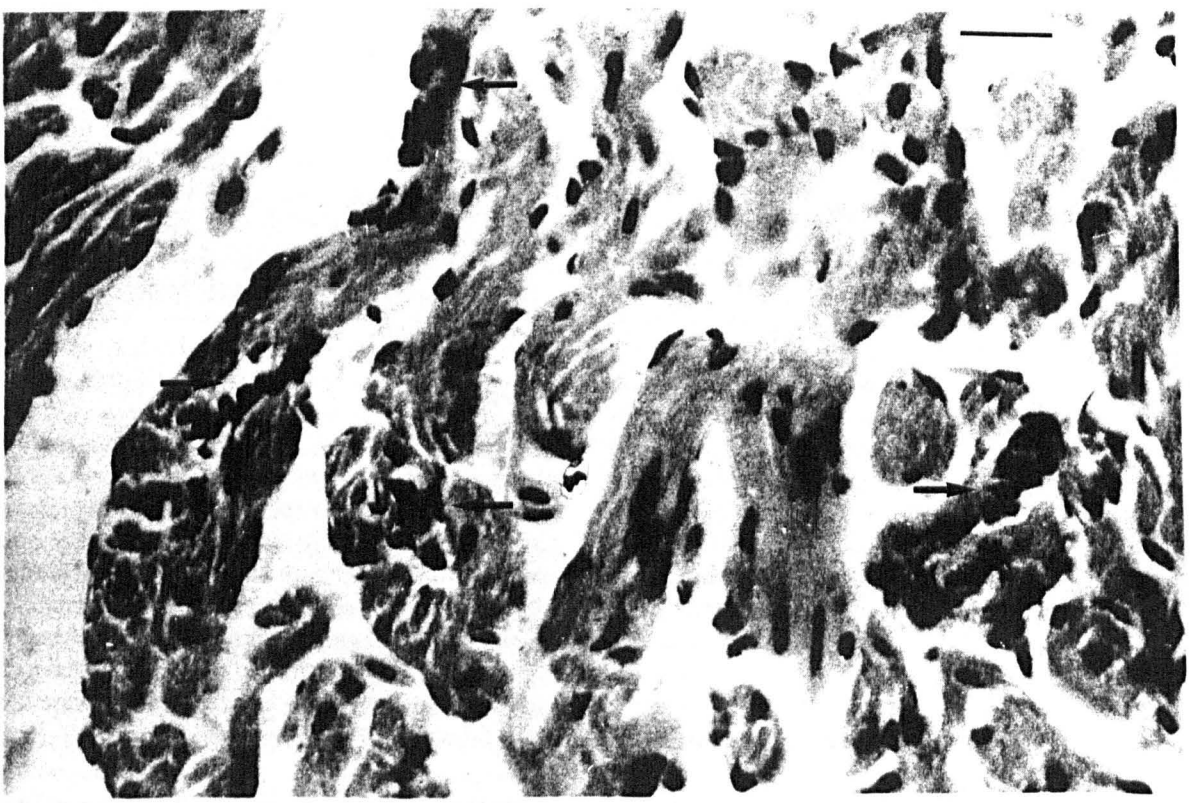

Figure Areas of myotomal degeneration (arrowed) in the heart ventricle of a salmon fed on SFO containing diet. Scale bar = 20 μm.

The levels of plasma PGE_2 and TXB_2 and production of these PGs by stimulated whole blood and cardiac myocytes are given in Table 4. There were no significant dietary-induced changes in levels of either eicosanoid in circulating plasma. In stimulated blood cells TXB_2 was significantly increased in fish given SFO compared to those given LO, whereas PGE_2 was significantly decreased in fish given LO compared to those given either FO or SFO. In stimulated cardiac myocytes TXB_2 production was not affected by dietary treatment whereas PGE_2 was significantly increased in fish given both SFO and LO compared to those given FO.

The lipid class composition of heart is shown in Table 5. The amount of phosphatidylinositol (PI) was significantly reduced in fish given SFO compared to those given FO and LO and PC was significantly reduced in fish given LO compared to both other dietary treatments. The latter observation significantly reduced the ratio PC:PE in LO-fed fish compared to the other two dietary treatments so that in LO-fed fish PE was the major polar lipid class.

Table 4 PGE_2 and TXB_2 concentrations in plasma and extracts from blood and cardiac myocytes stimulated with calcium-ionophore A23187 in salmon given diets containing FO, SFO or LO

Eicosanoid	FO diet	SFO diet	LO diet
Plasma TXB_2 (ng/ml)	1.24 ± 0.35	0.77 ± 0.55	1.02 ± 0.67
Plasma PGE_2 (pg/ml)	109.0 ± 15.5	125.3 ± 46.1	165.7 ± 75.3
Stimulated blood TXB_2 (ng/ml)	2.54 ± 2.0^{ab}	12.27 ± 6.20^{a}	2.20 ± 2.17^{b}
Stimulated blood PGE_2 (ng/ml)*	1.39 ± 0.33^{a}	2.93 ± 0.88^{a}	0.58 ± 0.21^{b}
Stimulated cardiac myocytes TXB_2 (pg/mg protein)	112.2 ± 48.4	108.7 ± 27.1	98.0 ± 56.8
Stimulated cardiac myocytes PGE_2 (pg/mg protein)	268.0 ± 141.9^{b}	857.0 ± 141.9^{a}	706.3 ± 157.9^{a}

Values are mean ± SD for 4 fish per treatment.
Values in the same row with different superscipt letters are significantly different.
* Values were subjected to log transformation before ANOVA.

Table 5 % lipid class compositions of hearts from salmon fed diets containing either FO, SFO or LO

Lipid	FO diet	SFO diet	LO diet
		g/100 g lipid	
Total Phospholipids	60.1 ± 8.6	54.5 ± 8.3	53.5 ± 3.5
Total Neutral lipids	39.8 ± 8.6	45.3 ± 8.3	46.3 ± 3.7
SM	3.0 ± 0.5	2.4 ± 0.4	2.6 ± 0.2
PC*	22.2 ± 3.1^{a}	20.4 ± 3.1^{a}	16.1 ± 0.9^{b}
PS	3.3 ± 0.6	2.7 ± 0.4	3.1 ± 0.2
PI	3.5 ± 0.6^{a}	3.0 ± 0.5^{b}	3.7 ± 0.3^{a}
PA/CL	8.7 ± 1.4	7.9 ± 1.5	8.8 ± 0.8
PE	19.4 ± 2.7	18.1 ± 2.7	19.3 ± 1.6
PC:PE ratio*	1.14 ± 0.03^{a}	1.13 ± 0.08^{a}	0.84 ± 0.04^{b}
Triacylglycerol	27.8 ± 9.8	32.8 ± 8.8	33.6 ± 5.1
Free fatty acids	0.4 ± 0.1	0.5 ± 0.1	0.8 ± 0.6
Cholesterol	11.0 ± 1.8	12.3 ± 1.7	12.0 ± 1.6

Values are means ± SD for 12 fish per treatment.
Values in the same row with different superscript letters are significantly different.
*Values subjected to arc sine square root transformation before ANOVA.
* Value subjected to log transformation before ANOVA.
PA: phosphatidic acid. CL: cardiolipin.

The fatty acid compositions of heart PC are shown in Table 6. Fish given SFO had significantly reduced total saturates compared to FO-fed fish. SFO-fed fish had significantly increased 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, AA and total n-6PUFA compared to both other dietary treatments. Fish given LO had increased 18:2n-6, 20:2n-6, 20:3n-6 and total n-6PUFA compared to fish given FO but had significantly reduced AA compared to both other treatments. Fish given SFO had significantly decreased EPA, total n-3PUFA and n-3/n-6 ratio compared to both other treatments. The 20:4/20:5 ratio was also significantly increased compared to both other treatments while total PUFA was significantly increased compared to FO-fed fish. Fish given LO had significantly increased 18:3n-3 and 20:4n-3 but decreased EPA, DHA and n-3/n-6PUFA ratio compared to FO-fed fish.

The fatty acid compositions of heart PE are shown in Table 7. Total monoenes were significantly different for each dietary treatment with the highest levels in FO-fed fish and the lowest in SFO-fed fish. SFO-fed fish had significantly increased 18:2n-6, 20:2n-6, 20:3n-6, AA and total n-6PUFA compared to the other two dietary treatments. Fish fed LO had significantly greater 18:2n-6, 20:2n-6 20:3n-6 and total n-6PUFA compared to FO-fed fish. 18:3n-3, 20:3n-3, 20:4n-3 and total n-3PUFA were significantly increased in LO-fed fish and significantly reduced in SFO-fed fish compared to fish fed FO. EPA was significantly reduced in fish fed SFO compared to the other two treatments. 22:5n-3 and the n-3/n-6PUFA ratio were significantly different in all dietary treatments with the highest level in FO-fed fish and the lowest in SFO-fed fish. Total n-3 PUFA were also significantly different in each dietary treatment with the highest level in LO-fed fish and the lowest in SFO-fed fish. The 20:4/20:5 eicosanoid precursor ratio was significantly increased in SFO-fed fish. Total PUFA were significantly increased

Table 6 Fatty acid composition of PC from hearts of salmon fed diets containing either FO, SFO or LO

Fatty acid	FO diet	SFO diet	LO diet
		mol%	
Total saturates	33.1 ± 0.8^{a}	31.3 ± 0.1^{b}	32.4 ± 0.3^{ab}
Total monoenes	12.9 ± 0.8	11.6 ± 0.9	12.4 ± 0.5
18:2(n-6)	0.5 ± 0.1^{c}	7.3 ± 0.1^{a}	2.3 ± 0.1^{b}
20:2(n-6)	0.1 ± 0.0^{c}	0.7 ± 0.0^{a}	0.2 ± 0.0^{b}
20:3(n-6)	0.1 ± 0.0^{c}	1.3 ± 0.1^{a}	0.3 ± 0.1^{b}
20:4(n-6)	1.8 ± 0.2^{b}	2.6 ± 0.2^{a}	1.4 ± 0.1c
Total (n-6)[1]	2.8 ± 0.3^{c}	12.2 ± 0.3^{a}	4.4 ± 0.1^{b}
18:3(n-3)	0.5 ± 0.2^{b}	0.2 ± 0.0^{b}	5.3 ± 0.3^{a}
18:4(n-3)	0.3 ± 0.1^{a}	0.1 ± 0.0^{b}	0.4 ± 0.0^{a}
20:4(n-3)	0.4 ± 0.1^{b}	0.3 ± 0.1^{b}	0.9 ± 0.0^{a}
20:5(n-3)	10.4 ± 0.1^{a}	6.6 ± 0.1^{c}	8.8 ± 0.5^{b}
22:5(n-3)	1.1 ± 0.1	0.9 ± 0.1	1.1 ± 0.1
22:6(n-3)	25.7 ± 1.3^{a}	24.0 ± 0.3^{ab}	22.8 ± 1.0^{b}
Total (n-3)	38.4 ± 1.0^{a}	32.1 ± 0.2^{b}	39.3 ± 0.8^{a}
(n-3)/(n-6)*	13.7 ± 1.5^{a}	2.6 ± 0.1^{c}	8.9 ± 0.4^{b}
20:4/20:5	0.2 ± 0.0^{b}	0.4 ± 0.0^{a}	0.2 ± 0.0^{b}

Values are means ± SD for 3 fish per treatment. SD < 0.05 are tabulated as 0.0.
Values in the same row with different superscript letters are significantly different.
[1]Includes 18:3(n-6), 22:4(n-6) and 22:5(n-6). *Values were subjected to log transformation before ANOVA.

Table 7 Fatty acid composition of PE from hearts of salmon fed diets containing either FO, SFO or LO

Fatty acid	FO diet	SFO diet	LO diet
		mol%	
Total saturates	15.2 ± 0.2	14.9 ± 0.4	14.9 ± 0.6
Total monoenes	11.3 ± 0.4[a]	7.0 ± 0.2[c]	8.3 ± 0.2[b]
18:2(n-6)	1.0 ± 0.1[c]	7.5 ± 0.3[a]	2.9 ± 0.1[b]
20:2(n-6)	0.2 ± 0.1[c]	0.8 ± 0.0[a]	0.3 ± 0.0[b]
20:3(n-6)	0.1 ± 0.0[c]	0.9 ± 0.1[a]	0.3 ± 0.1[b]
20:4(n-6)	1.9 ± 0.3[b]	2.6 ± 0.2[a]	1.4 ± 0.1[b]
Total (n-6)[1]	3.9 ± 0.2[c]	12.7 ± 0.4[a]	5.6 ± 0.2[b]
18:3(n-3)	0.9 ± 0.1[b]	0.4 ± 0.1[c]	5.2 ± 0.1[a]
20:4(n-3)	0.6 ± 0.1[b]	0.3 ± 0.0[c]	1.0 ± 0.0[a]
20:5(n-3)	5.2 ± 0.1[b]	4.0 ± 0.2[c]	5.6 ± 0.3[a]
22:5(n-3)	3.1 ± 0.1[a]	2.6 ± 0.1[b]	2.8 ± 0.1[b]
22:6(n-3)	37.8 ± 0.5	36.7 ± 0.4	36.1 ± 1.3
total (n-3)	47.6 ± 0.5[b]	44.0 ± 0.4[c]	50.7 ± 1.0[a]
Total PUFA	51.5 ± 0.3[b]	56.7 ± 0.3[a]	56.3 ± 1.1[a]
(n-3)/(n-6)	12.2 ± 0.5[a]	3.5 ± 0.1[c]	9.1 ± 0.2[b]
20:4/20:5	0.4 ± 0.1[b]	0.7 ± 0.1[a]	0.3 ± 0.0[b]
Total dimethyl acetals	5.4 ± 0.5[a]	4.6 ± 0.1[b]	4.7 ± 0.2[ab]

Footnotes as described for **Table 6**.

in SFO and LO-fed fish compared to those given FO. Total dimethyl acetals derived from plasmalogens were significantly reduced in SFO-fed fish compared to FO-fed fish.

Heart phosphatidylserine (PS) fatty acid composition was less affected by dietary lipid than PC or PE. 18:2n-6 and 18:3n-3 levels reflected dietary intake and 20:2n-6, 20:3n-6 and total n-6PUFA levels were significantly increased in SFO-fed fish (data not shown). The n-3/n-6PUFA ratio was significantly reduced in SFO-fed fish (data not shown). The fatty acid compositions of heart PI are shown in Table 8. Total monoenes were significantly reduced in fish fed SFO and LO compared to those fed FO. 18:2n-6 was significantly different in each dietary treatment with highest levels in SFO and lowest levels in FO-fed fish. 20:2n-6, 20:3n-6, AA and total n-6PUFA were significantly increased in SFO-fed fish compared to the other two treatments. 18:3n-3 was significantly increased in fish fed the LO containing diet.

Table 8 Fatty acid compositions of phosphatidylinositol from hearts of salmon fed diets containing either FO, SFO or LO

Fatty acid	FO diet	SFO diet	LO diet
		mol%	
Total saturates	29.8 ± 1.7	32.6 ± 0.8	31.9 ± 2.5
Total monoenes	9.9 ± 0.7[a]	7.8 ± 0.2[b]	7.5 ± 0.2[b]
18:2(n-6)	0.4 ± 0.0[c]	3.3 ± 0.2[a]	1.0 ± 0.1[b]
20:2(n-6)	0.2 ± 0.1[b]	0.4 ± 0.0[a]	0.1 ± 0.1[b]
20:3(n-6)	0.2 ± 0.0[b]	1.0 ± 0.1[a]	0.8 ± 0.2[a]
20:4(n-6)	16.6 ± 1.8[b]	19.6 ± 0.5[a]	15.9 ± 0.7[b]
Total (n-6)[1]	17.4 ± 1.5[b]	24.3 ± 0.6[a]	17.8 ± 0.6[b]
18:3(n-3)	0.4 ± 0.2[b]	0.2 ± 0.0[b]	2.8 ± 0.2[a]
20:4(n-3)	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.1
20:5(n-3)	8.8 ± 0.4[a]	5.6 ± 0.4[b]	8.8 ± 0.7[a]
22:5(n-3)	1.0 ± 0.1[a]	0.7 ± 0.1[b]	0.9 ± 0.1[ab]
22:6(n-3)	13.3 ± 2.2	10.6 ± 0.6	10.4 ± 0.4
Total (n-3)	23.9 ± 2.1[a]	17.4 ± 0.4[b]	23.4 ± 1.3[b]
Total PUFA	41.3 ± 1.0	41.7 ± 0.3	41.2 ± 1.6
(n-3)/(n-6)	1.4 ± 0.2[a]	0.7 ± 0.1[b]	1.3 ± 0.1[a]
20:4/20:5	1.9 ± 0.2[b]	3.5 ± 0.2[a]	1.8 ± 0.1[b]

Footnotes as described for **Table 6**.

EPA, 22:5n-3, total n-3PUFA and n-3/n-6PUFA ratio were all significantly reduced in SFO-fed fish compared to fish fed the other two dietary treatments. The 20:4/20:5 ratio was significantly increased in fish fed SFO.

The fatty acid compositions of leucocyte PC and PE and shown in Table 9. The dietary induced changes in fatty acid compositions are largely similar for both phospholipid classes. In general feeding SFO increased 18:2n-6 and all 20-carbon n-6PUFA while reducing the all n-3PUFA compared to FO-fed fish. This resulted in a decreased n-3/n-6PUFA ratio and an increased 20:4/20:5 eicosanoid precursor ratio compared to FO-fed fish. Feeding LO also increased 18:2n-6, 20:2n-6 and 20:3n-6 but decreased AA compared to FO-fed fish. EPA was increased in LO-fed fish compared to FO-fed fish and the resulting n-3/n-6PUFA ratio and 20:4/20:5 ratio were similar. The fatty acid compositions of leucocyte PS and PI are shown in Table 10. Dietary induced changes in PS were minimal although 18:2n-6 and 20-carbon n-6PUFA were increased in SFO-fed fish compared to the other two treatments. In LO-fed fish all n-3 PUFA were increased compared to the other two treatments. In PI, feeding LO did not reduce AA but did increase EPA such that the resulting 20:4/20:5 ratio was similar to that in FO-fed fish but was considerably less than SFO-fed fish.

DISCUSSION

Feeding both SFO and LO results in increased levels of 18:2n-6 and 20:3n-6 (the product of Δ-6-desaturation and elongation) into phospholipids of heart and leucocytes, compared to fish fed fish FO. However, in most phospholipid classes, the incorporation of AA (the product of

Table 9 Fatty acid compositions of leucocyte PC and PE from salmon fed diets containing either FO, SFO or LO

Fatty acid	PC			PE		
	FO diet	SFO diet	LO diet	FO diet	SFO diet	LO diet
				mol%		
Total saturates	33.0	35.4	34.5	21.5	14.6	14.2
Total monoenes	27.4	23.2	25.4	14.4	9.4	13.7
18:2(n-6)	1.6	8.8	2.4	1.8	10.4	4.0
20:2(n-6)	0.2	1.4	0.3	0.2	1.3	0.4
20:3(n-6)	0.3	1.4	0.4	0.2	0.8	0.3
20:4(n-6)	1.8	2.6	1.4	2.5	4.0	2.2
Total (n-6)[1]	4.3	14.4	4.9	4.9	16.9	7.2
18:3(n-3)	0.5	0.2	2.8	0.3	0.3	3.0
20:4(n-3)	0.4	0.2	0.6	t	t	0.3
20:5(n-3)	7.6	5.2	7.9	3.4	2.7	4.2
22:5(n-3)	1.2	0.6	0.7	0.9	0.8	0.9
22:6(n-3)	12.9	10.6	12.0	28.6	28.6	30.8
Total (n-3)[2]	22.8	20.6	24.6	33.2	32.4	39.6
Total PUFA	27.1	35.0	29.5	38.1	49.3	46.8
(n-3)/(n-6)	5.3	1.4	5.0	6.8	1.9	5.5
20:4/20:5	0.2	0.5	0.2	0.7	1.5	0.5
Total dimethyl acetals	—	—	—	10.1	8.8	10.4

Results are obtained from pooled leucocyte samples from 6 fish per treatment. t = trace < 0.05%. [1]Includes 22:4(n-6) and 22:5(n-6). [2]Includes 18:4(n-3) and 20:3(n-3).

Table 10 Fatty acid compositions of leucocyte phosphatidylserine and phophatidylinositol from salmon fed diets containing either fish oil, sunflower oil or linseed oil

Fatty acid	PS			PI		
	FO diet	SFO diet	LO diet	FO diet	SFO diet	LO diet
	mol%					
Total saturates	37.8	43.1	40.4	38.6	40.6	40.4
Total monoenes	17.1	10.0	8.7	21.3	7.1	5.5
18:2(n-6)	1.6	2.4	0.9	1.1	1.4	0.6
20:2(n-6)	t	0.7	0.3	t	0.4	0.1
20:3(n-6)	t	0.5	0.2	t	0.8	0.6
20:4(n-6)	0.5	0.9	0.6	16.5	27.6	30.2
Total (n-6)[1]	2.4	4.9	2.2	19.4	30.7	31.7
18:3(n-3)	0.5	0.2	0.9	0.6	t	0.5
20:4(n-3)	t	t	0.2	t	t	0.4
20:5(n-3)	1.3	1.1	1.7	1.0	0.8	1.5
22:5(n-3)	1.3	1.3	1.7	0.5	0.9	1.0
22:6(n-3)	24.3	24.9	29.9	6.5	5.3	4.8
Total (n-3)[2]	28.0	27.5	34.8	8.6	7.5	8.2
Total PUFA	30.4	32.4	37.0	28.0	38.2	39.9
(n-3)/(n-6)	11.7	5.6	15.8	0.4	0.2	0.3
20:4/20:5	0.4	0.9	0.4	16.2	36.1	19.8

Footnotes as described for **Table 9**.

Δ-5-desaturation) was increased when SFO was fed but was reduced when LO was the dietary lipid. Therefore, it appears that dietary LO, which is rich in 18:2n-6 but much richer in 18:3n-3, has an inhibitory effect on the Δ-5-desaturase enzyme responsible for converting 20:3n-6 to AA. Since there is more n-3PUFA than n-6PUFA in both 18 and 20-carbon fatty acids in LO-fed fish compared to SFO-fed fish, it is inevitable that 18:2n-6 is metabolized to AA in SFO-fed fish whereas the same does not occur in LO-fed fish to the same extent. That is, in LO-fed fish, there is competition at the Δ-6-desaturase between 18:2n-6 and 18:3n-3 and also at the Δ-5-desaturase between 20:3n-6 and 20:4n-3 the net result of which is an apparent inhibition of Δ-5-desaturation. The ability of 18:3n-3 to inhibit conversion of 18:2n-6 to AA has been observed previously in mammals (23). The SFO and LO containing diets both contained a similar concentration of EPA (as well as DHA) which was approximately half that provided by the FO diet. However, heart and leucocyte phospholipid fatty acid compositions indicated that, while SFO-fed fish had reduced levels of EPA, those fed LO had EPA values greater than or equal to those found in FO-fed fish. These results suggest appreciable conversion of 18:3n-3 to EPA via Δ-6- and Δ-5-desaturation and elongation. The ability of post-smolt Atlantic salmon to elongate and desaturate 18:2n-6 to AA has been identified previously (11). Except for leucocyte PS and PE, which showed small increases in DHA content, phospholipids from fish fed LO did not have increased DHA levels compared to fish fed FO. While parr of salmonid fish have been shown to desaturate and elongate 18:3n-3 to DHA (2), it is likely that the diets used in this experiment contained enough long-chain highly unsaturated fatty acids to satisfy the DHA requirement of salmon.

While considerable data have been accumulated, from both mammals and fish, on the effects of different dietary lipids on tissue fatty acid compositions, the effects on lipid class composition are less well documented. In the present study salmon fed LO had reduced levels of PC compared to the other two dietary treatments resulting in a PC:PE ratio less than unity. In the vast majority of fish species and tissues studied PC is the dominant phospholipid class (2), although, as in mammals, some fish brain tissue has PE in excess of PC (24). Acclimation to low environmental temperature can alter the PC:PE ratio in fish tissues (25) which is accompanied by increased incorporation of n-3PUFA, principally DHA, into membrane phospholipids, especially PE. In the present experiment DHA was not significantly increased in heart membrane phospholipids although there was an overall increase in n-3PUFA in PE largely as a result of increased 18:3n-3. While the exact reason for the altered PC:PE ratio in fish fed LO is unclear, the consequences in terms of membrane physiology and biochemistry could be important.

One aspect of membrane biochemistry which can be influenced by fatty acid composition is the activities of membrane associated enzymes, including those involved in phospholipid metabolism. Nalbone et al (15) demonstrated that cardiomyocytes grown in the presence of n-6PUFA had higher phospholipase A activity and lower lysophospholipase activity compared to those grown with n-3PUFA. In the present experiment the activity of phospholipase A was significantly increased in fish fed 18:2n-6-rich SFO compared to fish fed either FO or LO. Increased phospholipase activity towards endogenous phospholipids could increase turnover of membrane fatty acids, provide increased substrate for eicosanoid synthesis and generally have a destabilising effect on membrane integrity. Although lysophospholipase activity was not measured in this experiment, increased hydrolysis of PC could result in increased production of lysoPC which has potent cytolytic activity (26).

In a previous study we identified an extensive cardiomyopathy in salmon fed SFO which was largely absent in those fed FO (11). We speculated that the changes in membrane phospholipid fatty acid composition arising from feeding SFO could alter the species, concentration and thus the biological efficacy of resulting eicosanoids. Considerable evidence exists that EPA and DHA from dietary FO can modulate production of AA-derived eicosanoids either by inhibition of cyclooxygenase activity (27) or by production of EPA-derived eicosanoids of reduced biopotency relative to their AA homologue (10). Suppression of AA-derived eicosanoids by dietary 18:3n-3 has also been observed in mammals (28). In the present study focal cardiac myopathy was observed in fish fed SFO and while some degeneration of cardiac tissue was also observed in fish fed FO these were much less severe. Cardiac myopathy was virtually absent in fish fed LO.

The AA-derived PGs, TXB_2 and PGE_2 were measured in plasma, stimulated cardiac myocytes and stimulated blood cells. TX is an important mediator of platelet

aggregation, vascular tone and intracellular calcium concentration (10), while PGE_2 is a mediator of inflammatory activity (29), is inhibitory to muscle fibre formation and can stimulate protein catabolism (14, 30).

Whole blood was chosen in preference to isolated leucocytes since this most closely resembles the in vivo situation where the eicosanoid spectrum is influenced by interactions between cell types. Studies with salmonid blood indicate that erythrocytes are not capable of eicosanoid synthesis (31). In stimulated blood cells, fish fed LO produced significantly less TXB_2 than those fed SFO, and PGE_2 production in the LO group was significantly less than both the SFO and FO fed treatments. Thus, in general, production of AA-derived prostanoids by leucocytes was considerably reduced by feeding dietary LO. There was no difference in TXB_2 production by stimulated cardiac myocytes between dietary treatments but PGE_2 production was increased in myocytes from fish fed SFO and LO compared to those fed FO. It would appear therefore that increased production of PGE_2 by cardiac myocytes is not fundamental in the development of cardiac lesion. Feeding increased levels of purified 18:3n-3 similarly depressed AA-derived eicosanoid production in rats (32). The reasons for depressed AA-derived prostanoid production by leucocytes can be explained by the fatty acid compositions of leucocyte phospholipids. In general, fish fed LO, had increased EPA in all leucocyte phospholipids and decreased AA in PC and PE compared to both other dietary treatments. Fish which developed the histopathological cardiac lesion showed extensive leucocyte infiltration at the lesion site. Thus increased production of highly bioactive AA-derived eicosanoids by leucocytes may be resposible for the severity of lesion development.

This experiment suggests that feeding diets high in 18:2n-6 can result in undesirable pathophysiological conditions that may, in part, be due to alterations in fatty acid composition and metabolism. However feeding diets high in 18:3n-3 may increase the anti-inflammatory activity to a greater degree than feeding FO alone and thus attenuate undesirable cardiac pathology.

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Effect of Diets Rich in Linoleic or α-Linolenic Acid on Phospholipid Fatty Acid Composition and Eicosanoid Production in Atlantic Salmon (*Salmo salar*)

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Atlantic salmon post-smolts were fed diets rich in linoleic acid (sunflower oil, SO), α-linolenic acid (linseed oil, LO) or long-chain polyunsaturated fatty acids (fish oil, FO) for a period of 12 wk. In the liver phospholipids of fish fed SO, the levels of 18:2n-6, 20:2n-6, 20:3n-6 and 20:4n-6 were significantly elevated compared to both other treatments. In choline phospholipids (CPL), ethanolamine phospholipids (EPL) and phosphatidylserine (PS) the levels of 22:4n-6 and 22:5n-6 were significantly elevated in fish fed SO. In liver phospholipids from fish fed LO, 18:2n-6, 20:2n-6 and 20:3n-6 were significantly elevated but 20:4n-6, 22:4n-6 and 22:5n-6 were similar or significantly decreased compared to fish fed FO. Liver phospholipids from fish fed LO had increased 18:3n-3 and 20:4n-3 compared to both other treatments while EPL and phosphatidylinositol (PI) also had increased 20:5n-3. In fish fed LO, 22:6n-3 was significantly reduced in CPL, PS and PI compared to fish fed FO. Broadly similar changes occurred in gill phospholipids. Production of 12-lipoxygenase metabolites in isolated gill cells stimulated with the Ca^{2+}-ionophore A23187 were significantly reduced in fish fed either SO or LO compared to those fed FO. However, the ratio 12-hydroxy-5, 8, 10, 14-eicosatetraenoic acid (12-HETE)/12-hydroxy-5, 8, 10, 14, 17-eicosapentaenoic acid (12-HEPE) was significantly elevated in stimulated gill cells from SO-fed fish. Although mean values of thromboxane B_2 (TXB_2) and prostaglandin E_2 (PGE_2) were increased in fish fed SO, they were not significantly different from those of the other two treatments. *Lipids 28,* 819–826 (1993).

The so-called essential fatty acids, linoleic acid (18:2n-6) and linolenic acid (18:3n-3), are the precursors of the long-chain n-6 and n-3 species which are important components of the phospholipid bilayer of cell membranes. It is generally accepted that both the n-6 and n-3 fatty acids are metabolized by the same sequence of desaturating and elongating enzymes (1). In these pathways the desaturation steps are generally rate-limiting while elongation is rapid (2), and thus competition between substrate fatty acids at desaturase binding sites will determine the nature of the resulting polyunsaturated fatty acids (PUFA) and ultimately the composition of cellular membranes. In competitive terms, the n-3 PUFA are much more potent inhibitors of n-6 PUFA metabolism than *vice versa* although the relative concentrations of the two substrates will also determine the products that result (3,4). Many species of freshwater fish, including Atlantic salmon (*Salmo salar*), possess the enzymes necessary to elongate and desaturate 18:3n-3 to docosahexaenoic acid (DHA, 22:6n-3) (5) and are capable of metabolizing 18:2n-6 similarly, resulting in increased arachidonic acid (AA; 20:4n-6) in membrane phospholipids (6).

In mammals, AA is the major precursor for biologically active eicosanoids and in humans consuming a typical "Western-type" diet, overproduction of AA-derived eicosanoids may explain the prevalence of the many inflammatory conditions occurring in the developed world (7). Fish provide a useful model system for the study of eicosanoid metabolism since, although AA may be the preferred eicosanoid substrate (8,9), fish contain considerable amounts of eicosapentaenoic acid (EPA, 20:5n-3) and DHA, which can attenuate the production and efficacy of AA-derived eicosanoids (10). EPA can be metabolized by both cyclooxygenase and lipoxygenase enzymes resulting in products of lower bioactivity when compared to their AA homologues (11,12). EPA can also competitively inhibit the prostaglandin synthetase enzyme complex, thereby reducing production of AA-derived prostanoids (13). A number of recent studies have suggested that feeding oils rich in 18:3n-3 can reduce production of AA-derived eicosanoids by increasing levels of EPA in membrane phospholipids (14,15). In addition 18:3n-3 can directly inhibit cyclooxygenase activity (16). In previous studies with salmon we have shown that a high level of dietary 18:2n-6 can result in development of a severe cardiomyopathy involving active necrosis of both atrium and ventricle (17) and altered eicosanoid metabolism in blood leucocytes and gill cells (18).

The objective of the present study was to investigate the metabolism of 18:2n-6 and 18:3n-3 in salmon fed diets containing either sunflower oil (SO), linseed oil (LO) or fish oil (FO) by measuring phospholipid fatty acid compositions in liver and gill. The 12-lipoxygenase products and the cyclooxygenase products prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2) were measured in isolated gill cells stimulated with the calcium ionophore A23187.

MATERIALS AND METHODS

Animals and diets. Three hundred and thirty Atlantic salmon S1 smolts (salmon undergoing transition to seawater in one year) were obtained from the S.O.A.F.D. Fish Cultivation Unit (Aultbea, Wester Ross, Scotland) and distributed randomly into three tanks of 2000 L capacity each, which were supplied with seawater at a rate of 26 L/min. The fish (mean weight *ca.* 86 g) were subject to natural photoperiod, and the water temperature during the experimental period (August-November) varied from 15-10°C. Diets were supplied by automatic feeders which were adjusted to provide 20 g/kg biomass per day. Fish were weighed every 28 d and the ration adjusted accordingly.

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Abbreviations: AA, arachidonic acid; BHT, butylated hydroxytoluene; CPL, choline phospholipids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EPL, ethanolamine phospholipids; FO, fish oil; HDHE, hydroxy-4, 7, 10, 13, 16, 19-docosahexaenoic acid; HEPE, hydroxy-5, 8, 10, 14, 17-eicosapentaenoic acid; HETE, hydroxy-5, 8, 10, 14-eicosatetraenoic acid; HPLC, high-performance liquid chromatography; LO, linseed oil; PGE_2, prostaglandin E_2; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SO, sunflower oil; TLC, thin-layer chromatography; TXB_2, thromboxane B_2; UV, ultraviolet.

The diets were formulated to meet the nutritional requirements of salmonid fish (19) and contained 47% protein and 16% lipid. The composition of the basal diet has been described in detail previously (20) and contained fishmeal (650 g/kg) (LT94, Ewos Ltd., Westfield, Lothian, Scotland), pre-cooked starch (150 g/kg), vitamin mix (10 g/kg), mineral mix (24 g/kg), α-cellulose (65.5 g/kg) and choline chloride (4 g/kg). The dietary lipid (100 g/kg) was supplied either as fish oil (Fosol, Seven Seas Ltd., Hull, United Kingdom), sunflower oil (Tesco, Cheshunt, United Kingdom) or linseed oil (ICN Biomedical Ltd., High Wycombe, United Kingdom). An antioxidant mix (0.4 g/kg) was mixed with the oil before adding to the other diet components (20). The fatty acid compositions of the diets are shown in Table 1.

Lipid extraction and fatty acid analysis. Samples were collected after 12 wk of the dietary trial and stored at −80°C until analyzed. Lipids were extracted from liver and gill tissue by the method of Folch *et al.* (21). Total lipid extracts were separated into choline phospholipids (CPL), ethanolamine phospholipids (EPL), phosphatidylserine (PS) and phosphatidylinositol (PI) fractions by thin-layer chromatography (TLC) as described by Vitiello and Zanetta (22). The plates were sprayed with 0.1% 2′,7′-dichlorofluorescein in 97% methanol containing 0.05% butylated hydroxytoluene (BHT), and the lipid bands were visualized under ultraviolet (UV) light. Acid-catalyzed transmethylation was carried out overnight at 50°C as described by Christie (23). The fatty acid methyl esters were separated and quantified by gas-liquid chromatography (Carlo Erba Vega 6000, Fisons Ltd., Crawley, United Kingdom) on a 50 m × 0.32 mm capillary column (CP-Wax 51, Chrompak Ltd., London, United Kingdom). Hydrogen was used as carrier gas, and temperature programming was from 50°C at 35°C/min to 150°C and then to 225°C at 2.5°C/min. Individual methyl esters were identified by comparison with known standards and by reference to published data (24).

Preparation of isolated gill cells. The procedure used for isolating gill cells has been described in detail previously (18). Minced gill filaments were incubated in a Hanks' medium specially formulated for use with salmonid fish (25) containing 0.1% collagenase (type IV, Sigma Chemical Co., Poole, United Kingdom) for 45 min at room temperature with constant stirring. After filtering through nylon gauze, the cells were collected by centrifugation at 400 × *g* for 2 min, washed twice in Hanks' medium and finally resuspended in 1 mL of the same medium containing 1 mM $CaCl_2$.

Ionophore challenge and eicosanoid extraction. Isolated gill cells prepared as described above were placed in glass tubes pre-coated with Sigmacote and incubated in a shaking water bath at 18°C for 10 min. Calcium ionophore was added in 2 μL of dimethyl sulfoxide at a final concentration of 10 μM and the incubation continued for a further 20 min. The cells were sedimented by centrifugation

TABLE 1

Fatty Acid Composition of Diets

Fatty acids	Fish oil diet	Sunflower oil diet (wt%)	Linseed oil diet
14:0	6.6	1.8	1.8
16:0	14.8	9.5	9.1
18:0	2.4	4.3	3.5
Total saturated[a]	24.6	15.9	14.9
16:1n-7	5.1	2.0	2.0
18:1n-9	9.7	17.8	17.4
18:1n-7	2.0	1.0	1.2
20:1n-9	10.5	3.1	3.1
22:1n-11	16.6	4.8	4.4
24:1	1.0	0.6	0.5
Total monoenoic[b]	45.2	29.5	28.7
18:2n-6	1.4	40.3	12.2
18:3n-6	0.2	0.1	t[d]
20:2n-6	0.3	0.1	0.1
20:3n-6	0.1	t	t
20:4n-6	0.6	0.5	0.5
Total n-6[c]	2.8	41.1	12.9
18:3n-3	1.3	0.5	31.8
18:4n-3	3.1	1.0	0.9
20:4n-3	0.7	0.2	0.2
20:5n-3	6.5	3.6	3.4
22:5n-3	0.9	0.5	0.5
22:6n-3	8.8	4.9	4.5
Total n-3	21.3	10.7	41.3
Total PUFA[e]	25.0	51.8	54.2
n-3/n-6 ratio	7.6	0.3	3.2

[a]Includes 15:0, 17:0, 20:0 and 22:0.
[b]Includes 20:1n-11, 20:1n-7 and 22:1n-9.
[c]Includes 22:4n-6 and 22:5n-6.
[d]t = Trace value <0.05%.
[e]PUFA, polyunsaturated fatty acids.

(12000 × *g*, 2 min) and the eicosanoids extracted from the supernatant using C_{18} Sep-Pak minicolumns (Millipore Ltd., Watford, United Kingdom) according to Powell (26)

High-performance liquid chromatography (HPLC). The hydroxy acids 12-hydroxy-5, 8, 10, 14-eicosatetraenoic acid (12-HETE), 12-hydroxy-5, 8, 10, 14, 17-eicosapentaenoic acid (12-HEPE) and 14-hydroxy-4, 7, 10, 13, 16, 19-docosahexaenoic acid (14-HDHE) were separated and quantified by reverse-phase HPLC using a Spherisorb 5 μm octadecyl silane (ODS 2) column (25 cm × 4.6 mm, Anachem, Luton, United Kingdom). The chromatographic system was equipped with Waters Model M-45 pumps (Waters Chromatography, Warford, United Kingdom) and the effluent was monitored at 235 nm using a Pye-Unicam LC-UV detector (Pye-Unicam, Cambridge, United Kingdom). An isocratic solvent system containing acetonitrile/methanol/water/acetic acid (40:29:30:0.5, by vol) was used at a flow rate of 1 mL/min. Quantification was based on use of external standards of 12-HETE and 12-HEPE. Identification of 14-HDHE was done as described previously (18).

Measurement of PGE_2 and TXB_2. PGE_2 and TXB_2, the stable metabolite of thromboxane A_2, were measured by enzyme immunoassay using kits supplied by Cascade Biochemicals Ltd. (Reading, United Kingdom).

Materials. All solvents were of HPLC grade and were obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland, United Kingdom). TLC plates (20 cm × 20 cm × 0.25 mm), pre-coated with silica gel 60 were obtained from Merck (Darmstadt, Germany). 12(*R,S*)-HETE and 12(*S*)-HEPE were obtained from Cascade Biochemicals Ltd. (Reading, United Kingdom). Sigmacote and A23187 were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, United Kingdom).

Statistical analysis. Significance of difference ($P < 0.05$) between dietary treatments was determined by analysis of variance using a Statgraphics (system 3.0) computer package. Data which were identified as nonhomogeneous were subjected to either arcsine square root or log transformation before analysis. Differences between means were determined by Tukey's test.

RESULTS

The fatty acid compositions of liver CPL are shown in Table 2. Total monoenoic fatty acids were significantly reduced in SO-fed fish compared to FO-fed fish. In SO-fed fish 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6 were all significantly increased compared to both other dietary treatments. Fish fed LO had significantly increased 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6 and total n-6 PUFA but significantly reduced 22:5n-6 compared to fish fed FO. LO fish had significantly increased 18:3n-3, 18:4n-3, 20:3n-3 and 20:4n-3 compared to both other dietary treatments but had significantly reduced DHA compared to FO-fed fish. In fish fed SO 18:3n-3, 20:4n-3, EPA, 22:5n-3 and total n-3 PUFA were significantly reduced compared with both other dietary treatments while DHA was significantly reduced compared to FO-fed fish. The n-3/n-6 PUFA ratio was significantly different in the three dietary treatments with the highest value in FO-fed fish and the lowest in SO-fed fish.

The fatty acid compositions of liver EPL are shown in Table 3. Total monoenoic fatty acids were significantly different for each dietary treatment with the highest levels in FO-fed fish and the lowest levels in SO-fed fish. Also, 18:2n-6, 20:2n-6, 20:3n-6 and total n-6 PUFA were

TABLE 2

Fatty Acid Compositions of Choline Phospholipids from Salmon Liver[a]

Fatty acids	Fish oil diet	Sunflower oil diet (wt%)	Linseed oil diet
Total saturated	29.3 ± 1.6	29.9 ± 3.5	31.7 ± 3.7
Total monoenoic	16.0 ± 1.6^b	13.2 ± 0.8^c	14.6 ± 1.1^{b,c}
18:2n-6	1.1 ± 0.3^d	10.6 ± 1.7^b	4.0 ± 0.5^c
18:3n-6	t^d	0.5 ± 0.3^b	0.2 ± 0.0^c
20:2n-6	0.3 ± 0.1^d	1.7 ± 0.5^b	0.6 ± 0.1^c
20:3n-6	0.3 ± 0.1^d	6.4 ± 1.0^b	2.0 ± 0.5^c
20:4n-6	0.7 ± 0.1^c	5.5 ± 2.2^b	0.6 ± 0.1^c
22:4n-6	0.1 ± 0.0^c	0.7 ± 0.4^b	0.1 ± 0.0^c
22:5n-6	0.3 ± 0.1^c	0.7 ± 0.3^b	0.1 ± 0.0^c
Total n-6	2.8 ± 0.3^d	26.0 ± 0.4^b	7.4 ± 0.4^c
18:3n-3	0.3 ± 0.1^c	0.1 ± 0.0^d	3.6 ± 1.2^b
18:4n-3	0.1 ± 0.0^c	0.1 ± 0.0^c	0.7 ± 0.2^b
20:3n-3	t^c	t^c	0.4 ± 0.1^b
20:4n-3	0.8 ± 0.2^c	0.2 ± 0.1^d	2.9 ± 0.8^b
20:5n-3	9.8 ± 1.0^b	3.8 ± 0.4^c	9.5 ± 1.2^b
22:5n-3	3.0 ± 0.6^b	2.0 ± 0.5^c	3.3 ± 0.4^b
22:6n-3	35.9 ± 2.6^b	23.8 ± 3.2^c	25.5 ± 1.7^c
Total n-3	49.6 ± 1.8^b	29.7 ± 3.1^c	45.8 ± 2.2^b
Total PUFA	52.4 ± 1.8	55.7 ± 3.2	53.1 ± 2.1
n-3/n-6	18.0 ± 1.6^b	1.2 ± 0.1^d	6.2 ± 0.5^c
20:4/20:5	0.1 ± 0.0^c	1.5 ± 0.6^b	0.1 ± 0.0^c

[a]Results are % by weight ± SD from four fish per treatment; t = trace value <0.05%. SD <0.05 are recorded as 0.0. PUFA, polyunsaturated fatty acids. Values in the same row with different superscript letters b, c, d are significantly different ($P < 0.05$).

TABLE 3

Fatty Acid Compositions of Ethanolamine Phospholipids from Salmon Liver[a]

Fatty acids	Fish oil diet	Sunflower oil diet (wt%)	Linseed oil diet
Total saturated	12.6 ± 0.7	12.7 ± 0.6	13.6 ± 1.4
Total monoenoic	25.2 ± 1.8[b]	15.5 ± 2.5[d]	21.1 ± 0.4[c]
18:2n-6	1.7 ± 0.4[d]	12.0 ± 2.1[b]	4.7 ± 1.1[c]
18:3n-6	0.1 ± 0.0	0.2 ± 0.1	t
20:2n-6	0.4 ± 0.1[d]	2.3 ± 0.5[b]	0.7 ± 0.1[c]
20:3n-6	0.3 ± 0.1[d]	5.1 ± 0.6[b]	1.5 ± 0.2[c]
20:4n-6	1.3 ± 0.4[c]	9.6 ± 2.7[b]	1.1 ± 0.1[c]
22:4n-6	t[c]	0.8 ± 0.4[b]	t[c]
22:5n-6	0.5 ± 0.0[c]	1.0 ± 0.3[b]	0.3 ± 0.1[d]
Total n-6	4.2 ± 0.4[d]	30.9 ± 2.5[b]	8.1 ± 1.1[c]
18:3n-3	0.8 ± 0.2[c]	0.3 ± 0.1[d]	3.0 ± 0.6[b]
20:3n-3	t[c]	t[c]	0.7 ± 0.1[b]
20:4n-3	0.5 ± 0.1[c]	t[d]	1.7 ± 0.4[b]
20:5n-3	8.8 ± 1.2[c]	3.6 ± 1.0	11.1 ± 1.1[b]
22:5n-3	2.1 ± 0.4[b,c]	1.8 ± 0.4[c]	2.8 ± 0.4[b]
22:6n-3	41.9 ± 1.9[b]	32.5 ± 2.7[c]	36.0 ± 0.9[c]
Total n-3	54.1 ± 0.8[b]	38.2 ± 2.2[c]	55.4 ± 0.3[b]
Total PUFA	58.3 ± 1.2[d]	69.2 ± 2.7[b]	63.5 ± 1.4[c]
n-3/n-6	12.9 ± 1.2[b]	1.3 ± 0.1[d]	6.9 ± 0.9[c]
20:4/20:5	0.2 ± 0.1[c]	2.9 ± 1.4[b]	0.1 ± 0.0[c]

[a]Results are % by weight ± SD from four fish per treatment; t = trace value <0.05%. SD <0.05 are recorded as 0.0. PUFA, polyunsaturated fatty acids. Values in the same row with different superscript letters b, c, d are significantly different ($P < 0.05$).

significantly affected by dietary treatment with the highest levels in SO-fed fish and the lowest levels in FO-fed fish. Levels of 22:5n-6 were also significantly different in each dietary group with highest values in SO-fed fish and lowest in LO-fed fish. AA and 22:4n-6 were significantly increased in fish fed SO compared to both other dietary treatments. Also, 18:3n-3, 20:4n-3 and EPA were significantly different in each dietary treatment with highest levels in LO-fed fish and lowest in SO-fed fish. 22:5n-3 was significantly increased in LO-fed fish compared to SO-fed fish whereas DHA was significantly greater in FO-fed fish compared to both other treatments. Total n-3 PUFA were significantly lower in SO-fed fish while total PUFA were significantly different in each dietary treatment with the highest levels in SO-fed fish and the lowest in FO-fed fish. The ratio of n-3/n-6 PUFA was significantly different in each dietary treatment with the highest value in FO-fed fish and the lowest in SO-fed fish. The AA/EPA ratio of eicosanoid precursors was significantly increased in SO-fed fish compared to both other treatments.

The fatty acid compositions of liver PS are shown in Table 4. Total saturated fatty acids were significantly increased whereas total monoenoic fatty acids were significantly decreased in fish fed SO and LO compared to those fed FO. The level of 20:3n-6 was significantly different in each dietary treatment with the highest levels in SO-fed fish and the lowest in FO-fed fish. AA and 22:5n-6 were also significantly different in each treatment with the highest levels in SO-fed fish and the lowest in LO-fed fish. 18:2n-6, 20:2n-6, 22:4n-6 and total n-6 PUFA were all significantly increased in fish fed SO compared to the other dietary treatments. Levels of 18:3n-3 and 20:4n-3 were significantly greater in fish fed LO than in the two other treatments while EPA was significantly reduced in SO-fed fish compared to LO-fed fish. Total n-3 PUFA were significantly reduced in SO-fed fish compared to both other treatments while DHA was reduced in fish fed SO compared to those fed FO. Total PUFA were significantly greater in SO-fed fish compared to LO-fed fish. The n-3/n-6 PUFA ratio was significantly lower and the AA/EPA ratio significantly greater in SO-fed fish compared to both other treatments.

The fatty acid compositions of liver PI are shown in Table 5. Total saturated fatty acids were significantly elevated in fish fed SO and LO compared with those fed FO. Total monoenoic fatty acids were significantly different in all treatments with the highest levels in fish fed FO and the lowest in those fed SO. Levels of 18:2n-6 and total n-6 PUFA were significantly increased in SO-fed fish compared to the other two treatments whereas 20:3n-6 was significantly greater in LO-fed fish compared to the other treatments. AA was significantly different in all dietary treatments with the highest level in SO-fed fish and the lowest level in LO-fed fish. Also, 18:3n-3, 20:4n-3 and total n-3 PUFA were significantly reduced in fish fed SO compared to the other two treatments. EPA was significantly different in all treatments with the highest level in LO-fed fish and the lowest in SO-fed fish. Levels of 22:5n-3 and DHA were significantly different in all treatments with the highest levels in FO-fed fish and the lowest in SO-fed fish. Total PUFA were significantly greater in SO-fed fish compared to LO-fed fish. The n-3/n-6 PUFA ratio was significantly lower and the AA/EPA ratio significantly greater in fish fed SO compared to both other treatments.

The major PUFA in gill CPL and EPL are shown in Figure 1. in CPL 18:2n-6 and 20:3n-6 were significantly

TABLE 4

Fatty Acid Compositions of Phosphatidylserine from Salmon Liver[a]

Fatty acids	Fish oil diet	Sunflower oil diet (wt%)	Linseed oil diet
Total saturated	31.7 ± 1.5[c]	35.5 ± 1.4[b]	35.8 ± 1.8[b]
Total monoenes	10.3 ± 0.8[b]	6.2 ± 0.5[c]	6.7 ± 1.0[c]
18:2n-6	0.5 ± 0.1[c]	1.5 ± 0.2[b]	0.6 ± 0.1[c]
18:3n-6	0.2 ± 0.2	0.2 ± 0.1	t
20:2n-6	0.2 ± 0.0[c]	1.0 ± 0.3[b]	0.3 ± 0.1[c]
20:3n-6	0.2 ± 0.1[d]	1.6 ± 0.5[b]	0.6 ± 0.1[c]
20:4n-6	0.5 ± 0.1[c]	1.7 ± 0.3[b]	0.2 ± 0.1[d]
22:4n-6	t[c]	0.8 ± 0.5[b]	t[c]
22:5n-6	0.6 ± 0.1[c]	1.1 ± 0.4[b]	0.3 ± 0.0[d]
Total n-6	2.0 ± 0.2[c]	8.0 ± 0.7[b]	2.0 ± 0.1[c]
18:3n-3	0.4 ± 0.1[b,c]	0.2 ± 0.1[c]	0.6 ± 0.2[b]
18:4n-3	0.4 ± 0.1[b]	t[c]	t[c]
20:4n-3	t[c]	t[c]	0.5 ± 0.1[b]
20:5n-3	1.4 ± 0.5[b,c]	0.7 ± 0.3[c]	1.4 ± 0.3[b]
22:5n-3	2.7 ± 0.4	3.2 ± 0.8	3.8 ± 0.6
22:6n-3	47.3 ± 1.6[b]	43.2 ± 2.3[c]	44.6 ± 0.7[b,c]
Total n-3	52.3 ± 0.3[b]	47.2 ± 1.7[c]	50.9 ± 0.7[b]
Total PUFA	54.3 ± 1.0[b,c]	55.2 ± 1.3[b]	52.9 ± 0.7[c]
n-3/n-6	25.9 ± 2.1[b]	5.9 ± 0.6[c]	25.8 ± 1.3[c]
20:4/20:5	0.4 ± 0.2[c]	3.1 ± 1.6[b]	0.2 ± 0.1[c]

[a]Results are % by weight ± SD from four fish per treatment; t = trace value <0.05%. SD <0.05 are recorded as 0.0. PUFA, polyunsaturated fatty acids. Values in the same row with different superscript letters b, c, d are significantly different ($P < 0.05$).

different in all dietary treatments with the highest levels in SO-fed fish and the lowest in FO-fed fish. AA was significantly increased and EPA significantly decreased in SO-fed fish compared to the other treatments. Consequently, the AA/EPA ratio was significantly greater in SO fish compared to the other two treatments. DHA and total n-3 PUFA were significantly decreased in SO-fed fish compared to both other treatments. Largely similar effects occurred in gill EPL except that 20:3n-6 was significantly greater in SO-fed fish compared to both other treatments and DHA was significantly reduced in SO-fed fish compared to FO-fed fish.

The major PUFA of gill PS and PI are shown in Figure 2. In gill PS 18:2n-6 and 20:3n-6 were significantly

TABLE 5

Fatty Acid Compositions of Phosphatidylinositol from Salmon Liver[a]

Fatty acids	Fish oil diet	Sunflower oil diet (wt%)	Linseed oil diet
Total saturated	33.3 ± 1.6[c]	38.1 ± 0.5[b]	38.4 ± 1.1[b]
Total monoenoic	12.5 ± 1.4[b]	6.7 ± 0.7[d]	9.2 ± 0.6[c]
18:2n-6	0.5 ± 0.1[c]	1.4 ± 0.3[b]	0.6 ± 0.1[b]
20:2n-6	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.1
20:3n-6	2.0 ± 0.3[c]	1.8 ± 0.7[c]	5.8 ± 1.2[b]
20:4n-6	28.4 ± 0.3[c]	41.8 ± 0.4[b]	21.0 ± 1.2[d]
Total n-6	31.5 ± 0.2[c]	46.1 ± 0.5[b]	28.3 ± 1.7[c]
18:3n-3	0.4 ± 0.1[b]	t[c]	0.5 ± 0.1[b]
20:4n-3	0.3 ± 0.1[b]	t[c]	0.5 ± 0.1[b]
20:5n-3	4.9 ± 0.9[c]	0.7 ± 0.3[d]	10.1 ± 2.5[b]
22:5n-3	2.2 ± 0.3[b]	0.6 ± 0.1[d]	1.7 ± 0.2[c]
22:6n-3	12.4 ± 1.2[b]	4.7 ± 0.5[d]	8.1 ± 0.4[c]
Total n-3	19.7 ± 0.4[b]	6.2 ± 0.6[c]	21.5 ± 2.9[b]
Total PUFA	51.4 ± 0.7[b,c]	52.2 ± 1.1[b]	49.7 ± 1.1[c]
n-3/n-6	0.6 ± 0.0[b]	0.1 ± 0.0[c]	0.7 ± 0.2[b]
20:4/20:5	7.0 ± 2.2[c]	76.1 ± 28.2[b]	3.2 ± 2.2[c]

[a]Results are % by weight ± SD from four fish per treatment; t = trace value <0.05%. SD <0.05 are recorded as 0.0. PUFA, polyunsaturated fatty acids. Values in the same row with different superscript letters b, c, d are significantly different ($P < 0.05$).

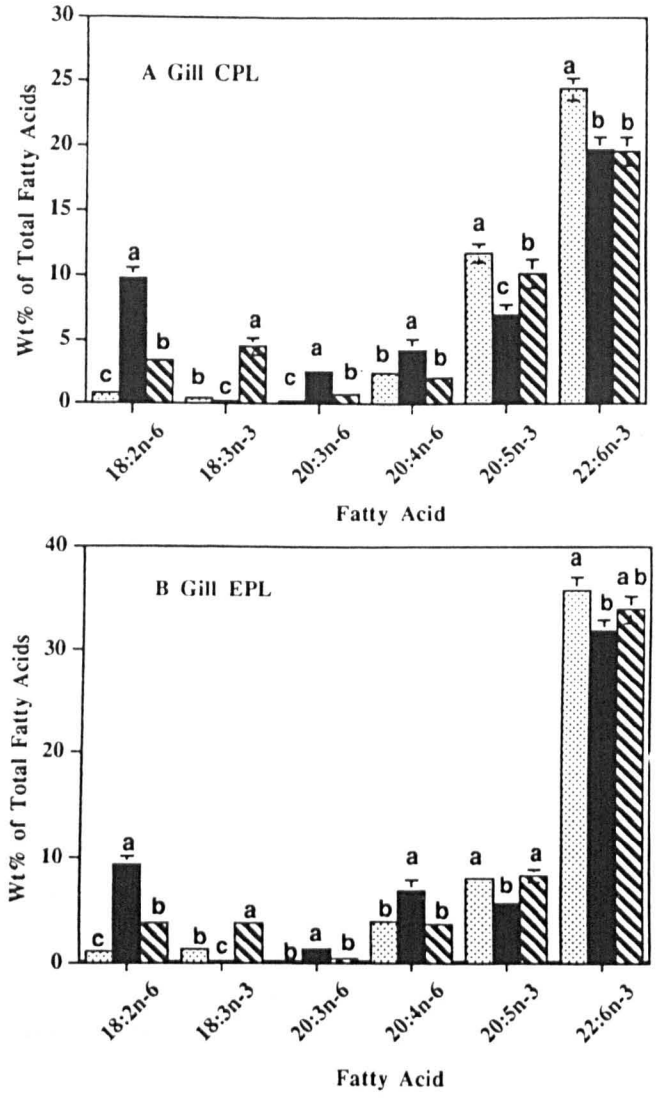

FIG. 1. The levels of the major polyunsaturated fatty acids of gill choline phospholipids (CPL) (A) and ethanolamine phospholipids (EPL) (B) from salmon fed diets containing either fish oil (dotted bars), sunflower oil (filled bars) or linseed oil (hatched bars). Values are means ± SD for four fish per treatment. Values for each fatty acid having a different column letter are significantly different ($P < 0.05$).

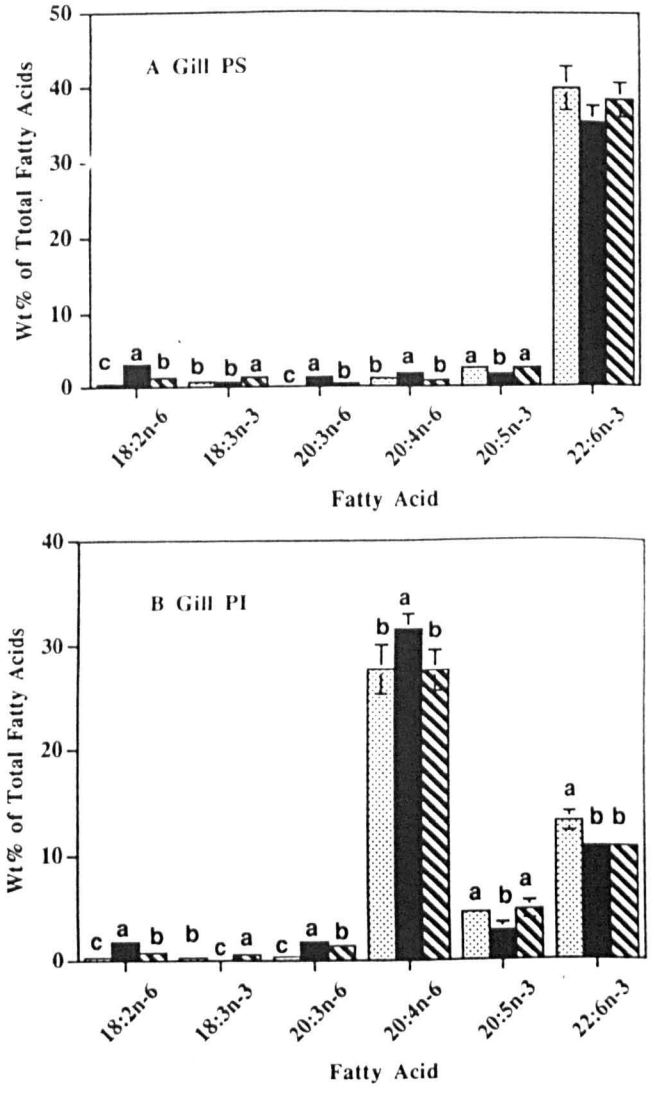

FIG. 2. Levels of the major polyunsaturated fatty acids of gill phosphatidylserine (PS) (A) and phosphatidylinositol (PI) (B) from salmon fed diets containing either fish oil (dotted bars), sunflower oil (filled bars) or linseed oil (hatched bars). Values are means ± SD for four fish per treatment. Values for each fatty acid having a different column letter are significantly different ($P < 0.05$).

different in all dietary treatments with the highest levels in SO-fed fish and the lowest levels in FO-fed fish. AA was significantly increased and EPA significantly reduced in SO-fed fish, resulting in an increased AA/EPA ratio when compared to fish fed either FO or LO. In gill PI the results were largely similar to PS except that DHA was significantly greater in fish fed FO than in both other dietary treatments.

The production of eicosanoids by isolated gill cells stimulated by A23187 is shown in Table 6. The production of 12-HETE, 12-HEPE and 14-HDHE were all significantly reduced in fish fed both LO and SO compared to those fed FO. However, the ratio of 12-HETE/12-HEPE was significantly increased in fish fed SO compared to both other treatments. While mean values of both TXB_2 and PGE_2 were greatest in fish fed SO, they were not significantly different from either of the other dietary treatments.

DISCUSSION

Feeding SO results in increased levels of 18:2n-6, 20:2n-6, 20:3n-6 and AA in all liver phospholipids while 22:4n-6 and 22:5n-6 were increased in CPL, EPL and PS compared to both other treatments. 20:3n-6, the product of Δ6 desaturation and elongation of 18:2n-6, was also increased in fish fed LO, whereas AA, the product of Δ5 desaturation, was generally decreased in those fish compared to fish fed FO. Similarly 22:5n-6, the product of Δ4 desaturation and elongation of AA was decreased in fish fed LO compared to those fed FO. Therefore it appears that feeding LO, which contains 18:2n-6 and 18:3n-3 in a ratio

TABLE 6

Eicosanoids from Isolated Salmon Gill Cells Stimulated with the Ca^{2+}-Ionophore A23187

Eicosanoid[a]	Fish oil diet	Sunflower oil diet	Linseed oil diet
12-HETE	17.7 ± 9.6[b]	5.7 ± 2.9[c]	5.8 ± 0.5[c]
12-HEPE	45.2 ± 12.4[b]	7.8 ± 3.2[c]	21.2 ± 8.2[c]
12-HETE/12-HEPE	0.354 ± 0.094[c]	0.791 ± 0.189[b]	0.327 ± 0.138[c]
14-HDHE	34.1 ± 11.8[b]	6.8 ± 3.8[c]	10.4 ± 1.9[c]
TXB_2	142.8 ± 80.4	229.0 ± 120.9	172.0 ± 59.6
PGE_2	192.3 ± 54.0	807.3 ± 562.3	510.3 ± 177.9

[a]Values are mean ± SD from four fish per treatment. Values for 12-HETE, 12-HEPE and 14-HDHE are ng/mg protein, whereas those for TXB_2 and PGE_2 are pg/mg protein. [b,c]Values in the same row with different superscript letters are significantly different ($P < 0.05$). HETE, hydroxy-5, 8, 10, 14-eicosatetraenoic acid; l HEPE, hydroxy-5, 8, 10, 14, 17-eicosapentaenoic acid; HDHE, hydroxy-4, 7, 10, 13, 16, 19-docosahexaenoic acid; TXB_2, thromboxane B_2; PGE_2, prostaglandin E_2.

of 1:3, has an inhibitory effect on Δ5 (and possibly Δ4) desaturase which is responsible for AA production. The ability of 18:3n-3 to reduce the conversion of 18:2n-6 to AA, by inhibition of Δ6 desaturase, has been recorded previously in mammals (27,28). The differences in 20:3n-6 and AA production by fish fed either SO or LO can be explained by considering the competitive influences of the different dietary fatty acid compositions. In SO-fed fish the high 18:2n-6 level makes AA production inevitable whereas the presence of 18:3n-3 in LO-fed fish results in competition which reduces 20:3n-6 production from 18:2n-6. At the same time Δ6 desaturation and elongation of 18:3n-3 results in increased 20:4n-3 in LO-fed fish, and this, coupled with reduced 20:3n-6, results in reduced AA production and an apparent inhibition of Δ5 desaturase. Similar competitive effects presumably operate to increase or reduce 22:5n-6 levels in SO- and LO-fed fish, respectively.

In a previous study using the related salmonid, the rainbow trout, no increase in elongated and desaturated products of 18:3n-3 were observed in the polar lipid fraction of fish fed 18:3n-3 enriched diets (29). However, in the present study with Atlantic salmon, although dietary EPA levels were similar in both SO and LO diets, the fatty acid compositions of phospholipid classes of both liver and gill showed decreased EPA in SO-fed fish, but LO-fed fish had EPA values greater than or equal to those of FO-fed fish. These results suggest that salmon are capable of EPA production from 18:3n-3 utilizing the pathways of Δ6 and Δ5 desaturation and elongation. Although some phospholipid classes from liver of LO-fed fish had elevated 22:5n-3, there was no apparent increase in DHA compared to fish fed FO. Previous studies with Atlantic salmon parr have demonstrated that they are capable of converting 18:3n-3 to DHA (5) but in the larger post-smolts used in the present study Δ4 desaturation is either very low or, more likely, the requirement for DHA is met by dietary input.

Both AA and EPA can be metabolized by cyclooxygenase to yield prostaglandins of the 2 and 3 series and by lipoxygenase to yield leukotrienes of the 4 and 5 series and a number of HETE and HEPE isomers (14,30). It is generally regarded that EPA is a poorer substrate for cyclooxygenase than AA and thus acts as a competitive inhibitor (16). The biological activity of the EPA-derived prostanoids and leukotrienes is considerably less than that of their AA-derived equivalents (12,31) which explains the ability of so-called 'MaxEPA' preparations to attenuate many of the pathophysiological conditions which occur in humans (7). In the present study feeding LO decreases AA and increases EPA in tissue phospholipids of Atlantic salmon and might therefore be expected to alter the spectrum of eicosanoids produced by these fish.

Gill cells were chosen since they are known to possess a highly active 12-lipoxygenase (18,32) and also to contain cyclooxygenase activity (5). While overall production of 12-lipoxygenase products was greatest in fish fed FO, the ratio of 12-HETE/12-HEPE was increased in SO-fed fish and was similar in both FO- and LO-fed fish. Although no specific physiological role has been identified for products of 12-lipoxygenase, a number of recent studies have implicated 12-HETE as a modulator of ion channels (33,34). Clearly this activity would be particularly important in gills which, along with posterior kidney, are vital in controlling osmoregulation in fish. Diet-induced changes in gill lipoxygenase products might therefore affect the ability of salmon to adapt to varying salinity.

In a previous study Atlantic salmon given increasing dietary linoleic acid produced decreasing amounts of gill 12-lipoxygenase products (18). In the present study salmon fed LO also produced significantly less 12-lipoxygenase products compared to fish fed FO. A similar decrease was also observed in platelet 12-lipoxygenase products from rats fed 18:3n-3 compared to those fed FO (14). One possible explanation is that changes in membrane phospholipid fatty acid composition might affect the activity of phospholipase A_2 which provides the precursors for eicosanoid production. Recent studies have established that phospholipase A_2 activity can be either increased or decreased in different tissues as a result of decreasing the membrane n-3/n-6 PUFA ratio (35,36).

The tendency for the PI fraction to accumulate 20-carbon fatty acids in fish has resulted in the hypothesis that this phospholipid might be the source of precursor fatty acids for eicosanoid production (5,37). However, the ratio of 12-HETE/12-HEPE produced by stimulated gill cells in the current experiment was most similar to the AA/EPA ratio present in gill CPL (0.20, 0.61 and 0.20 for FO, SO and LO diets, respectively). Comparison of the precursor fatty acid ratios of both CPL and PI would make the former class the more likely source of lipoxygenase substrate fatty acids. A similar result has been recorded in mammalian platelets (38) and cultured umbilical cells (39).

The present study demonstrated that while diets rich in 18:2n-6 result in increased AA in membrane lipids of

salmon, the fatty acid compositions can be 'normalized' to the position in fish fed FO, by feeding 18:3n-3. In this context linseed oil could be useful, when used in conjunction with marine oils, as an inhibitor of inflammatory activity in fish.

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