The Influence of dietary polyunsaturated fatty acids on the immune response of Atlantic salmon (*Salmo salar* L.)

Thesis presented for the degree of
Doctor of Philosophy
University of Stirling

by
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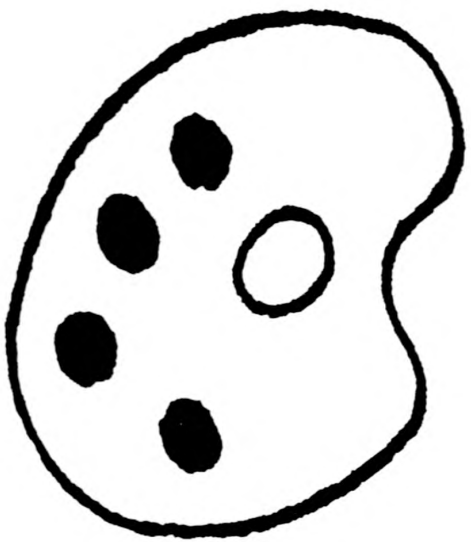
NUMEROUS ORIGINALS IN COLOUR



To my parents

Declaration

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

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

Kim Thompson.....

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ABBREVIATIONS

16:0	palmitic acid
18:0	stearic acid
18:1(n-9)	oleic acid
18:2(n-6)	linoleic acid
18:3(n-3)	linolenic acid
20:4(n-6)	arachidonic acid
20:5(n-3)	eicosapentaenoic acid
22:6(n-3)	docosahexaenoic acid
2-ME	2-mercaptoethanol
ANOVA	analysis of variance
APS	ammonium persulphate
BHT	butylated hydroxytoluene
BKD	bacterial kidney disease
BSA	bovine serum albumin
C.F.	condition factor
CI	cardiolipin
CMI	cell mediated immunity
CE	cholesterol ester
Con A	concanavalin A
CPM	counts per minute
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
DPG	diphosphatidylglycerol
DPM	disintegrations per minute
EFA	essential fatty acids
ELISA	enzyme-linked immunosorbent assay
EMEM	minimum essential medium modified with Earles salts
EWL	hen egg white lysozyme
FAMEs	fatty acid methylesters
FCS	foetal calf serum
FFA	free fatty acids
FITC	fluorescein isothiocyanate
GLC	gas-liquid chromatography
HBSS	Hanks buffered salt solution
HEPE	hydroxyeicosapenteanoic acid
HEPES	4-[2-hydroxyethyl-1-piperazine-ethanesulfonic acid]
HETE	hydroxyeicosatetraenoic acid
HI	humoral immunity
Hp.I.	hepatosomatic index
HPEPE	hydroperoxyeicosapentaenoic acid
HPETE	hydroperoxyeicosatetraenoic acid
HPTLC	high performance thin-layer chromatography
HRP	horse radish peroxidase
IP	intraperitoneally
I.U.	international units
IFAT	indirect fluorescent antibody technique
IFN-γ	gamma interferon
Ig	immunoglobulins
IL-1	interleukin-1
IL-2	interleukin-2
IP_3	inositol triphosphate
L-15	Leibovitz L-15 medium
LPC	lysophosphatidylcholine
LPS	lipopolysaccharide
LSM	lymphocyte separation medium

LTs	leukotrienes
LXs	lipoxins
MAG	monoacylglycerol
MAF	macrophage activation factor
MeOH	methanol
MHC	Major Histocompatibility Complex
MIF	macrophage inhibition factor
MLR	mixed leucocyte reactions
MTT	3(4.5-di-methylthiazoyl-2-yl)2.5 diphenyltetrazolium bromide
NBT	nitroblue tetrazolium
PAGE	polyacylamide gel electrophoresis
PBS	phosphate buffered saline
PC	phosphatidylcholine
PD	pancreas disease
PE	phosphatidylethanolamine
PEM	protein-energy malnutrition
pen/strep	potassium benzyl-penicillin / streptomycin sulphate
PFA	plaque forming assay
PGs	prostaglandins
Ph.I.	phagocytic index
PHA	phytohaemagglutinin
PI	phosphatidylinositol
PLT	primary lymphoid tissue
PMA	phorbol myristate acetate
PMN	polymorphonuclear leucocytes
PS	phosphatidylserine
PUFAs	polyunsaturated fatty acids
RBC	red blood cell
RPS	relative percentage survival
SC	subcutaneous
SE	sterol esters
S.I.	stimulation indices
SDS	sodium dodecyl sulfate
SF-1	serum-free medium
SFA	saturated fatty acids
sIg	surface immunoglobulins
SLT	secondary lymphoid tissue
SM	sphingomyelin
SOD	superoxide dismutase
Sp.I.	splenomatic index
SRBC	sheep red blood cells
TAG	triacylglycerol
TBS	tris-buffered saline
TCA	trichloroacetic acid
TD	thymus dependent
TEMED	N,N,N,N,-tetromethyl-ethylenediamine
T_H	T cell helper
TI	thymus independent
TNF	tumour necrosis factor
TNP-KHL	trinitrophenyl-keyhole limpet cyanin
TNP-LPS	trinitrophenyl-lipopolysaccharide
TSA	tryptic soya agar
TSB	tryptic soya broth
Tx	thromboxanes
WBC	white blood cell

ABSTRACT

The present work examined the effects of dietary (n-3)/(n-6) PUFA ratios in Atlantic salmon parr on, firstly, the fatty acid composition of lipids in peripheral blood cells and immunocompetent tissues and secondly, disease resistance. No notable differences in physiological and immunological parameters were observed between fish fed different (n-3)/(n-6) PUFA diets, except for a significantly higher number of responding B cells in kidney and spleen of the fish fed high (n-3)/(n-6) PUFA diet. The protective vaccination of the groups of fish on the different (n-3)/(n-6) PUFA ratio diets was inconclusive, but significantly more salmon died in the low (n-3)/(n-6) group when non-vaccinated fish were challenged with *Vibrio anguillarum*.

Lipid class composition of lipids from leucocytes, erythrocytes and serum were all found to be independent of diet, while component fatty acids were definitely influenced by dietary PUFA. Total fatty acids of the erythrocyte lipid were always high in (n-3) PUFA. Leucocyte lipid, by comparison, contained higher levels of saturated and monoenoic fatty acids, particularly 18:1 (n-9). The overall (n-6) PUFA unsaturation was higher in the lipid of leucocytes than the erythrocytes and leucocytes incorporated greater proportions of dietary 18:2(n-6) into their lipid than erythrocytes. Levels of 18:2(n-6) in the lipid of serum and leucocytes from (n-6) PUFA fed fish began to rise after four weeks on the diet, and increased steadily until a (n-3)/(n-6) PUFA ratio of 0.8 was maintained after 16 weeks. Erythrocytes did not show diet induced modification until 8-16 weeks, at which point 18:2(n-6) fatty acid levels were observed to plateau. Influences of dietary fatty acid composition were most evident in the PC and PE fractions.

Dietary 18:2(n-6) fatty acid incorporation was greater in the lipids of the lymphoid organ tissues than in leucocytes isolated from these tissues. Lymphoid tissues from (n-3) dietary group fish possessed higher (n-3)/(n-6) PUFA ratios than their leucocytes, while (n-3)/(n-6) PUFA ratios

were found to be similar between the lymphoid tissues and their corresponding leucocytes of the (n-6) PUFA dietary group. Lipid from headkidney macrophages of Atlantic salmon was higher in phospholipids than headkidney leucocytes and T and B-cells, while their fatty acid profiles were similar.

Comparisons of salmon parr were made with a warm water species of fish and a mammalian species. The fatty acid composition of rabbit cell lipid reflected their dietary 18:2(n-6) intake, whereas the fish had high levels of (n-3) PUFA. Erythrocytes and leucocytes of African catfish had similar fatty acid compositions, while Atlantic salmon erythrocytes contained a higher degree of 22:6(n-3) fatty acid than did the leucocytes. The leucocyte lipid of salmon contained higher levels of saturated and monoenoic fatty acids than the catfish leucocytes.

The lipid class and fatty acid composition of Atlantic salmon erythrocytes and leucocytes were unaffected by water temperature, except for a higher PE level in the erythrocytes of cold water acclimated fish.

When salmon leucocytes were cultured *in vitro*, the lipid composition of the leucocytes was unaffected by FCS or fish serum when these were used as medium supplements. The rate of incorporation of exogenous fatty acids into the peripheral blood leucocytes was influenced by metabolic temperature. Greatest incorporation of exogenous fatty acids by fish peripheral blood leucocytes occurred within the first day of incubation, but peaked around day 2 when cells were cultured at 15°C, and day 5 when incubated at 4°C. Kidney and blood leucocytes incorporated greater amounts of 20:4(n-6) and 18:1(n-9) fatty acid into their lipid than those from thymus and spleen, but all leucocytes displayed a preference for 20:4(n-6) and 18:1(n-9) fatty acids over 20:5(n-5), 18:2(n-6) and 18:3(n-3). The study suggests that the lymphoid system of salmon is predisposed to (n-6) PUFA despite the natural abundance of (n-3) PUFA in the lipids of salmon diets and tissues.

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CHAPTER 1 PROJECT AIMS

Fish farming in Scotland has developed rapidly over the past few decades to the point where Scotland is the second largest producer of Atlantic salmon after Norway. This expansion has produced the need for extensive research into methods of control for the associated disease problems which can cause significant financial losses to farmers. The organisms responsible for massive mortalities are ubiquitous in the fish farming environment and are especially prevalent when fish are maintained at high stocking densities.

Preventative measures against such diseases include chemotherapy and vaccination, but there are also indications that diet modification may help to prevent disease. The influence of diet on disease resistance is well documented in other animals but it is only recently that this research has focused on fish, especially the influence of dietary polyunsaturated fatty acids (PUFAs) on the immune response of fish.

The study described here was undertaken to examine the effects of dietary (n-3) and (n-6) polyunsaturated fatty acids on disease resistance of Atlantic salmon. It is well established that polyunsaturated fatty acids are essential components in the mammalian diet for the maintenance of health and research has indicated that eicosapentaenoic acid (20:5(n-3)), docosahexaenoic acid (22:6(n-3)) of the (n-3) series and arachidonic acid (20:4(n-6)) of the (n-6) series are the most physiologically active PUFAs. Large quantities of 20:5(n-3) and 22:6(n-3) can be obtained from fish oils. Cultured fish receive less (n-3) in their diets than their wild counterparts (van Vliet and Katan, 1990)

and it has therefore been suggested (Blazer, 1992) that by increasing dietary (n-3) in farmed fish, disease resistance in fish may, in turn, be increased.

Possible mechanisms for the influence of PUFAs on immune function are, firstly, structural changes in the lymphocyte membrane which influence cellular functions such as enzyme kinetics, ion transport and permeability, receptor expression and signal transmission and, secondly, via eicosanoids (chemical mediators of the immune response). Dietary lipids influence endogenous pools of lipids within the cell. The uptake of these fatty acids varies both with the species and organs investigated, and some PUFAs have significant effects on the elongation /desaturation of other fatty acids.

Low temperatures are known to immunosuppress fish and, in salmon at 4°C or below, the immune response is paralysed. This makes cold water vaccination difficult. In response to environmental temperature changes, fish cells undergo homeoviscous adaptation. At low temperatures, the degree of membrane fluidity is maintained by the preferential uptake of (n-3) PUFAs into cell plasma membranes.

The following thesis sets out to examine the effects of different dietary n-3/n-6 PUFA ratios on:- i) the fatty acid composition of lipids in peripheral blood cells and immunocompetent tissues of salmon and ii) the susceptibility of salmon to infectious pathogens and the protective immunity of vaccines.

CHAPTER 2 GENERAL INTRODUCTION

2.1 THE ATLANTIC SALMON

The Atlantic salmon (*Salmo salar* L.) is one of eight species of salmon in existence, but is unique to the Atlantic Ocean and most of its northern hemisphere tributaries. A cold water species, its optimal environmental temperature range is 10 - 17°C, but it can survive temperatures between 1 and 24°C. One of the largest salmon species, reaching up to 27 kg and 122 cm, it has a light brown dorsal surface, silver sides, and black spots on the dorsal, adipose, and anal fins.

The anadromous life cycle of the salmon can be divided into three stages. Firstly, they hatch from eggs after a 50-110 day incubation period in fresh water. They emerge as alevins feeding off their attached yolk sacs for several weeks before developing into fry, which then turn into parr and feed on plankton and insect larvae. At several centimetres in length, the juvenile fish are characterised by thirteen dark pigmented "parr marks" on each side.

The second stage in salmon development is the parr-smolt transformation (smoltification), which is accompanied by the disappearance of the "parr marks", a silvering in appearance, and an increased tolerance to salt water in preparation for migration to the sea. Smoltification may be influenced by fish size, possibly genetically determined, by photoperiod and by water temperature, and the metabolic changes during the metamorphosis are controlled by thyroid hormones.

In the southern parts of their habitat, parr can smolt after their first year, and are called potential S1 parr. In cooler regions it may take another year before smolting occurs and these are known as potential S2 parr, while in the cold waters of the Arctic it can take up to six years (Sedgwick, 1988). Potential S1 parr weigh typically 30 - 40 g, and potential S2 parr about 50 g respectively, at the start of smoltification (Sedgwick, 1988) (See Plate 2.1). Apart from land locked clusters of salmon which do not undergo smoltification, they continue their life cycle in the marine environment. Migration to the sea, between May and June, is a normal part of the second life-cycle stage of the salmon which typically remain at sea for between 2 to 5 winters.

The third and final stage of the life cycle is marked by the return between October and January of the now sexually mature Atlantic salmon to their native stream to spawn. After spawning, the salmon which are now known as kelts, return to the sea for another year. Only 5 % of kelts survive to repeat the spawning process the following year.

2.2 LIPIDS

2.2.1 Lipids; structure and function

Lipids can be defined as a group of water-insoluble biomolecules which are soluble in nonpolar solvents. Many different classes of lipids exist in nature but only those containing fatty acids will be discussed here. In animal systems, fatty acids are usually unbranched molecules, which contain a long hydrocarbon chain of even numbers of carbon atoms, between 14 and 24 in length, and a terminal carboxylic acid group. The chain may be saturated or unsaturated, containing double bonds in the *cis* position. In

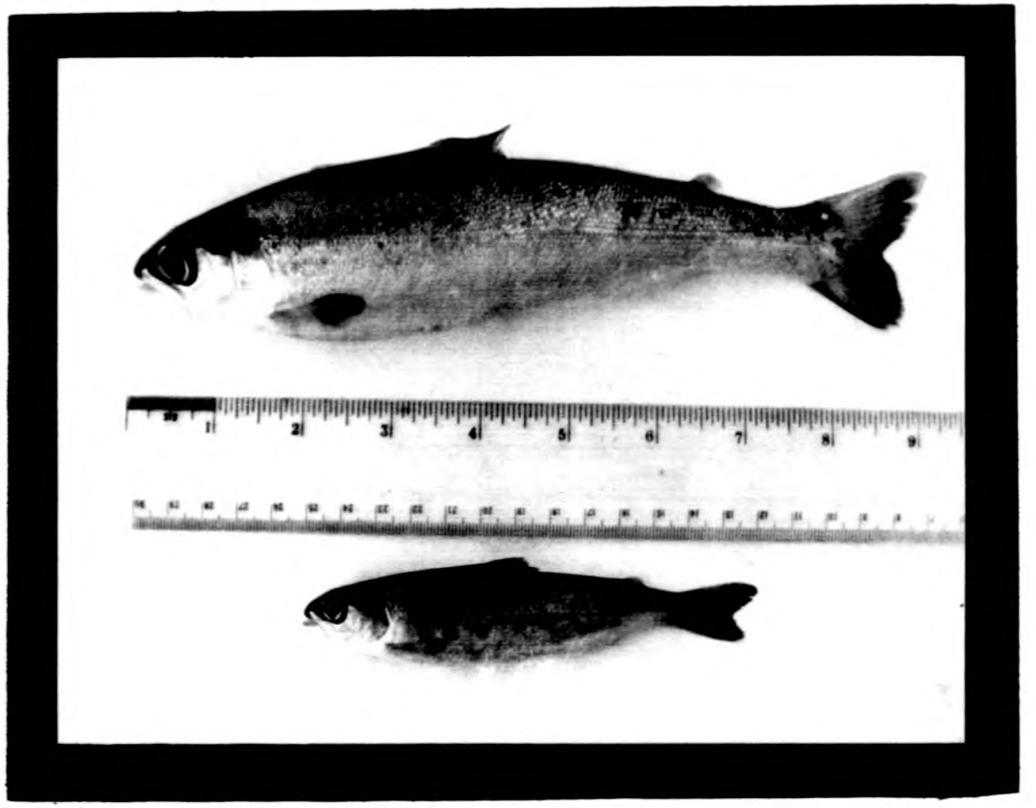
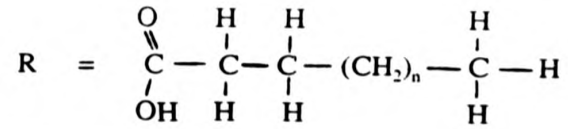


Plate 2.1. (a) Potential S1 and (b) Potential S2 Atlantic Salmon *(Salmo salar* L.)

polyunsaturated fatty acids, the double bonds are separated by at least one methylene group. The nomenclature of fatty acids can be confusing. In the present work,the trivial names of commonly occurring fatty acids will be used, coupled with a short hand notation in which the number of carbon atoms in the chain is separated from the number of double bonds by a colon, and the position of the first double bond relative to the methyl end is shown in brackets. For example, eicosapentaenoic acid (20:5(n-3)) has 20 carbons and five double bonds, the last of which is three carbons from the methyl terminal carbon. The official nomenclature for fatty acids is laid down by the International Union of Pure and Applied Chemistry and the International Union of Biochemistry and this substitutes the "e" at the end of the parental hydrocarbon with "oic acid" if it is saturated, "enoic acid" if it contains one double bond (i.e. a monosaturated fatty acid), and "dienoic acid" if it contains two double bonds, etc. Consequently, linoleic acid, (18:2(n-6)), can be written as (*cis, cis*) 9,12 octadecadienoic acid, or as *cis*(Δ-)9, *cis*(Δ-) 12- 18:2, indicating that the chain is 18 carbons long, with two *cis* double bonds at carbon 9 and carbon 12, numbering carbon atoms from the carboxyl end of the chain. Alternatively, linoleic acid can be written as (*cis, cis*) (n-6, 9) octadecadienoic acid when numbering from the methyl end. A "Δ" sign indicates numbering from the carboxyl end, whilst an "n" indicates counting from the methyl end. The general structure for fatty acids and those fatty acids which feature in this thesis are presented in Figure 2.1.

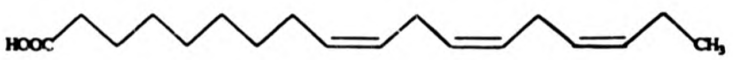
Neutral lipids are apolar and as such are completely soluble in nonpolar solvents. Phospholipids, on the other hand, are classed as polar lipids because of their polar phosphate and headgroup. Triacylglycerols, the predominant neutral lipid in fish,

fatty acid

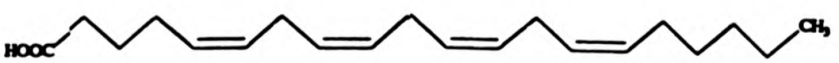


linoleic acid (18:2(n-6))

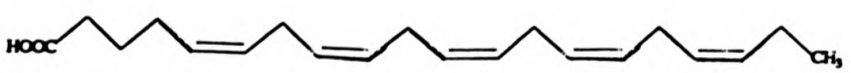
linolenic acid (18:3(n-3))



arachidonic acid (20:4(n-6))



eicosapentaenoic acid (20:5(n-3))



docosahexaenoic acid (22:6(n-3))

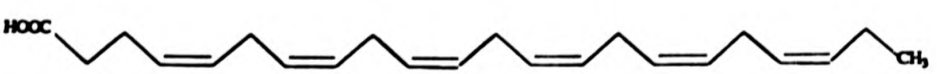
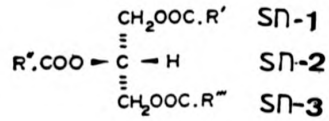
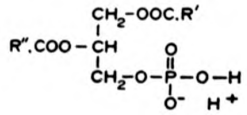


Figure 2.1(a) The structure of principle fatty acids involved in the present work

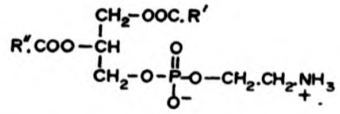
1,2,3-triacyl-*sn*-glycerol (TAG)



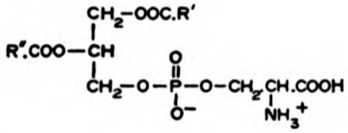
phosphatidic acid



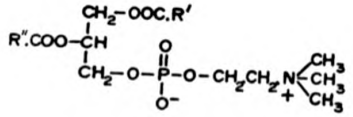
phosphatidylethanolamine (PE)



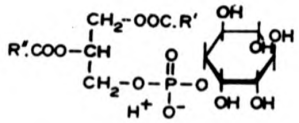
phosphatidylserine (PS)



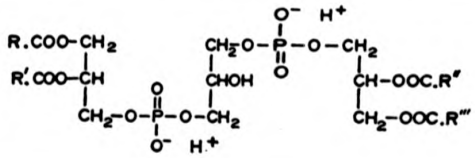
phosphatidylcholine (PC)



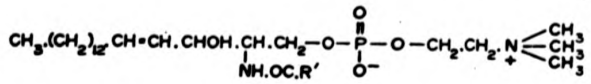
phosphatidylinositol (PE)



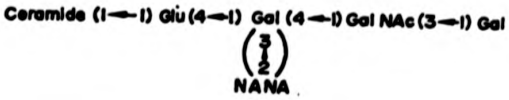
cardiolipin



sphingomyelin



ganglioside



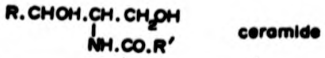


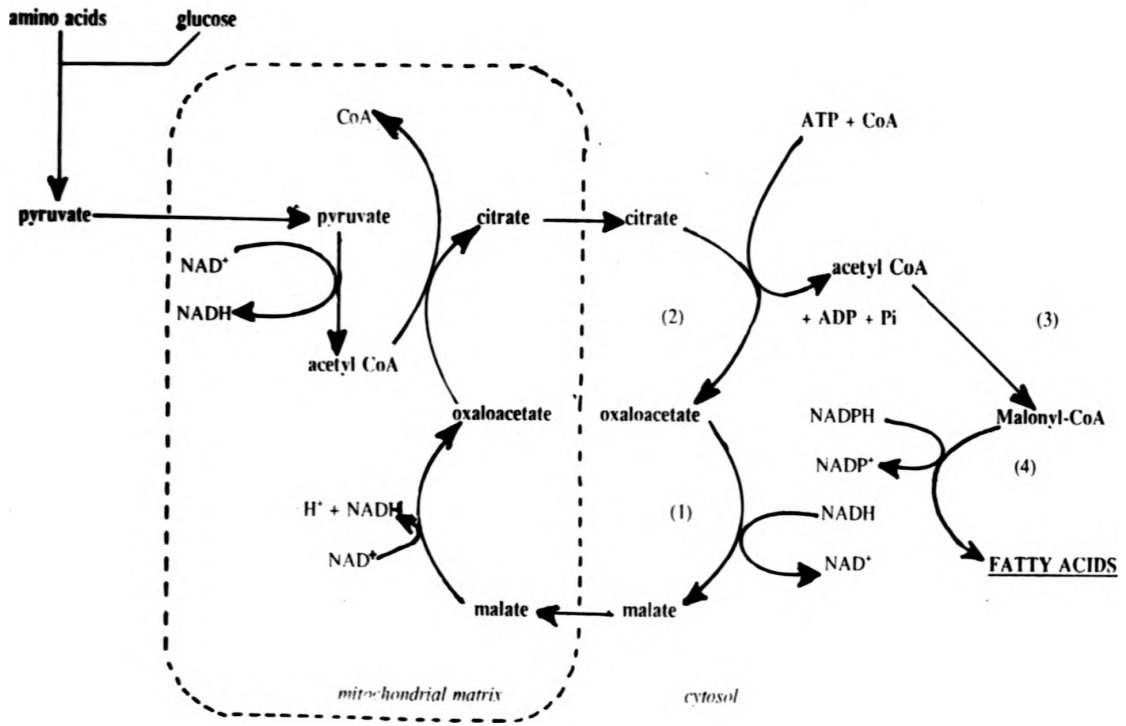
Figure 2.1 (cont.) The structure of principle lipids involved in the present work

consist of three fatty acids esterified to glycerol (see Figure 2.1). Phospholipids, the major lipid class of membranes, are structurally comprised of two fatty acids esterified to the "one" and "two" position hydroxy groups of glycerol. The third hydroxy group contains a phosphate group esterified to an alcohol. Other lipid classes found in membranes include cholesterol, glycolipids (containing a sugar moiety) and sphingomyelin, a phospholipid derived from sphingosine rather then glycerol.

The biochemical roles of phospholipids include functioning as components of membranes, providing structural support to cells, serving as chemical messengers, supplying signals during cell activity, and in special circumstances can provide the organism with important energy reserves, for example, in eggs.

The biosynthesis of fatty acids in fish, resembling that of mammals and outlined as Gurr and Harwood (1991), occurs mainly in the liver and to a lesser extent in the adipose tissue (Henderson and Sargent, 1985). Carbon derived from carbohydrate and amino acid catabolism, can be used for the formulation of citrate which is transferred to the cytosol and converted to acetyl-CoA and oxaloacetate (see Figure 2.2). Acetyl-CoA participates in the first step of the fatty acid synthesis by its irreversible carboxylation to malonyl-CoA by acetyl-CoA carboxylase. Malonyl-CoA is elongated by cycles of condensation and reduction reactions, utilizing NADPH as the source of energy. The reaction is dependent on a multienzyme complex, fatty acid synthetase, and is responsible for attaching 2 carbons atoms/cycle, donated by acetyl-CoA.

Palmitate and stearate are the major products of the fatty acid synthetase system in fish



(1) Malate Dehydrogenase; (2) ATP Citrate Lyase; (3) Acetyl-CoA Carboxylase; (4) Fatty Acid Synthetase.

Figure 2.2 The biosynthesis of fatty acids in vertebrates

(Wilson and Williamson, 1970; Warman and Bottino, 1978). The elongation and desaturation of these products require catalysis by enzymes present in the endoplasmic reticulum, which introduce 2 carbon units and double bonds into both fatty acids synthesised *de novo* and dietary fatty acids. Vertebrates lack the desaturase enzymes necessary to introduce double bonds beyond C9 (i.e. Δ^{12} and Δ^{15} desaturases) and therefore require linoleic (18:2(n-6)) and linolenic acid (18:3(n-3)), or longer chain derivatives of these fatty acids, from their diet. The concept of essential fatty acids is discussed later in Section 2.3. Freshwater fish are able to desaturate and elongate fatty acids by the pathway shown in Figure 2.3, but due to an inability to convert C18 PUFAs, marine fish require C20 and C22 in their diet (Sargent *et al.*, 1989). Fatty acid desaturase Δ^6 is an important rate limiting enzyme in this pathway and has a substrate preference for 18:3(n-3), before 18:2(n-6), before oleic acid (18:1(n-9)), with the result that the pattern of dietary fatty acids influences desaturation by this enzyme (Sargent *et al.*, 1989). Fatty acids, derived from either *de novo* synthesis or the diet, are esterified to glycerol 3-phosphate in the endoplasmic reticulum to form triacylglycerols or phospholipids.

2.2.2 Lipid composition of fish

The lipid composition of fish has been the topic of a number of reviews (Bell *et al.*, 1986; Henderson and Tocher, 1987; Yurkowski, 1989). In the review by Henderson and Tocher (1987), the authors state that the fatty acid composition of different species and different tissues depends on the predominant lipid classes which are present. The major lipid class in lipid rich tissues is most likely to be triacylglycerols, whereas in tissues of low lipid content, phospholipids are predominant. In wild fish, monoenes are the

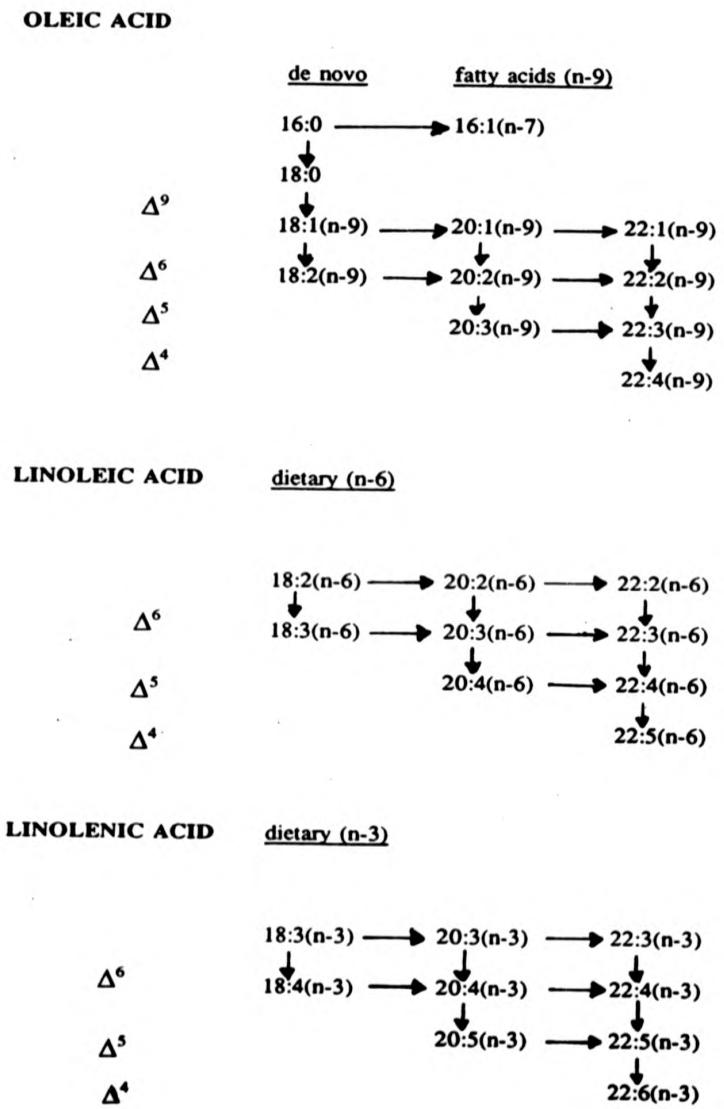


Figure 2.3

Elongation and desaturation pathways for the major fatty acid families found in fish.
Vertical lines represent desaturase activity (Δ), horizontal lines represent elongation.

most abundant fatty acid of the triacylglycerols, followed by saturates, followed by PUFAs, with the principal fatty acids normally being 18:1(n-9), palmitic acid (16:0), and 16:1(n-7) respectively (Henderson and Tocher, 1987). Phospholipids, by comparison, contain much higher levels of PUFAs and lower amounts of monoenes. The saturated fatty acid patterns between the two are similar. The PUFAs of phospholipids are of longer chain length than those of the triacylglycerols, with 22:6(n-3) being the predominant PUFA in total phospholipid.

The bulk of phospholipids are found in the membranes of cells. Plasma membranes contain approximately 25% lipid, of which the majority are phospholipids, predominantly phosphatidylcholine (PC) and phosphatidylethanolamine (PE). In trout liver, PC and PE both have a predominance of fatty acids 22:6(n-3), 16:0 and 18:1(n-9), but their relative amounts depend on the temperature (see Section 2.4.) (Hazel, 1979). Fatty acid compositions of PC and PE from other fish tissues are referred to by Henderson and Tocher, (1987). Saturated fatty acids are generally located in position 1 of PE and PC, while PUFAs tend to be found at position 2. Phosphatidylserine (PS) is also rich in 22:6(n-3) and 16:0, but contains more stearic acid (18:0) than oleic acid 18:1(n-9), cardiolipin (Cl) and sphingomyelin (SM) are high in 16:0 and 18:1(n-9), whereas phosphatidylinositol (PI) contains high levels of 18:0 and 20:4(n-6). The latter phospholipid may play an important role, in fish, in the transduction of signals through biomembranes as it does in mammals (Berridge, 1984).

The high (n-3) PUFAs content of fish has created considerable interest in fish lipids, especially as a source of (n-3) PUFAs for human nutrition. In recent years, (n-3)

PUFAs have been increasingly regarded as being beneficial to human health.

The extent to which lipids vary between different fish species depends on environmental influences, whether the species is freshwater or marine, wild or reared in captivity, and the water temperature due to latitude or seasonal fluctuations. The physiological state, such as the sex and maturity of the fish, also have a bearing on their lipid content (Castell, 1979; Sheridan *et al.*, 1985(a); Henderson and Tocher, 1987; van Vliet and Katan, 1990).

The origins of lipids in food chains in both marine and freshwater ecosystems have been evaluated by Sargent *et al.* (1989) who described their influence on the biochemical characteristics of fish lipids. The diet of marine fish tends to be rich in longer chain (n-3) PUFAs, whereas freshwater fish have a greater input from dietary 18:3(n-3). Hilditch and Williams (1964) determined that freshwater species have higher amounts of saturated fatty acids (SFAs) and C18 PUFAs, and lower amounts of C20 and C22 unsaturates than marine fish, with the result that freshwater species have a lower (n-3)/(n-6) PUFA ratio than marine fish. Reported (n-3)/(n-6) PUFA ratios for freshwater fish are typically in the range of 0.5 to 3.8 compared to values in marine fish of 4.7 to 14.4 (Henderson and Tocher, 1987).

Wild salmon receive their lipid requirements through the aquatic food web, whilst their cultured counterparts rely on fish nutritionists to supply them with a balanced diet. The total lipid content in cultured Atlantic salmon smolts is higher than wild Atlantic salmon, and contains less saturates and more monoenoic fatty acids (Bergström, 1989).

During the life cycle of anadromous salmonids changes occur in their lipid content. The fatty acid profiles of parr are typical of those found in fresh water fish, while smolts possess fatty acids more characteristic of marine fish (Hilditch and Williams, 1964). It was believed that this difference was a reflection of dietary influences (Saddler *et al.*, 1972), but evidence by Sheridan *et al.* (1985a) suggests that the lipids of parr change prior to smoltification, even when fish have been maintained on the same diet. They have shown that the fatty acid composition of freshwater steelhead trout parr has higher proportions of SFAs, particularly 16:0, and lower proportions of PUFAs, than the freshwater smolts, concurring with observations found in Atlantic salmon (Lovern, 1934), and masu salmon (Ota and Yamada, 1971). Sheridon (1989) suggests that the decrease in SFAs may be due to the fasting-like physiological state of the smolt. Saturated fatty acids have been shown to be mobilized in preference to unsaturated fatty acids in starved rainbow trout (Jezierska *et al.*, 1982). Monosaturated fatty acids have also been shown to increase in migrating coho smolts (Saddler *et al.*, 1972).

The depletion of total body lipids in association with smoltification has been the subject of numerous authors (Vanstone and Markert, 1968; Fessler and Wagner, 1969; Ota and Yamada, 1971; Sheridan, 1989), but why it occurs, and which of the biological steps during smoltification it supplies with energy, is unclear. This depletion occurs in the triacylglycerols, in tissues of high energy reserves (i.e. adipose tissue, dark and light muscle, liver and serum) (Sheridan *et al.*, 1983), and is the result of decreased lipid synthesis and increased lipase activity (Sheridan *et al.*, 1985b). Seasonal changes in the lipid content and fatty acid composition of wild cherry salmon (Ota and Yamada, 1974), and wild and hatchery-reared Atlantic salmon (Bergström, 1989), have been noted. In

the muscle of cherry salmon parr, the lipid content changes from 4.9 % in summer to 1.0 % in winter. In non-smolting parr, the lipid content rises again in the spring to 4.2 %, whereas it only increases to 1.9 % in smolts.

2.3 REQUIREMENT OF MAMMALS AND FISH FOR DIETARY (n-3) AND (n-6) PUFAs

As stated previously in Section 2.2.1., mammals and fish have a dietary requirement for (n-3) and (n-6) PUFAs. They are known as essential fatty acids (EFA) because they are essential components of the diets of vertebrates, which are unable to synthesis them *de novo*, but require them for cellular structure and function. In terrestrial animals, this requirement can be satisfied by dietary 18:2(n-6), and to a lesser extent 18:3(n-3) with their dietary lipid intake being more dependent on (n-6) fatty acids from seeds. Fish, on the other hand, in keeping with the high (n-3) content of their lipid, are more dependent on (n-3) fatty acids from algae and phytoplankton (Crawford *et al.*, 1989). In fresh water fish, EFA requirements are met by dietary long chain (n-3) PUFAs, 18:3(n-3), and to a much lesser extent 18:2(n-6). Carnivorous marine fish however, need longer chain dietary (n-3) PUFAs for growth, since they lack the Δ^5-desaturase enzyme indicated in Figure 2.3 (Castell, 1979). Rainbow trout seem to have a lower requirement for (n-6) than (n-3) (Castell *et al.*, 1972a), while the requirement for (n-6) PUFAs by Atlantic salmon is still unclear. The physiological importance of EFA is outlined in numerous reviews both for mammals (Lands, 1986; Hwang, 1989; Derby and Pelletier, 1991) and for fish (Cowey and Sargent, 1977; Castell, 1979; Watanabe, 1982).

Symptoms of (n-6) EFA deficiency were first observed by Burr and Burr (1929), when they placed young rats on fat free diets. The now classical symptoms of (n-6) EFA deficiency developed, as scaliness of the tail, dry skin, kidney malfunction and reproductive failure. Less is known of (n-3) EFA deficiency in mammals, and some authors believe their essentiality is still questionable (Tinoco *et al.*, 1971). The pathogenesis of (n-3) EFA deficiency is often concealed by a pronounced (n-6) EFA deficiency. Clinical symptoms of (n-3) EFA deficiency differ to those of (n-6) EFA deficiency, and include neurological and visual disturbances, haemorrhagic dermatitis and growth retardation (Derby and Pelletier, 1991).

The primary symptoms of EFA deficiency in fish include poor growth and reduced survival. Other reported symptoms, (reviewed by (Henderson and Tocher, 1987)), include an increased hepatosomatic index with the liver becoming pale and swollen, enlarged hearts with possible lipid protrusions of blood vessels, a lowered haemoglobin in blood, increased water content in muscle, and decreased fertility and poor hatch rates in eggs. When fish are deprived of lipid and EFA for several months, they "faint" in response to sudden shocks, a condition termed shock syndrome (Castell, 1979).

An intuitive guess as to the expected EFA requirement of fish can be made by considering the different influences on their fatty acid composition, as outlined in Section 2.2. Marine and cold water fish, for example, have greater unsaturation than freshwater and warm water fish, which suggests membrane adaptation to their environment. Generally, warm freshwater fish have a requirement for PUFAs from either the (n-3) and (n-6) series (Takeuchi and Watanabe, 1977a; Takeuchi *et al.*, 1980),

or only the (n-6) series (Kanazawa *et al.*, 1980; Takeuchi *et al.*, 1983; Teshima *et al*, 1982), whereas cold water, marine fish require predominately the n-3 PUFAs series (Castell *et al.*, 1972a; Yu and Sinnhuber, 1972). Most of the studies on dietary EFA requirements have been carried out on rainbow trout and their optimal range of dietary 18:3(n-3) was found to be between 0.8 - 1.7 % of diets in which the total lipid diet content was 5 % (Castell *et al.*, 1972a; Watanabe *et al.*, 1974). As the amount of lipid in the diet increases, the requirement for 18:3(n-3) also increases to around 20 % of dietary lipid (Takeuchi and Watanabe, 1977b). Takeuchi and Watanabe, (1977c), established that 0.25 % of both dietary 20:5(n-3) and 22:6(n-3) fatty acids could satisfy the EFA requirement of rainbow trout, proving them to be a more efficient source of EFA than 18:3(n-3). By comparison, salmon, or at least chum and coho salmon, do not have the same ability to elongate and desaturate 18:3(n-3), and thus may require longer chain (n-3) PUFAs in the diet (Takeuchi *et al.*, 1979; Takeuchi and Watanabe, 1979; Yu and Sinnhuber, 1979). Some studies have shown that excess dietary EFA (Yu and Sinnhuber, 1979; Takeuchi and Watanabe, 1979), or high levels of highly unsaturated (n-3) PUFAs, may actually be detrimental to fish growth (Greene and Selivonchick, 1990).

The possible pathways of elongation and desaturation of the essential fatty acids to form longer chain PUFAs are shown in Figure 2.3. The fatty acid desaturases metabolize all three families of fatty acids, with the greatest affinity for the (n-3) family (Brenner and Peluffo, 1966). Garg *et al.* (1988a,b, 1989) have shown that the amount and type of dietary fat is an important consideration in the regulation of this pathway in rats. For example, although (n-3) fatty acids replaced extensively (n-6) fatty acids in the serum

and liver of rats when diets containing linseed oil (high in 18:3(n-3) fatty acid) were combined with beef tallow, the replacement of (n-6) by (n-3) fatty acids was reduced considerably when diets containing linseed oil were combined with safflower oil (high in 18:2(n-6)). It has been suggested that the 18:3(n-3) content of lipid in the diets of rats has to be greater than 15 % to inhibit the conversion of 18:2(n-6) to arachidonic acid (20:4(n-6)) (Kramer, 1980). Garg *et al.* (1989, 1990) believe that the dietary ratio of 18:2(n-6) to SFA may be significant in determining the ability of the products of 18:3(n-3) to depress 20:4(n-6) and cholesterol production in rats.

The extent to which 22:6(n-3) or 20:5(n-3) can be produced in rats from 18:3(n-3) is questionable. Results suggest the conversion is limited, but can be increased if the rest of the dietary fat is saturated (Garg *et al.*, 1989). Linoleic acid (18:2(n-6)) on the other hand, is rapidly converted to the products outlined in Figure 2.3 particularly 20:4(n-6) (Dyerberg, 1986; Garg *et al.*, 1989). The long chain PUFAs in fish oil diets lower desaturase activities compared to diets high in either 18:3(n-3) or 18:2(n-6) (Christiansen *et al.*, 1991).

In fish, as described above for mammals, (n-3) and (n-6) PUFAs compete as substrate for the desaturation and elongation enzymes in the pathway shown in Figure 2.3. When rainbow trout were fed diets completely lacking in 18:2(n-6) and 18:3(n-3), 18:1(n-9) was converted to 20:3(n-9) (Castell, 1972b). The fatty acid preference for desaturase Δ^6 in fish, as mentioned earlier, is for 18:3(n-3), before 18:2(n-6), before 18:1(n-9) respectively (Sargent *et al.*, 1989). A similar pattern to the competitive inhibition found in mammalian Δ^6 desaturase, was also found in fish. Yu and Sinnhuber (1976, 1979),

proved that 18:3(n-3) acid was a better inhibitor of the enzyme in salmonids, than 18:2(n-6), and the longer chain (n-3) PUFAs also inhibited desaturase Δ^6 activity.

One of the roles of the "essentiality" of EFAs in fish is as phospholipid constituents. These in turn are important structural components of biological membranes. It is the biomembranes of poikilothermic fish which adjust to environmental changes in temperature and water salinity, (see Sections 2.2.2. and 2.4.). Membranes make necessary modifications to their lipid composition by taking fatty acids from a "fatty acid acyl-CoA pool", consisting of fatty acids synthesised *de novo* and essential fatty acids, to maintain their optimal membrane function. This will be discussed further in Section 2.6.3. Essential fatty acids are also the precursors for eicosanoids and this role of EFA will be discussed in Section 2.6.2.

2.4. EFFECTS OF TEMPERATURE ACCLIMATION ON LIPID COMPOSITION OF FISH

As a response to changes in environmental temperature, poikilothermic organisms frequently show alterations in their membrane lipid composition (Hazel, 1979). The thermodynamic state of the membrane is important with regard to membrane function (e.g. membrane bound enzyme kinetics, ion transport and protein synthesis by membrane bound ribosomes) (Singer and Nicolson, 1972; Hazel and Prosser, 1974; Cossins, 1977; Hazel, 1979; Hazel, 1988). In response to temperature changes, the proportions of membrane phospholipids and their degree of fatty acid unsaturation adjust in an attempt to preserve a constant optimal fluidity, a process known as homeoviscous adaptation (Sinensky, 1974). It is believed that this adaptation is essential for

poikilotherms to survive extreme body temperature changes.

Acclimation refers to the time taken by the membrane to complete the homeoviscous adaptation process. In fish, this can be anywhere from days to weeks (Sellner and Hazel, 1982). Bly and Clem (1988) found that red blood cells from channel catfish *Ictalurus punctatus* took 8 weeks to acclimate, T cells and thrombocytes took 3-5 weeks, whereas B cells took only 1-3 weeks.

Temperature-induced alterations in the lipid composition of membranes may occur by:- (a) changes to the type of lipid classes present in the membrane, (b) changes in the acyl chain structure, (c) molecular species restructuring and/or (d) varying the phospholipid / cholesterol ratio.

Of all the phospholipid classes, PE and PC, which together comprise 60-80 % of the total phospholipid classes in the plasma membrane, exhibit the greatest magnitude of change in response to temperature. In rainbow trout liver, the total phospholipid content did not change, but PE, which represents 16 % of the phospholipids in the livers of fish acclimated to 20°C, increased to 20.2 % in fish acclimated to 5°C (Hazel, 1979). Similarly, proportions of PE were observed to increase during cold water acclimation, and decline during warm water acclimation in trout kidney (Hazel and Landrey, 1988a), trout gill (Hazel and Carpenter, 1985), goldfish brain (Dreidzic *et al.*, 1976), goldfish intestine (Miller *et al.*, 1976) and goldfish gill mitochondria (Caldwell and Vernberg, 1970) to select only a few studies. Conversely, the proportion of PC increases with increasing water temperature, and decreases with cold water acclimation in trout kidney

(Hazel and Landrey, 1988a). The balance between PC and PE may play an intrinsic regulatory role in temperature acclimation in trout and significant alterations in the ratio of these two phospholipids occur with even small temperature changes (i.e. 4°C). The changes in the two phospholipid classes takes place more rapidly when adapting to cold water. With warm water acclimation, PE attenuates before a rise in PC levels, but in response to cold water, PE increases only after a decline in the level of PC (Hazel and Landrey, 1988a). Since PE has been found to be the most unsaturated phospholipid class within trout liver (Hazel, 1979), the increased PE content in relation to other phospholipid classes during cold water adaptation (Miller *et al.*, 1976), has the net effect of adding to the total degree of unsaturation of cell lipids. Cold water acclimation also results in increased unsaturation within PE itself (Hazel, 1979), and results in PE having a higher unsaturation index than PC.

Hazel (1979) found a decline in the SFA content from 28.7 % to 25.8 % in the phospholipids of liver from rainbow trout when transferred from 20°C to 5°C, while PUFAs increased from 27.5 % to 35.9 %. Changes within the PUFA pattern also occurred. During cold water acclimation the proportion of 22:6(n-3) increased in the PC fraction of the liver of rainbow trout, but not in the PE, SM, and PS; the content of 20:5(n-3) was found to increase in all these phospholipids (Hazel, 1979). Changes to (n-6) fatty acids also varied with the phospholipid class, by increasing in PC, PS, PI and cardiolipin, and attenuating in PE, SM and lysophosphatidylcholine (LPC) during cold acclimation.

Molecular species modifications within the phospholipid classes are present throughout

acclimation. Located in the membrane, enzymes of a deacylation /reacylation cycle rapidly adjust the phospholipid structure (Hazel *et al.*, 1987). Hazel and Zebra (1986) found that in temperature-induced restructuring of molecular species, only a few molecular species were responsible for the majority of observed changes. In the mitochondrial membranes of rainbow trout liver, an increase from 24 to 40 weight percent in the molecular species 16:0/22:6-PC occurred during cold acclimation. Another predominant species in PC of cold acclimated rainbow trout liver was 16:0/20:5. The incorporation of longer chain unsaturated PUFAs occurred by replacing di- and monoenoic species at the *sn*-2 position (Hazel, 1988).

One explanation why cell membranes should incorporate higher unsaturation at lower temperatures is that the membrane must largely stay in its liquid-crystalline phase, rather than its gel phase. PUFAs compared to SFAs have lower melting points and are less packed within the membrane; the resultant effect is a more dynamic membrane (Hazel, 1984). At lower temperatures, the acyl chains become more ordered in their packing and by introducing a bulkier unsaturated acyl chain, this order is disrupted. The introduction of a *cis* bond into an saturated acyl chain means, geometrically, that the chain length decreases, but its area within the membrane increases, resulting in the molecule being less compact within the membrane. It is interesting to note the effect that adding one double bond into a saturated acyl chain can have on the physical properties of the fatty acid. The melting point of 18:0 is 70.1°C, whereas that of 18:1 (n-9) is 16.3°C (Weast *et al.*, 1964). The addition of a second double bond into the molecule does not have such a marked effect on its melting temperature. Bell *et al.* (1986) however remark that the melting point of all PUFAs are well below

physiological temperatures, and that little difference would be made to membrane fluidity if 18:2(n-6) or 18:3(n-3) were replaced with longer chain PUFAs. Docosahexaenoic acid, (22:6(n-3)) in fact melts at a temperature 10°C higher than 20:5(n-3). Phospholipids in biological membranes contain a multitude of lipid species, all with different transition temperatures. As a result, a broad phase transition temperature exists within the membrane. Regions of fatty acids in the gel phase exist within the membrane, creating microenvironments of different fluidities.

Alterations to the headgroup, from PC to PE, the norm during cold water acclimation, also exert disrupting influences on membrane structure. PC is cylinder shaped, and as such, packs into the membrane. When PE is introduced into membranes, it disrupts the packing, because of its wedged shaped smaller head group and bulkier unsaturated fatty acids. Another important structural membrane lipid class, cholesterol, has been reported as increasing (Selivonchick and Roots, 1976), decreasing (Huang *et al.*, 1961), or being unaffected by environmental temperature changes (Di Luzio, 1972). The cholesterol : phospholipid ratio in cell plasma membranes influences membrane fluidity, by ordering the membrane packing.

Hazel *et al.* (1988 a,b) refer to an acclimation time course in the membranes of trout kidney, during which the above compositional adjustments occur. From 0-4 days, there is a change in the proportions of phospholipid classes with significant changes in that of PE within 16-24 hours. No changes are seen in the level of PC for at least 4 days when transferred from 5°C to 20°C, but if transferred to 20°C from 5°C, there are significant changes within 8 hours of the transfer (Hazel and Landrey, 1988a). The

profile of PUFAs change at a later stage in the acclimation process (i.e. 7-10 days), possibly due to a slow change in desaturase activity (Hazel and Landrey, 1988b).

Several regulatory mechanisms have been suggested to control the changes in the fatty acid composition of membrane lipid during cold water acclimation (Hazel and Zebra, 1986; Henderson and Tocher, 1987). Such mechanisms are complex and membrane specific, depending on the membrane lipid composition. The fatty acid acyl-CoA pool, supplies substrate for specific desaturases and chain elongation enzymes, whose rates of activity are important in temperature acclimation. Changes in the molecular species of phospholipids can be brought about by selection of particular fatty acids from the fatty acid acyl-CoA pool for positioning onto the *sn*-1 and *sn*-2 positions of the phospholipid glycerol moiety during phospholipid synthesis, or alternatively, by molecular species retailoring through deacylation-reacylation.

2.5. FISH IMMUNOLOGY

2.5.1. An overview of teleost immunology

While information on the immune system in fish is not as advanced as that of mammals and birds, their immune system appears to share many of the attributes of the higher vertebrates (Warr and Cohen, 1991). Available data on fish immunology are unfortunately based on several phylogenetically distinct genera, and as a result, species differences are evident. It has been demonstrated that fish have both cell mediated immunity (CMI) and humoral immunity (HI), based on their ability to reject allografts and make antibodies respectively. The following is an appraisal of the immune system

of teleosts.

Differences can be found between the major lymphoid organs of teleost fish and those of higher vertebrates (Ellis, 1982). In mammals, lymphocytes are produced in the primary lymphoid tissue (PLT), of which the thymus is responsible for T cell production, and bone marrow for B cell production. In fish, this division is not so obvious. The PLT appears to be the thymus, and to a lesser extent the kidney (Rowley *et al.*, 1988). The thymus, a paired lymphoid organ, is situated in the gill chamber beneath the pharyngeal epithelium. It produces large numbers of lymphocytes, but it is uncertain if both T and B cells are produced. Whether or not the thymus functions as a secondary lymphoid tissue (SLT) is debatable, since the presence of plasma cells has been observed in this organ (Fange and Pulsford, 1985). There is a high division rate of thymocytes, some of which migrate to seed other lymphoid tissues, particularly the spleen (Tatner, 1985). The kidney includes lymphocytes in all stages of development, suggesting that it has a role as both PLT and SLT, and it is the main producer of antibodies in fish (Ellis, 1982). Other SLT in teleosts are the spleen and gut associated lymphoid tissue (Rombout and van den Berg, 1989). Fish lack germinal centres for antigen trapping, but it has been suggested that the melano-macrophage centres found in the spleen and kidney fulfil this role (Ellis, 1988). In this respect, these organs resemble mammalian lymphnodes, which fish do not possess.

Fish contain three peripheral cell populations:- erythrocytes, thrombocytes and the leucocytes, comprising granulocytes, monocytes, and lymphocytes. The erythrocytes of fish are nucleated and are therefore larger than those of mammals, but their function is

the same. Thrombocytes resemble mammalian platelets in being responsible for clot formation, and several authors suggest that they are also phagocytic (see Rowley *et al.*, 1988). Romanowsky stains show four morphologically distinct groups of thrombocytes; spiked, spindled, oval and lone nucleus, thought to be produced by the rupture of the cell membrane during stain preparation (Ellis, 1977). They are difficult to differentiate from lymphocytes, but fluorescent staining of immunoglobulins (Ig) on the lymphocyte surface, can discriminate between the two populations. Conflicting reports of thrombocyte : lymphocyte ratios in rainbow trout, 1:25 (McCartney *et al.*, 1973), compared to 1:2 (Weinreb, 1958), illustrate the difficulties in distinguishing thrombocytes from lymphocytes.

The heterogeneity and function of fish granulocytes was the topic of a recent review by Hine (1992). Granulocytes in fish are composed of neutrophils, eosinophils, mast cells and basophils, but the relative proportions of the granulocyte populations is species dependent (Rowley *et al.*, 1988). They are generally the first cells to migrate to sites of inflammation, where they nonspecifically destroy invading organisms by phagocytosis or cytotoxic killing (Finn and Nielson, 1971; MacArthur *et al.*, 1984). Factors such as leukotrienes and complement fragments act as chemoattractants, inducing the granulocytes to sites of inflammation (Rowley *et al.*, 1988). Neutrophils exist in fewer numbers (1-4 % of total leucocytes) than in mammals, but increase with stress (Dunn *et al.*, 1989). Depending on the fish species, nuclei are either round, oval or lobed.

Monocytes from teleosts are similar to their mammalian counterparts, morphologically and functionally, making up some 1-4 % of total leucocytes. They act as phagocytic

cells, moving to sites of chronic and acute infection (Rowley *et al.*, 1988). Morphologically, they are eccentric, with kidney shaped nuclei which stain blue-purple with Romanowsky stain. Macrophages in fish, believed to be derived from the monocytes, are located in tissues and are rarely found in the blood. Some macrophages contain pigment such as melanin (Agius, 1985), and are known as melano-macrophages, some of which, in turn, develop into the melano-macrophage centres. Non-specific phagocytosis by macrophages has been observed both *in vivo* (Ellis, 1976; Hunt and Rowley, 1986) and *in vitro* (Braun-Nesje *et al.*, 1981; Secombes, 1986). Opsonisation of the ingestants by antibodies and complement fragments, facilitate their recognition and engulfment by macrophages (Sakai, 1984; Johnston and Smith, 1984).

As with mammals, the role of macrophages in fish is more than simply that of phagocytosis. Macrophages, found in the lymphoid tissues, are thought to trap, process and present antigen to lymphocytes. Evidence suggests that macrophages interact with, and stimulate lymphocytes, and for this reason are termed accessory cells. The spleen of fish is composed of highly phagocytic macrophages, enveloped by a reticulin fibre network, called an ellipsoid, in which immune complexes are trapped (Ellis, 1982). Small metabolically active lymphocytes are associated with the ellipsoids and melano-macrophage centres of the spleen, and melano-macrophage centres of the kidney (Ellis, 1980). Interleukin-1 (IL-1) is secreted by activated fish macrophages (Ellsaesser, 1989), which in turn may stimulate T lymphocytes to secrete interleukin-2 (IL-2). As with mammalian IL-2, it is thought that fish IL-2 can promote the clonal expansion of T cells which in part is responsible for T and B cell cooperation. Another recognised cytokine in fish is interferon (Secombes, 1987; Graham and Secombes, 1988; Graham and

Secombes, 1990b). A recent review by Cohen and Haynes (1991) discusses further the function of fish cytokines. Activated fish macrophages are also the main producer of a series of regulatory molecules from unsaturated fatty acids called eicosanoids. Fish produce, by the action of cyclooxygenase, various prostaglandins (Pgs) and thromboxanes (Txs) of the 1, 2 and 3 series (Rowley, 1991). Products of lipoxygenase activity are also found, and these include lipoxins (Lxs), leukotrienes (Lts) and hydroxy-eicosatetraeoic acids (HETE) (Rowley, 1991). Further information about eicosanoid structure and function is given in Section 2.6.2.

It is thought that macrophages are activated in a two step process (Ruco and Meltzer, 1978). Firstly, inactivated macrophages are "primed" by the stimulus of low-dose LPS, thioglycolate, complement, lymphokine or IFN-γ. In the primed state macrophages have increased spreading, alterations in cell surface receptors and an increase in metabolic activity. They also become more susceptible to stimulation by the above and other cytokines, which trigger full activation. Unstimulated macrophages can be fully activated *in vitro* with phorbol myristate acetate (PMA), mitogens concanavalin A (Con A) or lipopolysaccharide (LPS), or by calcium ionophore A23187 (West 1990). In the "activated" state, macrophages have decreased chemotaxis and enhanced spreading and adherence. They show increased phagocytosis, elevated metabolic activity with heightened respiratory burst activity, eicosanoid and cytokine production and the release of lysosomal enzymes. The result is an increase in bactericidal activity, and tumour and parasite cytotoxicity (West 1990).

When normal "unactivated" macrophages are cultured with antigen-sensitized

lymphocytes for 2-3 days the macrophages undergo the physiological changes mentioned above resulting in an enhanced ability to kill ingested bacteria. This observed activation of the macrophages is not dependent on the physical presence of sensitized lymphocytes, but rather a factor produced by the sensitized lymphocytes. If supernatant from cultures of activated lymphocytes is added to normal macrophages in the absence of the lymphocytes, macrophage activation will still occur (Secombes, 1987). The responsible factor is called macrophage activation factor (MAF) and attempts to characterize it suggest it may be a gamma interferon (IFN-γ) (Graham and Secombes, 1990b).

Once the macrophage has arrived at the site of infection, its migration is arrested by the presence of another factor produced by lymphocytes. If fish kidney leucocytes from an antigen-sensitized animal are packed into a capillary tube, then placed into culture in the absence of antigen, macrophages will migrate out of end of the tube. If, however, antigen is added to the culture medium, migration by the macrophages will be inhibited by the sensitized lymphocytes via a substance known as migration inhibition factor (MIF).

Morphological resemblances between mammalian and fish lymphocytes exist, but the degree to which heterogeneity occurs in fish, compared to mammals, is questionable. Mammalian B cells, but not T cells, express Ig on their surface (sIg), and these serve as receptors for antigen. In mammalian T cells, a surface molecule similar to the light chain molecules of Ig, act as receptors for antigen. Although sIg is found on all fish lymphocytes, a population of cells with only the heavy chain regions of Ig molecules on their surface, have been extracted from thymocyte membranes of carp *Cyprinus*

carpio (Secombes *et al.*, 1983). Two distinct cell populations have been separated by panning the lymphocytes with monoclonal antibodies (Lobb and Clem, 1982; Deluca *et al.*, 1983). In one study, monoclonals raised against channel catfish serum IgM reacted with 35-40 % of lymphocytes (Lobb and Clem, 1982). Lymphocytes with sIg on their membranes were termed sIg^+ lymphocytes, while those with only heavy chain molecules were termed sIg^- lymphocytes. It is also possible to separate the two cell populations by adherence to nylon wool, and by density centrifugation (Rowley *et al.*, 1988), allowing functional assays on the two cell types.

Mitogen studies on the two populations (sIg^+ or sIg^- lymphocytes) from channel catfish (Sizemore *et al.*, 1984), and trout (Deluca *et al.*, 1983) show that sIg^+ cells respond to LPS, a mammalian B cell mitogen, while sIg^- cells respond to Con A, a mammalian T cell mitogen. SIg^- cells respond in mixed leucocyte reactions (MLR), but both sIg^+ and sIg^- can act as stimulator cells (Miller *et al.*, 1986). Other functional similarities which fish sIg^+ cells have in common with mammalian B cells, is their ability to respond to thymus independent (TI) antigens. Thymus dependent (TD) antigens require both sIg^+ and sIg^- lymphocytes for a response (Miller *et al.*, 1985; Miller *et al.*, 1987). Cuchens and Clem (1977), divided lymphocytes from blue gill anterior kidney, thymus and spleen into the two cell populations. They called one of them T-like cells because they responded to phytohaemagglutinin (PHA), Con A and exhibited a MLR at 32°C. This population of cells also exhibited surface antigens to blue gill brain tissue, equivalent to mammalian Thy.1, a cell surface marker on mammalian T lymphocytes which cross-reacts with brain tissue. The above responses were suppressed at 22°C in this cell population. At this temperature however, the second population, the B-like cells reacted

with LPS and formed spontaneous rosettes with erythrocytes.

Hapten carriers have helped to elucidate the functions of the two lymphocyte subpopulations (Ellis, 1982; Miller *et al.*, 1985; Miller *et al.*, 1987). Antibody production to trinitrophenyl-LPS (TNP-LPS) (a mammalian TI antigen) requires sIg^+ cells and monocytes, while TNP-keyhole limpet (TNP-KHL) (a mammalian TD antigen) requires sIg^+ cells, sIg^- cells and monocytes (Miller *et al.*, 1985). This is analogous to the TI and TD antigen response seen in mammals. Although suppressor, helper and cytotoxic activities have been demonstrated in teleosts, the identification of separated cell populations responsible for these functions, has not been conclusively achieved for fish.

Histocompatibility restriction of antigen recognition seems evident in teleost fish and has been recently reviewed by Stet and Egberts (1991). Fundamentally, the Major Histocompatibility Complex (MHC) is involved with the recognition of self and non-self. In mammals, class I MHC loci code for a series of gene products involved in cytotoxic T cell responses, the class II MHC loci code for a series of gene products which regulate T cell recognition of antigens presented to them by accessory cells, while class III loci code for some of the products of the complement cascade.

As previously mentioned, the kidney of fish is the main antibody producer (Ellis, 1982). Fish, unlike mammals which produce IgM, IgG, IgD, IgE and IgA, produce only one class of Ig - IgM. Tetra and monomeric forms of this Ig have been found and they may execute different roles. Fish Ig has some of the attributes of the various mammalian

antibodies. IgG is transferred across the placenta and IgA is present in the milk of mammals giving maternal immunity to offspring. Immunoglobulins have been found in the ova of carp and plaice, but not salmon (van Loon, *et al.*, 1981; Bly *et al*, 1986c). Fish experience hypersensitivity, but do not produce any IgE (IgE mediates hypersensitivity in mammals), so IgM may mediate this process in fish. IgA, the secretory Ig, is present in saliva, mucus, tears etc. of higher vertebrates. Evidence supports Ig production in the body fluids of fish, either in cutaneous mucus, or fluids such as bile. Lobb (1987) lists several reports of such antibodies. IgG is the major Ig class in mammals and has a significant role in the secondary response. Fish, too, are known to possess immunological memory. The degree of the secondary response is dependent on the route of administration, the amount of antigen administered, the nature of the antigen and the environmental temperature (Ellis, 1988).

As with the mammalian immune system, that of the fish is divided into innate and adaptive immune responses. The innate immune response acts as the first line of defence against infectious agents and is often able to avert infection by potential pathogens. It is a nonspecific defence mechanism in which the fish's resistance to a pathogen does not improve with repeated infection. The combined activity of soluble factors such as complement, cytokines, acute phase proteins, and lysozyme, and that of cells such as macrophages and natural killer cells provide innate protection. The adaptive immune responses are based on specific defence mechanisms which improve the hosts resistance to a pathogen with repeated infections, resulting in a specific immunological memory. Adaptive immunity consists of both humoral and cell mediated immune responses. The former type pertains to antibody mediated responses which

occur within minutes to hours of exposure to antigen. The latter has a delayed response of hours to days after exposure to antigen. Innate and adaptive immune responses do not however work in isolation, but rather as an combined network of activities. The extent to which the two systems integrate in fish is not as well defined as it is in mammals. Non specific immunity in fish has been reviewed by Ingram (1980). Fish also possess nonspecific cytotoxic behaviour (Hayden and Laux, 1985).

2.5.2. The effect of temperature on the immune response of fish

Environmental temperature changes are known to influence the physiology of fish (Bly *et al.*, 1986a). In relation to fish immunology, low temperatures have been implicated in depressing CMI and HI responses of both cold and warm water species (Cuchens and Clem, 1977; Avtalion, 1981; Abuzzini *et al.*, 1982; Miller and Clem, 1984; Bly and Clem, 1988). The extent to which it influences these responses vary. Some studies have reported a lag in the response, be it normal or a depressed response, while others have observed a decrease or total suppression. There are recognised "permissive" temperature ranges in which the immune response will function, and "non permissive" temperatures at which the immune response is suppressed. These ranges vary between warm or cold water species (Bly and Clem, 1992), and for salmon the non permissive temperature is about 4°C (Fryer *et al*, 1976). The effects of temperature on the immune response of teleosts have recently been comprehensively reviewed by Bly and Clem, (1992).

Much of the evidence for temperature immunosuppression in fish has come from *in vitro*, rather than *in vivo* studies and it must be remembered that *in vivo* acclimation

temperatures can exercise effects on *in vitro* response (Avtalion, 1981; Miller and Clem, 1984; Clem *et al*, 1984). Using hapten carriers against TI and TD antigens, Miller and Clem (1984) showed in channel catfish that the primary antibody response to TD, and not TI antigens, was temperature sensitive, and that low *in vivo* temperatures increased the suppression of *in vitro* antibody responses to TD antigens. Responses to T cell mitogens and MLR, but not B cell mitogens, exhibited a similar temperature induced suppression (Cuchens and Clem, 1977; Clem *et al.*, 1984, Miller *et al*, 1986). This evidence suggests that it is a low temperature sensitive T cell function which is responsible for the suppression, rather than a B cell sensitivity (Faulmann *et al.*, 1983; Clem *et al.*, 1984). Clem's group established that it was the education of virgin T cells and not the activation of memory T cells which was blocked by low temperatures (Faulmann *et al.*, 1983; Clem *et al.*, 1984), and that antigen-specific tolerance or suppressor activity was not responsible for the immunosuppression (Bly and Clem, 1991). Using a series of "shift up" and "shift down" protocols, between "non permissive" and "permissive" temperatures, the group found the suppression to occur within 8 hours of cell activation (Clem *et al.*, 1984). By bypassing early events in lymphocyte activation with phorbol ester and calcium ionophore, temperature sensitivity was shown to occur pre-protein kinase C activation (Ellsaesser *et al.*, 1988; Lin *et al.*, 1992).

An inability of accessory cells to process and present antigen to the T cell was not responsible for the observed temperature induced suppression, since the addition of channel catfish IL-1 to proliferative assays, preformed at low culture temperatures, did not rescue the T cells (Ellsaesser *et al.*, 1988; Ellsaesser, 1989). Vallejo *et al* (1992),

by examining the ability of catfish monocyte cell lines to process antigen at varying *in vitro* temperatures, confirmed that antigen processing was not responsible for the observed T cell suppression.

An important early step in lymphocyte activation is the capping of antigen on the surface of mammalian cell membranes (Bourguigon and Bourguigon, 1984). It was postulated that lymphocyte plasma membrane viscosity, under the control of homeoviscous adaptation, discussed above, could play a crucial role in lymphocyte activation and function, and that the fatty acid composition of the T cell membrane may differ to that of the B cell, and hence effect the T cell membrane fluidity (Abuzzini *et al.*, 1982).

Bly *et al.*, (1986a; 1986b; 1987; 1988; 1990) undertook a series of studies to examine membrane fluidity and the effects of temperature on the capping properties of lymphocytes from channel catfish. Using a membrane fluidity probe, B cells were shown to require 1-3 weeks to acclimate, while T cells and thrombocytes required 3-5 weeks. The difference in acclimation was assumed to be insignificant with regard to cell activity. An inability of T cells to undergo homeoviscous adaptation was not the reason for the temperature suppression, since similar membrane fluidities were exhibited by all three cell populations (Bly and Clem, 1988). Bly *et al.* (1986a) also demonstrated that T cell temperature immunosuppression was not the result of an inability by surface receptors to bind antigen or Con A, nor was it a defect in sIg capping (Bly *et al.*, 1987), and T cells were as capable of capping surface antigen as B cells at lower assay temperatures (Bly *et al.*, 1988). When channel catfish were

acclimated between 12°C to 27°C, the low temperature T and B cells had significantly higher and lower proportions of 18:1(n-9) and 18:0 respectively, in their plasma membrane phospholipids (Bly *et al.*, 1986b). The phospholipid fatty acid compositions of T and B cells were similar, although they are known to differ in mammals (Buttke *et al.*, 1985). Exogenous 18:0 and 18:1(n-9) had no effect on B cell proliferation by LPS stimulation, but T cell responses to Con A were suppressed by 18:0 and enhanced by 18:1(n-9) *in vitro*. In fact, at "non-permissive temperatures" T cells responded to Con A in the presence of 18:1(n-9), suggesting that they could be rescued from low temperature suppression by the 18:1(n-9). T cells, unlike B cells, were unable to elongate the 18:0 to 18:1(n-9), resulting in a build up of 18:0 within the membrane. This in turn effected membrane fluidity and hence membrane function (Bly *et al.*, 1990). The effect of increased saturation within the T cell membrane was aggravated by low temperature. As explained in Section 2.4., membrane unsaturation usually increases at lower temperature. In the case of the T cell, saturation increased with the build up of 18:0. It was assumed that exogenous 18:1(n-9) increased membrane fluidity, thus restoring immune function, but these very subtle changes were not detectable by fluorescence polarisation probes (Bly and Clem, 1992).

As well as leucocyte function being temperature sensitive, cell numbers have also been reported to be affected (Dunn *et al.*, 1989). Increased levels of lymphocytes, neutrophils and eosinophils accompany increased temperatures. Dunn *et al.*(1989) recorded a 250% difference in goldfish lymphocyte cell count at 25°C (26.7×10^3 mm^{-1}), compared with those at 5°C (7.4×10^3 mm^{-1}). The life span of these cells is unknown in teleosts, and therefore their contribution to increased/decreased cell numbers experienced by

temperature changes is unknown.

The effects of temperature on the fish immune system pose practical problems to fish farms during vaccination. Thymus independent vaccines (e.g. against *Vibrio anguillarum*) protect at 6°C (Horne *et al.*, 1982), but the response against thymus dependent antigens (e.g. against *Vibrio salmonicida*) is depressed at these temperatures. Low temperature vaccination with thymus dependent vaccines produces short term protection, since secondary responses are absent (Rijkers *et al.* 1980; Stolen *et al.*, 1984). A normal secondary response can be achieved by vaccinating the fish at higher temperatures (Avtalion, 1981; Stolen *et al.*, 1984), or perhaps, as this study aimed to examine, through manipulation of the diet.

2.6. THE IMPORTANCE OF LIPIDS ON THE HEALTH OF ANIMALS

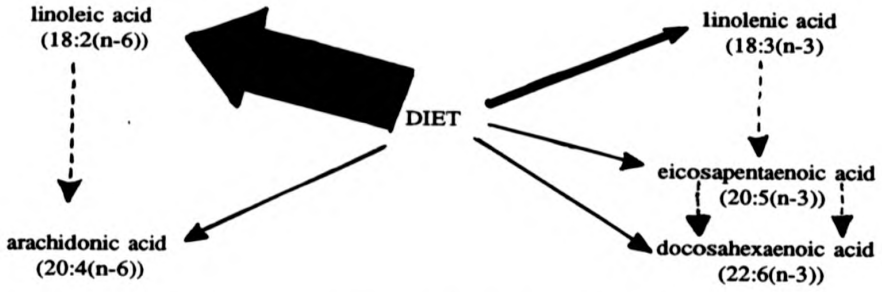
2.6.1. The influence of PUFAs on disease resistance

In recent years attention has focused on the influence of nutrition on human health. Malnutrition has been well documented in its ability to impair the immune response of mammals (Chandra and Newberne, 1977; Gross and Newberne, 1980; Philips and Baetz, 1981). Protein-energy malnutrition (PEM) for example, is associated with atrophy of lymphoid tissue and a decreased delayed type hypersensitivity response to antigens such as purified protein derivative or streptokinase-streptodornase. T helper cell numbers were found to decrease to half of their control level during malnutrition, but antibody levels to TI-antigens increased suggesting a possible reduction in T cell suppression or activity (Chandra, 1988). Non specific defences such as phagocytosis, intracellular

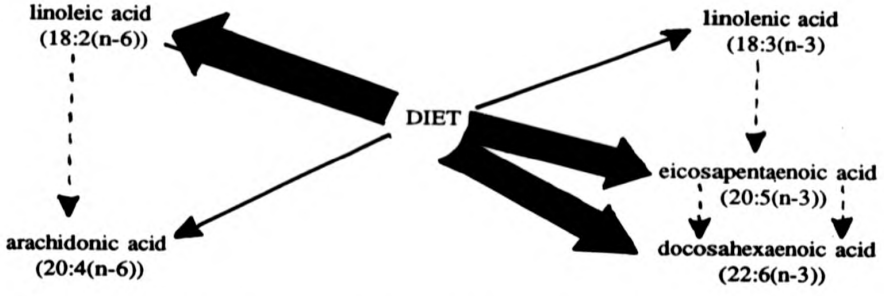
killing by macrophages and neutrophils, or complement activity are also seen to attenuate during PEM (Chandra, 1988). At the other extreme, the "western" diet has resulted in cases of immunosuppression due to obesity and high fat intake (Ferandes, 1989).

Investigations of the impact of dietary lipids on mammalian disease resistance have increased since the observation of low mortality rates due to heart disease and low incidences of breast cancer amongst Greenland Eskimos and Japanese, attributed to their high sea food diet (see Figure 2.4) (Bang *et al.*, 1976). PUFAs have been found to act as hypocholesterolemia agents, an important attribute since atherosclerosis (a build up of cholesterol in the arteries) is the primary cause of heart attacks. The (n-3) PUFAs lower serum triglyceride and very low density lipoprotein concentrations in the blood, and eicosapentaenoic acid 20:5(n-3) in particular, acting via eicosanoids, helps to prevent coronary heart disease through its antithrombotic effects. Two to three meals of fish per week has been suggested as being able to reduce heart attack mortalities by up to 50 % (Leaf and Weber, 1988). Simopoulos (1986), outlines the history behind the developing interest in PUFAs, particularly 20:5(n-3) and 22:6(n-3) found in abundance in fish lipids.

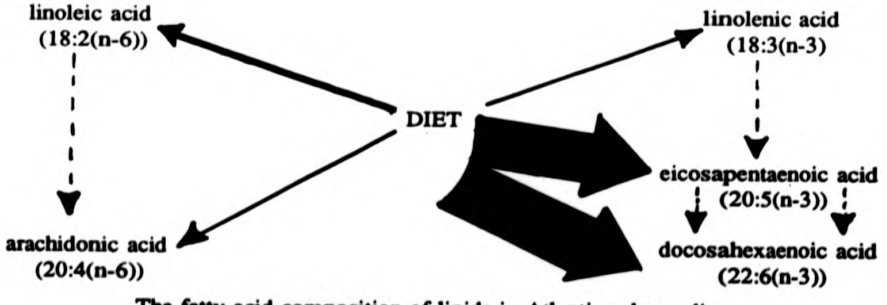
Increased levels of dietary (n-3) PUFAs have been shown to be beneficial in the treatment of several immune related pathophysiologies listed in Table 2.1., whereas dietary (n-6) PUFAs accentuate the pathological conditions of such diseases (Kinsella *et al.*, 1990). In rheumatoid arthritis the rheumatoid factor, which is produced by B



The fatty acid composition of lipids in the "Western" diet



The fatty acid composition of lipids in the Greenland Eskimo diet



The fatty acid composition of lipids in Atlantic salmon diet

Figure 2.4 A comparison of fatty acid composition of the Western diet, the Eskimo diet and the Atlantic salmon diet.

cells, is under the regulation of suppressor T cells. Excessive levels of PGE_2, derived from 20:4(n-6) (see Section 2.6.2.) inhibit suppressor T cell activity, and hence there is an over production of this factor. Similarly, an excess of antibody in systemic lupus erythematosus may result from a loss of suppressor activity due to increased levels of PGE_2. Delayed rejection times of skin grafts have also been attributed to increased dietary (n-6) PUFAs (Mertin and Hunt, 1976). These examples suggest that certain pathological conditions are due to (n-6) PUFAs suppressing the immune response. Further evidence of this is seen in (n-6) PUFA's ability to act more effectively as promoters of tumorigenesis than SFAs and fish oils (Vitale and Broitman, 1981; Vitale, 1988; Carroll, 1991). In several dietary studies, fish oils inhibited tumour promotion (Jurkowski and Cave, 1985; Braden and Carroll, 1986). Cave and Jurkowski (1987) showed that a high level of fish oil was needed to suppress the promoting effects of (n-6) when mixtures of dietary fats were used. Mice fed diets of low (n-6) PUFA/SFA ratios produced less antibody because of higher T cell suppresser activity, whereas high (n-6) PUFA/SFA diets resulted in increased antibody levels in the presence of lower suppressor activity (Ponnappan *et al.*, 1988). Current evidence suggests that eicosanoids are responsible for many of the observed effects of dietary fatty acids on the immune response. Exactly how (n-6) PUFAs exert their immunosuppression, and how (n-3) PUFAs alleviates it, is outlined in Section 2.6.2.

Diets enriched in (n-3) fatty acids may prevent, impede or ameliorate some of the conditions exacerbated by (n-6) PUFAs, but high levels of dietary (n-3) have also been implicated in disease.

Table 2.1: Pathophysiologies known to be influenced by dietary PUFA

Hodgkins disaease
Systemic lupus erythematosus
Rheumatoid factors
Autoimmune disease
Tumour growth
Anergy
Burn injury and trauma
Post-operative infections
Blood transfusion
Organ failure
Sepsis; endotoxins
Cachexia; onorexia
General immune suppression (burn, transfusion,post-sugery trauma)

Increased levels of eicosanoids especially PGE_2 are associated with several pathophysiologies

Abbreviations: PGE_2 Prostaglandin E2

Kinsella *et al.*, (1990) Nutr.(Suppl) 6:24-44

Increased free radical activity was observed in animals given high (n-3) PUFAs diets (Hu *et al.*, 1989). The oxidation of longer chain PUFAs to eicosanoids generates free radicals (Hill, 1981), and the unstable nature of the highly unsaturated PUFA molecules makes them susceptible to increased peroxidation *in vivo*. There is increasing evidence to indicate that these peroxides are able to promote tumour growth (Pritchard *et al.*, 1989) and are involved in other human diseases (Southern, 1988).

The effects of dietary lipids on the immune response of mammals has been the topic of numerous reviews in which the possible mechanisms by which dietary lipids influence the immune response have been discussed (Meade and Mertin, 1978; Goodwin and Webb, 1980; Vitale and Broitman, 1981; Johnston, 1985; Erickson, 1986; Vitale, 1988; Johnston, 1988; Hwang, 1989; Kinsella *et al.*, 1990). Some suggestions are that the effects may be due to changes in the levels of serum lipoproteins or of eicosanoids (see Section 2.6.2.) or possibly through alterations in cholesterol and/or phospholipid concentrations within the cell membrane (Vitale, 1988), since alterations to the cell membrane result in changes in receptor expression, enzyme activity and intracellular signalling (see Section 2.6.3.).

Investigations of dietary PUFAs on the immune response have led to confusing, if not contradictory, conclusions. In experiments where fatty acids were administered to either animals or to lymphocyte cultures, the data on T cell mitogen stimulation has been variable, with either no observed immunostimulation (De Deckere *et al.*, 1988), positive immunostimulation (Kelly and Parker, 1970) or immunosuppression being observed (Mertin and Hughes, 1975; Weyman *et al.*, 1977). Results suggest that both the

quantity and quality of dietary lipids play significant roles in the observed effects (Di Luzio, 1972; Locniskar *et al.*, 1983). High levels of (n-6) PUFAs (>20 μM) inhibit CMI, and while high levels of saturated fatty acids do not inhibit CMI to the same extent as (n-6) PUFAs, they are still inhibitory (Chandra, 1988). Low amounts of (n-6) PUFAs (<10 μM) on the other hand, have been found to enhance lymphocyte function and proliferation (Erickson *et al.*, 1983; Chandra, 1988; Buttke *et al.*, 1989). Chandra (1988) showed similar effects on the immune response by varying the lipid quality and source. When mice were fed 0-20 % fat with either (n-6) PUFAs (safflower oil) or saturated fatty acid (coconut oil) as a source of fat, mortalities to *Listeria monocytogenesis* occurred in the mice fed PUFA deficient or high PUFA diets, and decreased antibody levels to *Listeria monocytogenesis* were observed in these mice. Diets low in (n-6) PUFAs however, were again shown to enhance the immune response. Further *in vivo* and *in vitro* studies cited by Kinsella *et al.*, (1990), showed low concentrations of (n-6) PUFA stimulate the immune response, whereas high levels depress it.

It is generally accepted that CMI rather than HI is inhibited by high levels of PUFAs (Buttke *et al.*, 1989). It may not be a defect in the T cell, but rather one in the presentation of antigen to the lymphocyte by the macrophage (macrophages play an intrinsic role in lymphocyte activation). For example, increased cellular cholesterol within the macrophage can inhibit phagocytic function (Berken and Benacerraf, 1968; Dianzani *et al.*, 1976). Buttke *et al.*, (1989) believe that the difference in effects seen between T and B cells in mice lies in a fundamental difference in their lipid metabolism. They showed T cells to be deficient in stearoyl-CoA desaturase activity

and thus deficient in monosaturated fatty acid synthesis. This deficiency resulted in an accumulation of distearoyl phosphatidylcholine within the T cell, which was five times greater than that found in the B cell, whereas dietary oleic acid reduced distearoyl phosphatidylcholine content within the T cell.

The results of some studies suggest that it may in fact be serum lipoproteins which regulate the immune response, and it is thought that they do this through controlling specific cell receptor expression (Ho *et al.*, 1970; Edgington and Curtis, 1981; Stenbaek, 1984), and cholesterol metabolism (Brown and Goldstein, 1976). Serum lipoproteins have been found to inhibit lymphocyte proliferation (Ho *et al.*, 1970; Edgington and Curtis, 1981; De Deckere *et al.*, 1988). The composition and concentration of lipoproteins is controlled by the nutritional state (Natio *et al.*, 1976).

It is important to remember that although *in vitro* studies give an indiction of *in vivo* events, they do not themselves give a true representation since other mediators which possibly operate *in vivo* may have been excluded from the *in vitro* model. Johnston (1985), when presenting a synopsis of dietary trials (some of which gave contradictory results), emphasized the need for good experimental design. The observed effects depend on the duration of the feeding, the nature of the fats used in the trial and the immune organs used to assess immunocompetence.

2.6.2. The role of eicosanoids in disease

Eicosanoids are synthesised from PUFAs such as 20:4(n-6) and 20:5 (n-3) by the action of cyclooxygenase which forms prostaglandins and thromboxanes as products, and

lipoxygenase which forms leukotrienes and lipoxins. These eicosanoids are important in cell physiology since they function as intercellular signals (Gerrard, 1985). They are not stored by body tissues and are synthesised in nanomolar or picomolar amounts on demand. The major eicosanoids resulting from the two enzymes are shown in Figure 2.5. Cyclooxygenase produces 1-series PGs from 20:3(n-6), 2-series PGs from 20:4(n-6) and 3-series PGs from 20:5(n-3), while lipoxygenase produces 3-series LTs from 20:3 (n-6), 4-series LTs from 20:4(n-6), 5-series LTs from 20:5(n-3) and 7-series LTs from 22:6(n-3). The products of 5, 12 and 15- lipoxygenase are 5-hydroxyeicosatetraenoic acid (HETE), 12-HETE and 15-HETE, respectively.

The major precursor of eicosanoids in mammals is 20:4(n-6), giving rise to the 2-series PGs (see Figure 2.5). There are two ways in which eicosanoid production from 20:4(n-6) can be regulated; either by competitive substrate inhibition of cyclooxygenase and lipoxygenase or inhibition by the product. Increasing dietary (n-3) PUFA results in a reduction of 20:4(n-6) production from 18:2(n-6) due to competitive inhibition of the Δ^6-desaturase enzyme (Morgrum and Johnston, 1983; Hadden, 1988) and subsequently results in a decreased biosynthesis of eicosanoids from 20:4(n-6) and at the same time leads to an increased production of 3-series PGs, and 5 and 7-series LTs. It has been shown in mammals that 20:5(n-3) and 22:6(n-3) are more effective than 18:3(n-3) in suppressing levels of 20:4(n-6) in phospholipids (Hwang, 1989), with 20:5 (n-3) being the preferred substrate for Δ^5-desaturase enzyme over 20:4(n-6) (Ochi *et al.*, 1983). The result is that 20:5(n-3) and 22:6(n-3) give rise to a greater synthesis of lipoxygenase products. High dietary levels of 20:5(n-3) thus result in a reduction of LBT_4 and an increase in LBT_5. The potencies of lipoxygenase products from 20:5(n-3) on the

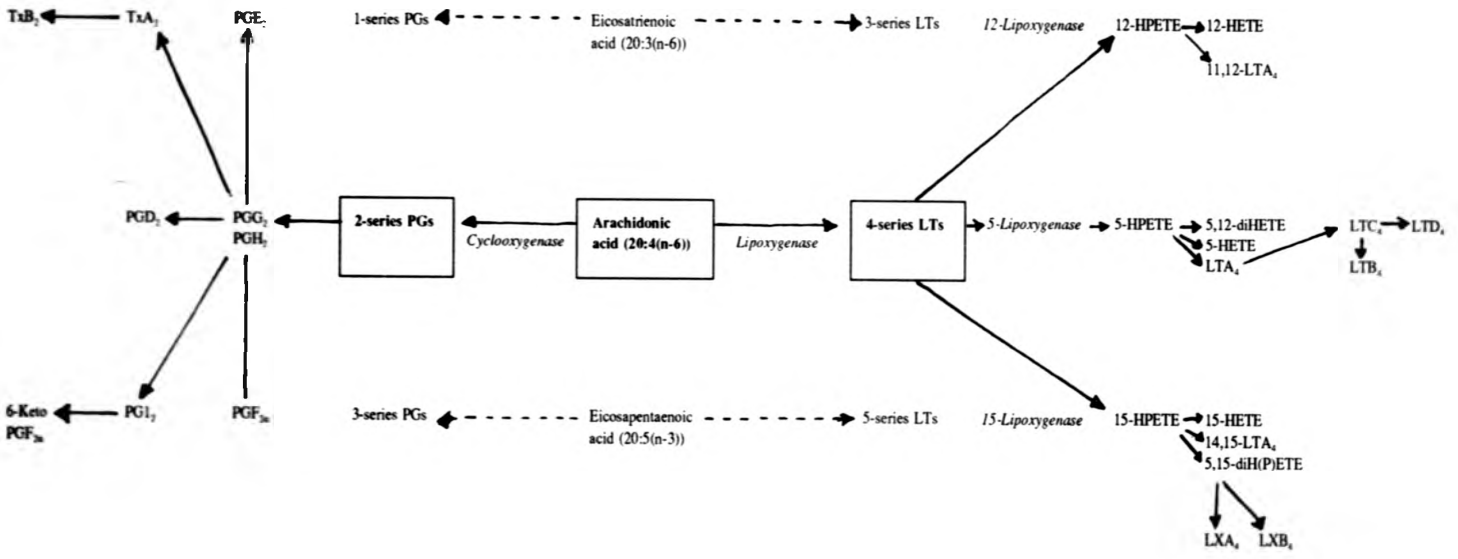


Figure 2.5 Pathway of eicosanoid formation from arachidonic acid (20:4(n-6)).

Abbreviations: HPETE-hydroperoxyeicosatetraenoic acid; HETE-hydroxyeicosatetraenoic acid; LT-leukotrienes; PG-prostaglandins; TX-thromboxanes

immune system have not been fully elucidated. Eicosapentaenoic acid (20:5(n-3)) on the other hand, is a poorer substrate for cyclooxygenase than 20:4(n-6) (Hwang, 1989).

An imbalance of eicosanoid synthesis can lead to pathological states as seen in inflammation and autoimmune diseases described in Table 2.1. Eicosanoids regulate the immune response through a feedback loop system, and is illustrated in Figure 2.6 for the eicosanoids derived from 20:4(n-6). Lipoxygenase products from 20:4(n-6) stimulate the immune response at low physiological levels by activating T helper cells (T_H) which in turn activate B cells, but higher concentrations ($>10^{-9}$ M) induce $T8^+$ cells (thymus subset) to become suppressor cells (Goetzl *et al*, 1988). The cyclooxygenase products from 20:4(n-6) stimulate the immune response at low physiological levels and suppress it at high physiological levels. Low levels of PGE_2 (10^{-10} M) induce mature T cells, whilst higher levels of PGE_2 ($>10^{-8}$ M) exert negative feedback on the system (Goodwin, 1985); thus PGE_2 regulates rather than inhibits suppressor T cell activity (Kinsella *et al.*, 1990). For further details on the antagonistic effects of lipoxygenase and cyclooxygenase on the immune system, several reviews are available (Johnston, 1985; Bailey, 1985; Hwang, 1989; Kinsella *et al.*, 1990). Putative effects of PGE_2 and lipoxygenase products on the mammalian immune response are shown in Table 2.2.

There is a bidirectional effect between eicosanoids and cytokines. Eicosanoids influence the production of cytokines which in turn can stimulate the synthesis of eicosanoids. Synthesis of IL-1, IL-2, and tumour necrosis factor (TNF) can be inhibited by PGE_2 (Kunkel *et al.*, 1982; Rappaport and Dodge, 1982), and IL-2 can regulate the immune

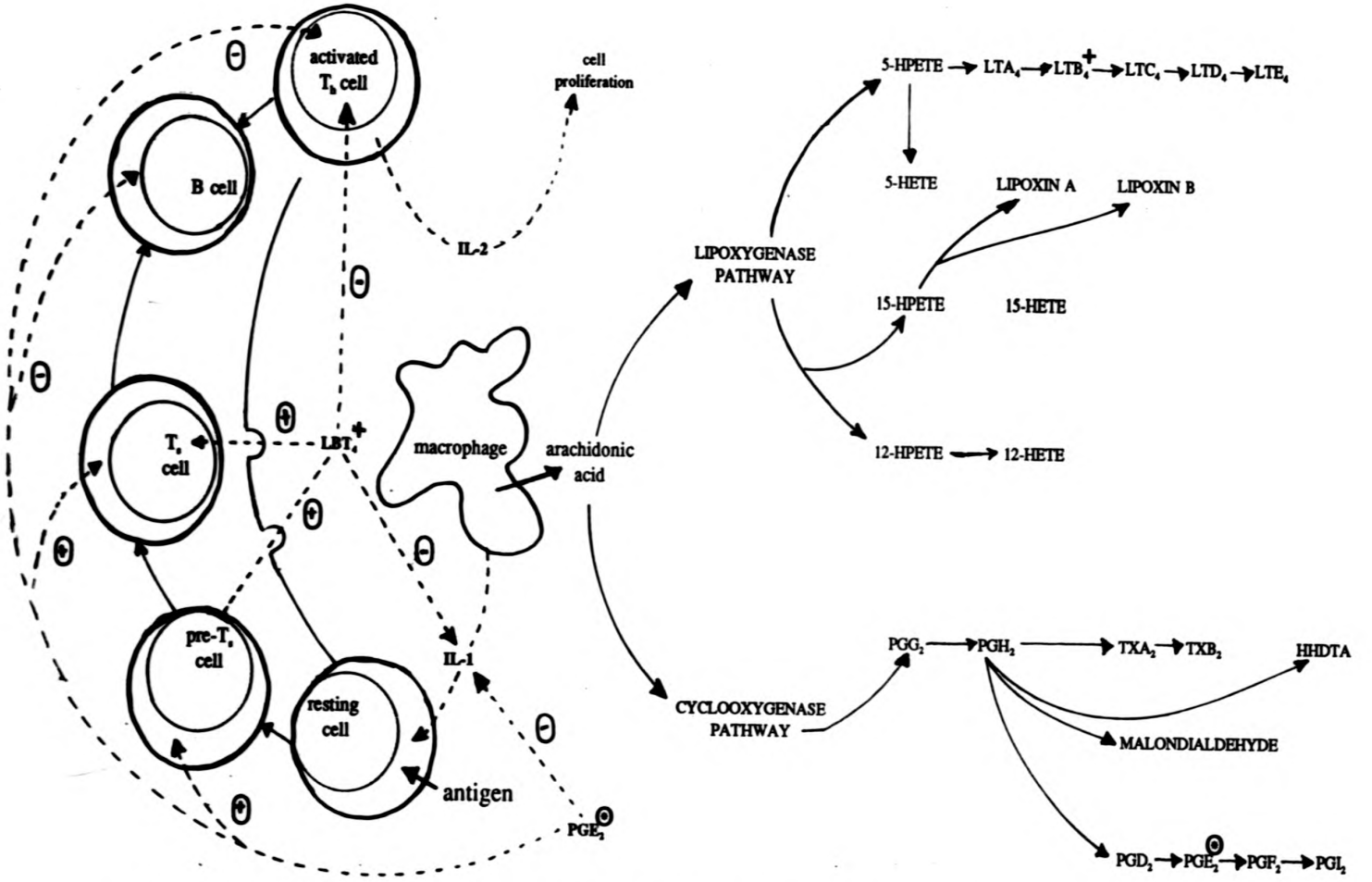


Figure 2.6 The role of PGE_2 and LBT_4 in the feedback loop of the immune response to antigens. Concentrations of PGE_2 and LTB_4 $>10^{-9}$ M inhibit activation of T_H cells, Kinsella *et al.*, 1990 **Abbreviations:** HPETE-hydroperoxyeicosatetraenoic acid, HETE-hydroxyeicosatetraenoic acid, HPEPE-hydroperoxyeicosapentaenoic acid, HEPE-hydroxyeicosapenteanoic acid, LT-leukotriene, PG-prostaglandin, TX-thromboxane, T_s-suppressor cell, IL-interleukin, HHDTA-hydroxy-heptadecatrienoic (+) positive feedback, (-) negative feedback

Table 2.2: Putative effects of PGE_2 and lipoxygenase products on the mammalian immune response.

Some reported effects of PGE_2 on immunological function	Very low concentrations ($<10^{-9}$M) required for normal immune and T cell function differentiation High concentrations ($<10^{-8}$M) are immunosuppressive increases intracellular c-AMP, inhibits protein kinase suppress macrophage and lymphocyte mediated reactions lymphocyte (T and B cell) proliferation lyphocyte migration natural killer cell activivty clonal proliferation and E-rosette formation reduce lymphokine production (IL-1 and IL-2) decreased antibody IgG production reduce mitogen induced proliferation regulate macrophage generation of active oxygen affect tumoricidal potency of macrophages reduce macrophage proliferation inhibits release of lysosomal enzymes from granulocytes reduce production of lymphocyte activating agents modification of macrophage membrane receptor function facilate excess production of rheumatoid factor
Some putative effects of lipoxygenase products on the immune system	LBT_4:- At low concentrations ($<10^{-9}$M) it induces IL-2 synthesis acts via C-GMP -activates leucocytes (PMN, suppressor T cell -induces interferon synthesis by murine cells At concentrations above $<10^{-9}$M it induces cell aggregation causes cell migration, chemotaxis, and lysosomal enzyme release -enhances adherence of lymphocytes to endothelium -suppress PHA mitogenesis of lymphocytes and E-rosette formation -inhibit antibody formation (10^{-7}M) and activivty of helper cells 5-HETE -stimulates guanylate-cyclase and mitogenesis

Abbreviations: HETE-hydroxyeicosatetraenoic acid, LT-leukotriene, PHA-phytohaemagglutinin, PMN-polymorphonuclear leucocytes, IL-Interleukin.

Kinsella, *et al.*, (1990) Nutr.(Suppl) 6:24-44

response by inducing immunosuppressive PGE_2 (Remrick *et al.*, 1987). The complexity of these interactions is reviewed by Goodwin (1989), who postulates that PGE_2 may activate suppressor cells, which in turn limit IL-2 production from activated T cells.

The inhibition loop can be manipulated by dietary fatty acids (Hwang, 1989). Figure 2.4 shows the distribution of dietary fatty acids in the "Western" diet where the consumption of vegetable oils has increased to 1-4 % levels higher than recommended (Johnston, 1985). Since high 18:2(n-6), 20:4(n-6) and prostaglandins of the 2 series are seen to depress the immune response by producing high levels of immunosuppressive eicosanoids, an argument exists for reducing (n-6) PUFA intake and fat level generally. Dietary (n-3) PUFA can displace 20:4(n-6) from the " fatty acid acyl-CoA pool" and membrane phospholipids and thus reduce the 20:4(n-6) products of lipoxygenase and cyclooxygenase. The eicosanoids resulting from the 3-series PGs, and 5 and 7-series LTs have attenuated activities to those of the 2 series.

2.6.3. The role of PUFAs in the activation and activity of lymphocytes and macrophages

Cell membranes, which are in a fluid state, consist of lipids and integral and peripheral proteins (Singer and Nicolson, 1972). Some of these proteins require a particular micro-environment for optimal activity, for example insulin receptors, membrane 5'-nucleotidase and Ca-ATPase (Spector and Yorek, 1985), and changes to this environment alters their function (Bell *et al.*, 1986). Membrane fluidity also effects the arrangement of the cell surface receptor molecules, important in lymphocyte activation. Lymphocytes alter their total lipid content, and their membrane fatty acid and lipid class

composition in response to changes to dietary fatty acids *in vivo* and exogenous fatty acids *in vitro* (Cinader *et al.*, 1983; Erickson *et al.*, 1983; Erickson, 1986). Diet modification effects membrane dynamics in a similar way to those described for temperature in Section 2.4. As the PUFA:SFA ratio within the cell membrane decreases, so does membrane fluidity which has been assessed in the lymphocyte membrane by several methods. One commonly reported in the literature is the depolarization of the membrane probe 1,6-diphenyl 1,3,5,hexatriene (Klausner *et al.*, 1980), but critics of this technique think the probe might perturb the membrane, or only reflect the fluidity of the micro-environment of the probe.

Lymphocytes are activated by two signals: the binding of antigen/mitogen to the appropriate receptor which promotes the G1 phase of the cell cycle, and the binding of IL-1 which promotes the S phase. In the S phase, the cell divides and produces IL-2. Subsequent daughter cells do not require mitogen for activation, only IL-2 (Hadden and Coffey, 1987). For lymphocyte activation to proceed, antigen is capped on the membrane and is then internalized, although the formation of a cap is not necessary in T lymphocyte activation (Hadden, 1988). Figure 2.7 illustrates early events associated with lymphocyte activation. A number of membrane changes occur within the first few minutes which are thought to alter membrane fluidity and thus cell function. When intracellular signalling occurs, the composition of the membrane phospholipids, fatty acids and cholesterol change (Mertin and Hunt, 1976; Ip *et al.*, 1980), and the membrane becomes permeable to ions, particularly Ca^{2+} (Bourguigon and Bourguigon, 1984).

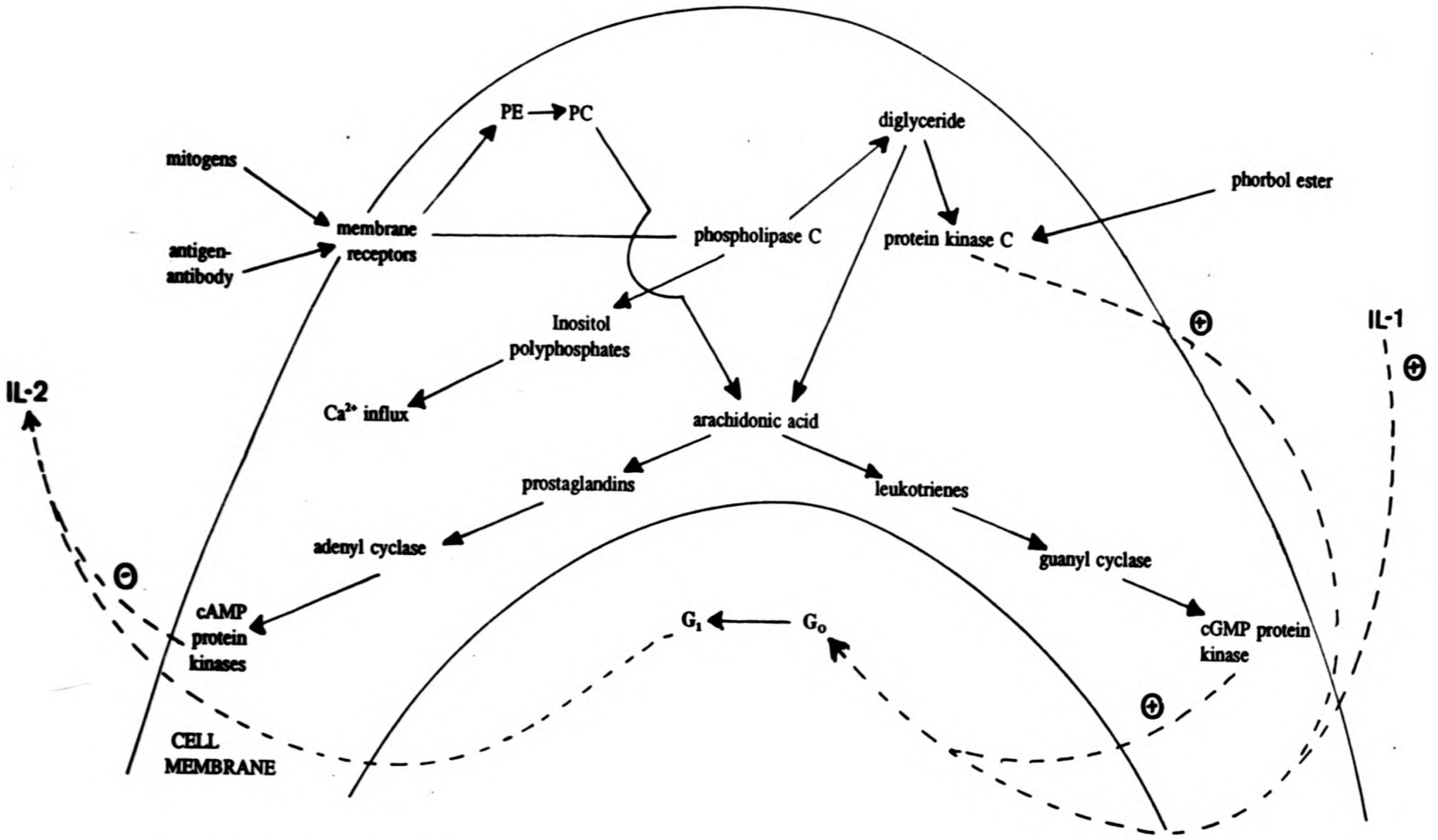


Figure 2.7 A schematic representation of the early events in T cell activation. **Abbreviations:** IL-Interleukin, PE-phosphatidylethanolamine, PC-phospatidylcholine, G_0- prior to cell division, G_1- after mitosis, prior to DNA synthesis

Early activation events include the hydrolysis of PI by phospholipase C to form inositol triphosphate (IP_3) and diglyceride. It is the IP_3 which mobilizes intracellular Ca^{2+}. Arachidonic acid (20:4(n-6)) is liberated from diacylglycerols by diacylglycerol lipase, although 20:4(n-6) is also released from PC, albeit to a lesser extent, by the action of phospholipase A_2. The content of membrane 20:4(n-6) increases at the *sn*-2 position of the phospholipids of lymphocytes shortly after they have been stimulated (Ferber *et al*, 1975) resulting in increased unsaturation within the membrane. The proportion of PC is increased by the conversion from PE through trans-methylation reactions. There is a rapid turnover of PC fatty acids with their complete replacement within 10 hours of stimulation (Meade and Mertin, 1978). *De novo* fatty acid synthesis is low, but turnover is high and the increased PUFAs within the membrane come from intracellular sources (Johnston, 1985). It is interesting to note that cholesterol, which decreases membrane fluidity (Ip *et al.*, 1980), enhances proliferation, whereas the process of activation increases membrane fluidity. By inhibiting cholesterol synthesis with 25-hydroxycholesterol during T cell mitogen stimulation, a decreased proliferation occurs. This can be reversed, or even enhanced with the addition of cholesterol (Heiniger *et al.*, 1978; Ip *et al.*, 1980). Maccecchini and Burger (1977), however, showed increased stimulation with decreased membrane fluidity. Cholesterol is an important structural component to cell membranes, and a prerequisite for blastogenesis (Imbar and Shinitzky, 1974; Heiniger *et al.*, 1978).

The free fatty acids such as 20:4(n-6), 20:5(n-3) and 22:6(n-3), released from activated lymphocyte membranes, have been postulated as mediators in signal transduction and in macrophage and lymphocyte interactions (Hwang, 1989). It is also believed that

these fatty acids are used by macrophages for the synthesis of eicosanoids. Macrophage themselves have particularly high levels of 20:4(n-6) (25 % of all esterified fatty acids in the macrophage membrane) (Hwang, 1989). Controversy exists over the extent to which lymphocytes can synthesize eicosanoids, if at all, and macrophages and neutrophils are believed to be the main eicosanoid producers. The eicosanoid products differ depending on the type of stimuli, source of macrophages (i.e anatomical site) and species of animal (Hwang, 1989).

Vitale (1988) suggested possible changes to the T cell function, by the following alterations; (i) changes to cyclic adenosine 3'5'-monophosphate concentration, (ii) a decrease in receptor spatial configuration, (iii) macrophage function or (iv) decreased cholesterol synthesis.

2.6.4. The importance of diet in the immune response of fish

The relationship between dietary lipid and the immune response in higher vertebrates was discussed in 2.6.1 - 2.6.3, but little is known about these influences in fish.

Minerals and vitamins are a prerequisite for active immunity in fish, outlined by Landolt, (1989). Paterson *et al.* (1981, 1985), administered diets of different trace element concentrations (Fe, Cu, Mn, Co, I, F and Ca) to Atlantic salmon and examined the susceptibility to bacterial kidney disease in these fish. He found the lowest incidence of BKD in fish fed high I and F concentrations with the highest incidence in the group maintained on a commercial diet.

Bell *et al.* (1984), carried out a similar study with Mn and Zn in the presence of ascorbic acid, in sockeye salmon, *Oncorhynchus nerka* and found that the survival of fish, inoculated with BKD, was inversely related to dietary ascorbic acid when dietary Mn and Zn levels were low. When levels of Mn and Zn were high, ascorbic acid did not affect survival. The modes of transmission and detection of BKD in these studies, however, were questionable (Landolt, 1989).

Vitamins A, B, C and E have all been demonstrated through dietary studies to be beneficial to immune function in fish. A significant increase in protective immunity was seen in fingering chinook salmon, *Oncorhynchus tshawytscha*, when immunized with heat treated *Vibrio anguillarum* after being fed high concentrations of pyridoxine (20-40 mg kg^{-1} feed) in a high protein diet (65 %). This was not true if similar concentrations of pyridoxine were fed with a low protein diet (35 %) (Hardy *et al.*, 1979). In channel catfish infected with *Edwardsiella ictuluri* and fed diets containing different amounts of ascorbic acid, the vitamin C-deficient fish experienced 100 % mortalities, while fish on a high dietary level of ascorbic acid (3000 mg kg^{-1} diet) had no mortalities (Li and Lovell, 1985). After immunizing with the bacteria, fish on the high ascorbic diet produced significantly higher antibody titres. It was also noted that ascorbic acid deficiency reduced phagocytosis. Hardie *et al.* (1991) observed a significant increase in the mortality rates of vitamin C-depleted Atlantic salmon, when challenged with *Aeromonas salmonicida*. Serum complement levels of this group were lower than those on the control diet, while high levels of dietary vitamin C elevated serum complement levels. They similarly observed a significant increase in the mortality rates of vitamin E depleted Atlantic salmon when challenged with *Aeromonas*

salmonicida and serum complement levels were also reduced in these fish (Hardie *et al.*, 1990).

In rainbow trout on a reduced α-tocopherol diet, a significant decrease in CMI mediated MIF assays and plaque forming assays (PFA) occurred, as well as a decreased antibody response assessed by haemagglutination and haemolysis of sheep red blood cells (SRBCs) (Blazer and Wolke, 1984a). Non specific activity by phagocytes was also significantly reduced in this dietary group. Another trial undertaken by Blazer and Wolke (1984b), placed rainbow trout either on a commercial diet, or an experimental diet containing 12.5 times more vitamin E and 25 % more vitamin C. Fish on the former diet had a significantly lower immune response than those fed with the latter diet. Similar trends of immune suppression were seen with Atlantic salmon, challenged with pancreas disease (PD) after having been maintained on high/low PUFAs diets with high/low vitamin E levels (Raynard *et al.*, 1989). Vitamin E deficient fish had increased susceptibility to PD. The investigators suggest that the increased occurrence of PD could be due to oxidative stress caused by peroxidation of unsaturated fatty acids.

The importance of vitamin E in cell function lies in its ability to protect membrane fatty acids from oxidation. Unsaturation is necessary for membrane fluidity and cell function as previously discussed. The greater the unsaturation, the larger the cell requirement for vitamin E (Lucy, 1974). It is known that when human lymphocytes are activated, there is a rapid change in membrane fatty acids to a more unsaturated state (Weyman *et al.*, 1977). Nothing is known of these changes in lymphocyte membranes of salmonids during activation.

The role of dietary lipids on the immune response of channel catfish has been investigated by Blazer *et al.* (1989); Blazer and Sheldon, (1989). In one trial, fish were fed a (n-3)/(n-6) fatty acid ratio of 0.41 compared to commercial diets with ratios of 0.16 and 0.1 (Blazer *et al.*, 1989). Fish were maintained on these diets for 42 days and 116 days before immunization with *Edwardsiella ictaluri*, then challenged 14 days later with the bacterium. Macrophages from fish on the high (n-3)/(n-6) fatty acid ratio diet were more capable of phagocytosing latex beads and live bacteria than those on the commercial diet. A more marked effect was seen in the phagocytes ability to kill ingested bacteria. There was no significant difference in the level of circulating antibody between the groups after immunization. In another recent study, Blazer and Sheldon (1989) fed channel catfish on 10 % lipid diets of either menhaden oil, soybean oil or beef tallow for 3 months. Although no difference was seen between the groups in their ability to phagocytose bacteria, a difference was seen in the ability of macrophages from the menhaden oil fed fish to kill the bacteria (i.e. killing levels of 44.4 % on the beef tallow diet, 45.5 % on the soybean diet and 60.4 % on the menhaden diet). These rates increased for soybean diet macrophages after immunization, even at low temperatures of 18°C (i.e. low temperature for catfish).

The influence of dietary lipids on trout erythrocytes demonstrates both physical and morphological changes within the cell (Leray *et al.*, 1986). Increased saturated fatty acids in the membrane correlated with an increase in the osmotic haemolysis rate. Increased osmotic fragility of erythrocytes occurs in Hitra disease, resulting in haemolysis of the erythrocytes by the causative agent, *Vibrio salmonicida*. Applying this to a clinical situation, Salte *et al.* (1988) investigated the effects of high levels of

dietary (n-3) PUFAs (20:5(n-3) and 22:6(n-3)) on the physical properties of erythrocyte membranes from salmon fed at low water temperatures. They showed reduced membrane fragility and decreased mortalities to Hitra in the high (n-3) PUFAs population. In juvenile Atlantic salmon, Erdal *et al.*, (1991) found that increased dietary (n-3) PUFAs decreased membrane fragility. However, these fish had lower survival rates against *Yersinia ruckeri*, and appeared to be immunocompromised by the high levels of dietary (n-3) PUFAs, possibly due to oxidative stress as described above.

It has also been shown that xenobiotic metabolism in channel catfish was effected by menhaden oil enriched diets (Ankley *et al*, 1989). These fish, compared to fish maintained on soybean or beef tallow enriched diets, had increased monooxygenase and glutathione-S transferase activities. Thus, a fish's defence against pollutants and other xenobiotics may also be influenced by the level of (n-3) PUFA in its diet.

CHAPTER 3 GENERAL MATERIALS AND METHODS

The methodology outlined in this chapter consists of a general description of the techniques used throughout this study. Variations to experimental parameters and design to those described below will be discussed in the appropriate chapters, as will the environmental temperatures of fish prior to any analysis. Buffer formulations are given in Appendix 1 and suppliers of materials are given in Appendix 2.

3.1 FISH HUSBANDRY

3.1.1 Introduction

Stress has been implicated in modulating the immune system of fish, making them more susceptible to pathogens (Ellis, 1981). Stressful operations, such as transport, handling, and anaesthesia were inevitably experienced by the fish throughout the course of the experiments. Serious consideration was therefore given to minimising the stress levels experienced by the fish.

3.1.2 Experimental animals

Experimental fish used in the research included Atlantic salmon *Salmo salar* L. (see Plate 2.1), rainbow trout (*Oncorhynchus mykiss* Walbaum) and African catfish (*Clarias gariepinus* Burchell 1822). Fish were acclimatized for a minimum of 3 weeks before commencing experimentation. Rabbits (New Zealand albino males) were obtained from the University of Stirling animal house.

3.1.3 Tank system

Covered fibreglass tanks containing 150 L of flow through water were used to accommodate the fish (see Plate 3.1). Mains supply water was purified by passing through a Water Treatment Service (WTS) 24 sand filter, a WTS 24 organic scavenger with a connected brine tank, and then through a charcoal filter (WTS, Cumbernauld, Scotland). The charcoal filter was backwashed once a day. Chlorine levels of the purified water were monitored daily and did not exceed acceptable levels. Water circulated through tanks at a flow rate of 1.5 L min^{-1} and was aerated by compressed air delivered through pumice air stones. Water temperature followed seasonal fluctuations and illumination was set at cycles of 13 hours of daylight (provided by banks of 40 W fluorescent tubing), 11 hours of darkness.

3.1.4 Maintenance of fish

Stock fish were maintained on Ewos diets. The feeding schedules of both stock and experimental fish complied with Ewos' recommended daily feeding guide (Table 3.1), and took into consideration the size of the fish, with respect to pellet size and composition. Water temperature and fish weight dictated the daily amounts of diet. The biomass of fish was determined every 4 weeks and the daily rations of the fish were adjusted according to Table 3.1. Further details on the diets and the feeding schedule can be found in Section 3.6 and 5.3.1

Plate 3.1 Layout of tank system used to maintain fish

Table 3.1

Ewos' recommended daily feeding guide for Atlantic salmon

3.1 ATLANTIC SALMON

DAILY FEEDING GUIDE

Figures give the feeding rate in per cent of body weight per day
(i.e. the weight of feed in kgs per day for each 100 kg of fish)

	FRESH WATER PHASE						SEA WATER PHASE				
Fish Weight	- 0.5g	0.5g - 1.2g	1.2g - 5.0g	5.0g - 12g	12g - 40g	40g - Transfer	Transfer 120g	120g 350g	350g - 1,000g	1,000g - 3,000g	3,000g+
	STARTER FEED					GROWER FEED					
Feed Size / Water Temperature in °C	0	1	2	3	4	2.5 mm	2.5 mm	4 mm	6 mm	9 mm	12 mm
2	1.4	1.0	0.7	0.5	0.3	0.3	0.6	0.4	0.3	0.2	0.2
4	2.7	1.9	1.2	0.9	0.6	0.5	1.1	0.9	0.6	0.4	0.4
6	4.1	3.0	1.9	1.5	1.0	0.8	1.8	1.3	1.0	0.6	0.6
8	5.3	3.7	2.4	1.8	1.3	1.0	2.3	1.7	1.3	0.8	0.7
10	6.0	4.2	2.8	2.1	1.4	1.1	2.6	1.9	1.4	0.9	0.8
12	6.3	4.4	2.9	2.2	1.5	1.1	2.7	2.0	1.5	1.0	0.8
14	6.5	4.5	2.9	2.2	1.5	1.1	2.8	2.0	1.5	1.0	0.8

NOTE: Following transfer to sea water, fish will feed more heavily after acclimatisation. This table is designed for our expanded and fry feeds. Farmers using the pelleted feed should increase feeding levels by 15%. Amounts given are only a guide and should be varied according to individual circumstances.

The size codes on grower feeds will change from 1/3/1989, and the above codes are the new codes applicable after that date. The size code for grower feeds (pellets) now correspond to the nominal pellet diameter in mm.

The pelleted diets were fed to fish at 15 % higher levels than indicated in Table 3.1 since the experimental diets were pelleted as opposed to expanded.

3.1.5 Disease prevention of fish

Tank cleanliness was an important factor in disease prevention. Although a flow through water supply removed excess diet and debris, stand pipes had to be cleared daily, and tank walls wiped weekly. Fish needed periodical treatment with chloramine T or malachite green, especially during the summer months when the water temperature was higher. Both chemicals were given as bath treatments at a dose of 2 ppm. Fish were starved for 24 hours prior to treatment. Chloramine T was used as a general bactericidal treatment and malachite green was used for fungal infections.

3.2 TISSUE AND CELL ISOLATION

3.2.1 Introduction

It is possible to isolate leucocytes from a number of haemopoietic tissues in salmon; spleen, kidney, thymus and blood. The characteristics of the cell populations found in these tissues have been described in Chapter 2. Blood offers the most convenient method of separating erythrocytes and leucocytes, and wherever possible, was used as the source of cells. If insufficient cell numbers were obtained from blood, leucocytes from other tissues (mainly the kidney) were pooled. Cells from different fish were not combined in tissue culture, so as to prevent an allogenic mixed leucocyte reaction. They were, however, pooled for some of the fatty acid analysis and this will be signified where applicable.

3.2.2 Fish anatomy

The major immunocompetent tissues of Atlantic salmon used in the isolation of leucocytes, include blood, spleen, thymus and anterior head kidney (Tatner, 1990a). Plate 3.2 illustrates the position of these tissues in relation to each other. The thymus, a paired gland, lies directly beneath the operculum. The kidney is located along the length of the backbone and consists of head and trunk kidney. Only the anterior kidney was used, since it is this region of the kidney which contains the majority of the immunocompetent cells (Ellis, 1977).

3.2.3 Blood, tissue and serum isolation

Blood was collected from the caudal vein. Other areas, such as the gill arch vein or the heart, could have been used for this purpose, but the caudal vein proved the easiest region for blood extraction. It is found below the spinal column and its best access is from the region behind the anal fin. If fish were not to be sacrificed, care was taken during the procedure so as not to damage the spinal nerve which lies between the spinal column and the caudal vein. Fish which had been fasted for 24 hours, were anaesthetised with benzocaine (ethyl-*p*-aminobenzoate) by transferring into a bucket containing a 2.5 ml saturated ethanoic solution of benzocaine in 10 l of water (Lied *et al.*, 1975). The fish were steadied by wrapping in a paper towel, and the blood was withdrawn using a 23 gauge needle. Care was taken so as not to introduce air bubbles into the syringe, as this promotes clotting. The volume of blood obtained was 0.3 - 1.0 ml depending on fish size. The blood was immediately diluted 1 in 4 (v/v) with Hanks buffered saline salts (HBSS), containing 20 international units (I.U.) ml^{-1} heparin (ammonium salt). It was gently mixed and kept on ice. If serum was required,

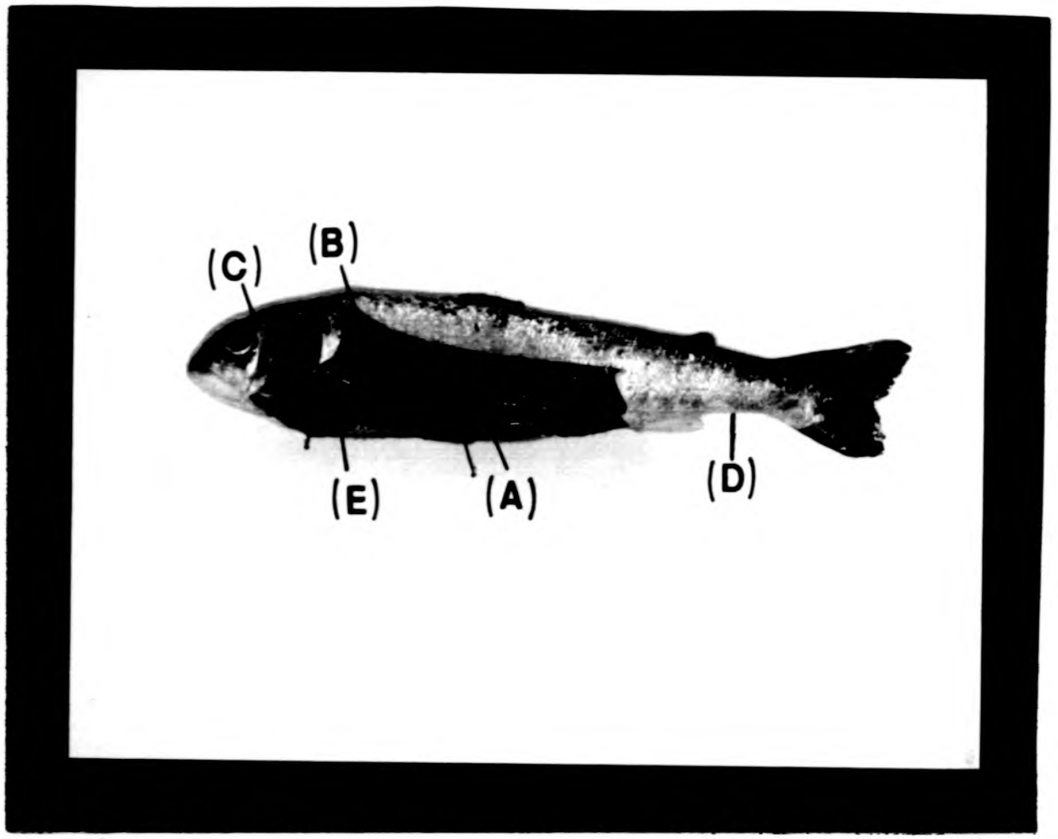


Plate 3.2 The position of major immunocompetent tissues of Atlantic salmon used in leucocyte isolation

(A) spleen; (B) headkidney; (C) thymus; (D) caudal vein; (E) liver

the blood was left undiluted, without heparin.

Removal of the thymus, spleen and kidney were carried out after severing the spinal cord. Procedures were carried out as aseptically as possible and the body cavity was carefully opened to prevent rupture of the alimentary canal. Tissues, placed in 4 ml of HBSS with 20 I.U. ml^{-1} heparin, were teased through a 100 µm nylon mesh to form a cell suspension, which was then kept on ice.

To prepare serum, blood was allowed to clot overnight at 4°C. The clot was detached from the sides of the sample tube with a pasteur pipette, and the serum was separated out by centrifugation at 13,000 g in a micro-centrifuge (Microcentaur, MSE) for 7 minutes. The serum was aliquoted and stored at -20°C.

3.2.4 Leucocyte isolation

Leucocytes were isolated from diluted blood and tissue suspensions by using density gradient centrifugation. The gradient chosen for separation was lymphocyte separation medium (LSM), an aqueous solution consisting of Ficoll and sodium metrizoate at a density of 1.077+0.001 gml^{-1} (Pettey and M^{c}Kinney,1983). Other gradients which have been applied for this purpose are discussed by Blaxhall (1981, 1985) and Rowley *et al.*, (1988); Rowley (1990).

Cell suspensions or diluted blood were layered on to 5 ml of precooled LMS, taking care not to disturb the interface. Samples were centrifuged (MSE 3000i chilspin, MSE., with swing-out rotor) at 2,000 g for 40 minutes. The leucocyte band, termed the

"buffy" coat, was found at the interface of the LSM and the medium, and contained predominately lymphocytes, granulocytes and thrombocytes, while the erythrocytes were pelleted at the bottom of the tube. If the red blood cell (RBC) contamination was greater than 2 %, the cells were replaced onto the LSM. The viability of the buffy coat was assessed with trypan blue and found to be 97.3 % ± 1.1 (n=3 fish)(see Section 3.2.6 for trypan blue exclusion). The buffy coat was washed twice with HBSS, at 1,000g, for 7 minutes. Cells were resuspended in Leibovitz L-15 medium (L-15) containing 2 mM glutamine, potassium benzyl-penicillin/streptomycin sulphate (pen/strep), 100 I.U. of each ml^{-1} and foetal calf serum (FCS) (0.5 -10 % depending on culture system).

3.2.5 Macrophage isolation and macrophage monolayers

A headkidney cell suspension was prepared by the method described in Section 3.2.3. Cell suspensions were placed onto previously prepared 34 % / 51 % Percoll gradients (Secombes, 1990). The 51 % fraction was prepared by mixing 5.1 ml of Percoll with diluted buffer (1 ml of x 10 HBSS and 3.9 ml distilled water). The 34 % fraction was carefully layered onto the 51 % fraction and then the cell suspension was layered onto the 34 % fraction taking care not to perturb the gradient. Macrophage separation resulted from the centrifugation of the gradient at 400 g for 25 minutes at 4°C. The band at the interface of the 34 % / 51 % fractions was enriched with macrophages and was further enriched by adhering washed macrophages from this band onto plastic. The cell concentration was adjusted to 2 $x10^7$ viable cells ml^{-1} in L-15 medium containing 0.1 % FCS, 2 mM glutamine and 100/100 I.U. ml^{-1} pen/strep. Concentrations of FCS greater than 0.1 % impeded the adherence of macrophages to plastic (Secombes, 1990). Cell suspensions (100 µl $well^{-1}$) were plated out in wells of a 96 well flat bottomed

microtitre plate. After two hours at 15°C, nonadhering cells were removed by two washes with L-15 medium. It was important to warm the medium to 15°C before washing, since cold medium facilitates the detachment of adherent cells. The resultant macrophage monolayers were fed with 100 µl of L-15 with 5 % FCS, 2 mM glutamine and 100/100 I.U. ml^{-1} pen/strep and maintained at 15°C in a humid atmosphere. Monolayers were used on the second day for enzyme assays and phagocytosis, but were cultured for longer when incubated with fatty acids.

3.2.6 Cell counts and cell viability

Measurements of cell counts were made using an improved Neubauer haemocytometer. Erythrocytes or leucocytes were diluted in HBSS, to give an approximate cell concentration of 200 cells in 4 large squares. The chamber under the coverslip was filled with the cell suspension, and the cells were allowed to settle for two minutes before counting all the cells in the four large squares. Cells which touched the upper and left-hand border were included in the count, while those on the lower and right-hand border were excluded. The average cell number was found per large square.

$$\text{cells } ml^{-1} = (\text{average cell number per large square}) \times \frac{1}{\text{dilution}} \times 10^4$$

The exclusion of trypan blue by viable cells was used to determine cell viability. Before use, 4 parts of 0.2 % trypan blue (aq) (w/v) was mixed with 1 part 4.25 % NaCl (aq) (w/v). The solution was added in equal volume to the cell suspension. A haemocytometer was used to count viable (unstained) and non-viable (stained) cells. It was important to count the cells within 3 minutes, since all cells would have

eventually taken up the dye.

3.2.7 Separation of T and B-like cells

Populations of T and B-like cells were separated using the panning method described by Sizemore *et al.*, (1984). Wells of a 6 well tissue culture plate were coated with 4 ml well^{-1} of monoclonal ascites (α-salmon IgM, clone 2G9, kindly provided by the Foundation of Applied Research at the University of Tromso, Norway), diluted 1/100 in bicarbonate buffer and incubated overnight at 4°C. The plate was allowed to adjust to room temperature before it was washed 3 times with phosphate buffered saline (PBS). Nonspecific binding sites were blocked with 10 % FCS in PBS, (4 ml well^{-1}) for 3 hours at room temperature. After washing the plate as described above, cell suspensions prepared as in Section 3.2.4 were added to the wells and the plate was incubated for 3 hours at room temperature, in a humidified chamber. The panning was repeated with the nonadherent fraction of the cell suspension. The adherent cells (B-cells) from the two pannings were pooled after dislodging them from the plastic into HBSS with a rubber policeman.

3.3 TISSUE CULTURE

3.3.1 Introduction

All subsequent procedures in this section were carried out in a laminar air flow cabinet using sterile materials. Numerous reports of fish cell culturing can be found in the literature and reported culture systems vary, depending on the fish species and the tissue being cultured (Faulmann *et al.*, 1983; Blaxhall, 1985; Tocher *et al.*, 1988; Tocher and

Dick, 1990a).

An atmosphere of CO_2 was unavailable for maintaining the cell cultures; the buffering of the tissue culture medium had, therefore, to reflect this. With insufficient buffering, most media become too alkaline (the optimal pH range for maintaining fish leucocytes is pH 7.2 - 7.4). L-15 does not require a CO_2 buffering system since it is buffered by free amino acid bases, instead of sodium bicarbonate. The addition of hepes (4-[2-hydroxyethyl-1-piperazine-ethanesulfonic acid]) to medium also acts as a strong buffer, both in the presence of air or CO_2. Blaxhall (1985), examined some of the factors influencing fish lymphocyte cultivation. He found that a CO_2 atmosphere of 5 %, required by mammalian cells, was inhibitory to fish leucocytes, whilst an atmosphere of air or 0.5 % CO_2 appeared optimal for fish lymphocytes.

3.3.2 Tissue culture medium and cell growth

Before commencing experimentation, a suitable culture medium for the *in vitro* culture of fish leucocytes was determined. The ability of either L-15, or minimum essential medium modified with Earles salts (EMEM), without sodium bicarbonate, to sustain Atlantic salmon headkidney leucocytes in culture for 10 days was examined. Each test medium contained glutamine at 2 mM, and pen/strep at 100/100 I.U. ml^{-1}, with and without hepes (20 mM), and either 2-mercaptoethanol (2-ME) ($5x10^{-5}$M), fish serum (2 % v/v), or foetal calf serum (2 % v/v and 10 % v/v). Serum-free medium (SF-1) containing glutamine at 2 mM and pen/strep at 100/100 I.U. ml^{-1}, was also tested. This medium was manufactured with hepes.

Leucocytes from Atlantic salmon headkidney, isolated according to Section 3.2.4, were resuspended at 1 $x10^6$ ml^{-1} cell in the various buffers, and plated out at 0.1 ml $well^{-1}$ in 96 well round bottom microtitre plates. The plates were incubated at 15°C in a humidified chamber. Cells were examined daily for 10 days, using an inverted microscope (Telaval 3, Jena). The decision of which culture medium to employ was based on the appearance of the cells in culture rather than on viability counts, (viability counts were higher than expected in wells where the appearance of cells were poor, possibly due to dead cells rupturing and not being included in the viability count). L-15, containing glutamine (2 mM), pen/strep at 100/100 I.U. ml^{-1} and 10% FCS, was considered the most suitable system for the *in vitro* work and was used throughout, unless indicated otherwise (Table 3.2).

3.3.3 Antigen proliferation assays

Antigen proliferation assays were carried out according to Tatner, (1990b). Headkidney leucocyte suspensions were prepared as reported in Sections 3.2.3 and 3.2.4. Washed cells were resuspended to concentrations of 0.5 $x10^7$ ml^{-1} in L-15 and additives. Cells were cultured in triplicate (0.1 ml $well^{-1}$) in a round bottom 96 well microtitre plate. Antigens (heat killed bacteria, see below) diluted in L-15 and additives were added to the wells at 0.1 ml $well^{-1}$. Controls of cells without antigen were also set up. Concentrations of antigens and incubation temperatures are given in Section 5.4.3.1.

After culturing the cells for three days, they were pulsed with 0.5 µCi ^{3}H-thymidine and incubated for 24 hours before harvesting them onto harvest mats with an automatic cell harvester (Titretex, Flow Ltd.). Harvested leucocytes were solubilized by heating

Table 3.2

Survival of Atlantic salmon headkidney leucocytes in various culture media

Medium	days 1	2	3	4	5	6	7	8	9	10
L-15	++	++	++	+	+	+	+	+	-	-
+ 2-ME	+	+	+	+	+	+	-	-	-	-
+ FCS (2%)	+++	+++	+++	+++	+++	+++	+++	+++	++	++
+ FCS (10%)	+++	+++	+++	+++	+++	+++	+++	++	++	++
+ FS	+++	+++	+++	++	++	++	+	+	+	+
+ hepes	++	+	+	+	+	+	-	-	-	-
+ hepes / FCS (2%)	+++	+++	++	++	++	++	++	++	+	+
+ hepes / FCS (10%)	+++	+++	+++	++	++	++	++	++	++	+
EMEM	++	±	±	-	-	-	-	-	-	-
+ 2-ME	+	-	-	-	-	-	-	-	-	-
+ FCS (2%)	+++	++	++	+	+	+	+	+	-	-
+ FCS (10%)	+++	+++	+++	++	++	++	++	++	+	+
+ FS	++	+	+	-	-	-	-	-	-	-
+ hepes	++	-	-	-	-	-	-	-	-	-
+ hepes / FCS (2%)	++	++	+	+	+	+	+	+	-	-
+ hepes / FCS (10%)	+++	++	++	++	+	+	+	+	+	-
SF-1	+++	++	++	++	+	+	-	-	-	-

Cells examined daily by inverted microscope and cell survival scored accordingly: +++ healthy cells; ++ cells beginning to appear granular; + visible cell death; ± few surviving cells; - all cells dead and lysed. (Mean of 3 fish). Abbreviations: Leibovitz L-15 (L-15) and Minimum Essential Medium, modified with Earles salts (EMEM), without sodium bicarbonate both contained glutamine at 2 mM and pen/strep at 100 / 100 I.U. ml^{-1}, with and without hepes (4-[2-hydroxyethyl-1-piperazine-ethanesulfonic acid]) (20mM), 2-mercaptoethanol (2-ME) ($5x10^{-5}$M), fish serum (2% v/v) or foetal calf serum (2% v/v and 10% v/v). Serum-free medium (SF-1)

the mats at 56°C for 30 minutes with 0.2 ml Soluene-350, in scintillation vials. Once cool, 2 ml of Ecoscint scintillation fluid was added. The vials were mixed thoroughly, and placed overnight at 4°C in the dark, before counting them in a liquid scintillation counter (TRiCarb 2000CA liquid scintillation counter (Canberra-Packard Instruments)). Triplicate samples were firstly converted from counts per minute (cpm) to disintegrations per minute (dpm) by reference to a quench curve, then expressed as stimulation indices (S.I.).

$$\text{S.I.} = \frac{\text{Mean dpm of stimulated cells}}{\text{Mean dpm of control cells}}$$

3.3.4 Binding of fatty acids to bovine serum albumin

Fatty acids were solubilized for use as tissue culture supplements, by binding them onto bovine serum albumin (BSA). Initially, the method of Spector and Hoak, (1969) (see a, below) was used with unlabelled fatty acids, but the method of Street et al (1990) (b, below), proving a simpler procedure, was therefore subsequently adopted for binding [1-^{14}C] fatty acids to BSA.

(a) 10 mg of each fatty acid [18:1(n-9); 18:2(n-6); 18:3(n-3); 20:4(n-6); 20:5(n-3) and 22:6(n-3)] was dissolved in enough hexane to cover 0.33 g of celite, spread as a thin layer in the bottom of a 50 ml conical stoppered flask. The fatty acids were protected from the light by wrapping the flasks in aluminum foil. After removing the solvent with oxygen-free nitrogen, the flasks were desiccated *in vacuo* for 30 minutes. The fatty acid/celite complex was added to 17.2 ml of binding buffer pH 7.4 (see Appendix

which contained 25 mg ml^{-1} BSA. The suspension was flushed with oxygen-free nitrogen and mixed on a magnetic stirrer at room temperature for 60 minutes before centrifuging at 13,000 g for 15 minutes. Particulates were removed by filtering through a 1.2 µm filter. Fatty acid/BSA solutions were filter sterilized with 0.22 µm filters, washing them through with L-15 medium. Samples were stored at -20°C under nitrogen.

(b) 10 mg of fatty acids [18:1(n-9); 18:2(n-6); 18:3(n-3); 20:4(n-6); 20:5(n-3) or 22:6 (n-3)), dissolved in 20 µl of 0.6 NaOH, were incubated in reaction vials with 1 ml of BSA (50 mg BSA dissolved in 1 ml distilled water). This resulted in a mixture with a ratio of 10 moles fatty acid:1 mole BSA. The vials were flushed with oxygen-free nitrogen, and solutions were gently stirred on a hot block with a magnetic stirrer for 30 minutes at 37°C. The pH was adjusted to 7.4 with 1 M NaOH (aq) before diluting the solutions with L-15 medium. Fatty acid/BSA complexes were filter sterilized though a 0.22 µm filter. Samples were stored at -20°C under nitrogen.

Concentrations of bound fatty acid were determined by extracting the BSA/fatty acid complexes with a Folch-Lees wash (see Section 3.8.2), transmethylating them in the presence of an internal standard (23:0) (5 mg ml^{-1}), and relating the peak areas of fatty acids from a gas-liquid chromatography (GLC) chromatogram, to that of the internal standard (see Sections 3.8.2 - 3.8.5).

3.4 HAEMATOLOGICAL EVALUATION AND IMMUNOLOGICAL ASSAYS

3.4.1 Introduction

The following methodology was used to study the ability of dietary lipids to modulate the immune response of the Atlantic salmon. A variety of physiological and mainly nonspecific immunological parameters within the dietary groups were also examined. Additional investigations of specific immunity in these fish are detailed in Section 3.3.3 (antigen stimulation) and Section 3.6.4 (vaccination and bacterial challenge). Some of these assays were also applied to investigations into the effects of exogenous fatty acids on Atlantic salmon leucocyte and macrophage function.

3.4.2 Determination of cell numbers

Erythrocyte numbers were determined on blood which had been diluted 1/5000 in PBS before counting them with a haemocytometer. Blood was diluted 1/20 in white cell counting fluid and leucocytes counted with a haemocytometer. Differential leucocyte counts were performed on Geimsa/ May-Grunwald stained blood smears (Section 3.7.3).

3.4.3 Haematocrit measurements

Heparin coated haematocrit capillary tubes were filled with blood and one end was sealed with cristaseal. The tubes were centrifuged at 10,000 rpm for 3 minutes in a haematocrit centrifuge (Hawksley and Sons Ltd., Lancing, W.Sussex). The haematocrit was expressed as the volume of packed RBC as a percentage of total blood volume.

3.4.4 Serum and cell protein concentrations

Serum and cell protein concentrations were evaluated using a Biorad protein assay. Cell pellets, resuspended in PBS, were first homogenized. A standard curve was prepared from a stock solution of 1 mg ml^{-1} BSA in PBS. Aliquots of 0.8 ml of test serum (diluted 1/100 and 1/1000 in PBS) or 0.8 ml of standard (100 $\mu g ml^{-1}$, 40 $\mu g ml^{-1}$, 20 $\mu g ml^{-1}$, 15 $\mu g ml^{-1}$, 10 $\mu g ml^{-1}$, 5 $\mu g ml^{-1}$ and 0 $\mu g ml^{-1}$) in PBS, were placed in tubes. Dye concentrate reagent (0.2 ml) was added, and the tubes were vortexed prior to incubating at room temperature for 5 minutes. Supernatants were read spectrophotometrically at 595 nm (UV/Visible 4050 Ultraspec II spectrophotometer, LKB Instruments, S.Croyden, Surrey), blanking against PBS/dye solution.

3.4.5 Serum complement levels

Serum complement levels were measured in dietary trial 2 fish by direct haemolysis of SRBCs (Hardie *et al.*, 1990). SRBCs were resuspended to 3 % (v/v) in HBSS and sensitized with a 1/100 dilution of trout anti-SRBC (titre 1/128), (courtesy of C. Finlay, University of Stirling) for 30 minutes at room temperature. No spontaneous haemolysis occurred at this dilution. An 0.5 ml aliquot of sensitized cells was added to 0.5 ml serum (diluted in HBSS to 10, 6, 5, 4, 3, 2 and 1, (%) by volume). Values for 100 % SRBCs lysis were obtained by lysing cells with distilled water, and for 0 % lysis by not adding test serum. Tubes were incubated at room temperature for 60 minutes, then centrifuged at 600 g for 10 minutes. The level of SRBC haemolysis was measured at 541 nm, blanking against the 0 % lysis sample. The volume of complement necessary for 50 % lysis (CH_{50}) was determined from a plot of percentage lysis against volume of serum added.

3.4.6 Kidney and serum lysozyme activities

The lysozyme activity in serum and kidney homogenates from dietary fish in trial 2 (Section 5.4.1.3) was assayed by a plate method developed by Ossermann and Lawlor (1966), and modified by Lie *et al.*, (1986). Assay methodology was as described by Ellis (1990a). Assay plates were prepared by pouring 15 ml of a 1 % w/v agarose solution, dissolved in 0.07 M NaH_2PO_4/Na_2HPO_4 buffer pH 6.2 at 50°C, onto an acetone cleaned, 10 x 10 cm glass plates. Before pouring the gel, 50 $\mu l ml^{-1}$ *Micrococcus lysodeikticus* was added. Once the gel had set, holes of 3 mm diameter were made in the agarose.

Kidneys were homogenized with one part tissue in twenty parts 0.004 M sodium phosphate buffer (pH 6.2) (w/v), while serum samples were diluted one in four. Samples were applied to plates at 9 μl $well^{-1}$. A standard curve was prepared using two fold serial dilutions of a stock solution of hen egg white lysozyme (EWL) at 1.6 μg ml^{-1} in PBS with 1 % w/v BSA. Plates were incubated for 17 hours at 20°C in a wet box and the next day washed for 30 minutes with distilled water. The gel was pressed by applying a 1 kg weight on top of one wet and seven dry filter papers. After drying, the gel was stained with 1.25 % w/v methylviolet solution for 1 minute and Lugol's iodine solution for 15 seconds. It was then destained with ethanol until zones of lysed bacteria could be seen around the wells. The diameters of the zones were measured, and the concentration of lysozyme in each sample was read off the EWL standard curve. The regions of lysed bacteria are proportional to the $\log_2$ in the range 1.6 - 0.1 μg ml^{-1} of lysozyme (Ellis 1990a).

3.4.7 Serum anti-trypsin activity

Serum anti-trypsin activity from dietary trial 2 salmon was measured by the method of Ellis (1990b). Trypsin (20 µl of a 300 µg ml^{-1} solution in 0.01 M Tris / NaCl pH 8.2) was incubated with 0 µl, 20 µl, 40 µl, 70 µl and 140 µl of test serum (see Section 3.2.3 for serum isolation) for 5 minutes, at room temperature. Trypsin activity had been previously titrated against sodium caseinate to 36 caseinase units. Sodium caseinate (2.5 ml of a 2.5 mg ml^{-1} solution in PBS) was added to the tubes before incubating for a further 15 minutes at room temperature. The reaction was stopped by the addition of 1.2 ml of trichloroacetic acid (TCA)(aq) 10 % v/v. Tubes were centrifuged at 2,000 g for 15 minutes and the supernatants were read at 280 nm. Blanks were prepared by mixing TCA and casein, prior to adding the enzyme and serum. Anti-trypsin activity was expressed as 0.001 units of OD_{280} change $minute^{-1}$.

3.4.8 Haemolytic plaque forming assay

Haemolytic plaque forming assay (PFA) was performed in microtitre plates (Kappler 1974). Spleen and kidney leucocytes were isolated from dietary trial 2 fish, two weeks after vaccination (see Sections 3.6.4 and 5.4.3.2). Cells were resuspended at 1 $x10^6$, 1 $x10^5$, 1 $x10^4$ and 1 $x10^3$ ml^{-1} in L-15 with additives and dispensed at 50 µl$well^{-1}$ into flat bottom 96 well tissue culture plates. The microtitre plate was centrifuged for 10 minutes at 250 x g and the culture medium was removed from the wells. Cell pellets were resuspended by vortexing the plate. Labelled SRBCs (see below) were added to the leucocytes at a final concentration of 1 % v/v in L-15 with additives (50 µl $well^{-1}$). Salmon serum had been added to the SRBC at a 1/20 dilution as the complement source. The plates were incubated for 4 hours at 4°C, then placed at 15°C overnight

before examining plates for plaque formation. A control of labelled SRBCs in the presence of complement, but in the absence of sensitized leucocytes was included.

SRBC labelling with antigen:- SRBCs (4 % v/v) were incubated in the presence of heat killed *Aeromonas salmonicida* (Section 3.6.3), at 10 mg ml^{-1} and 2.5 % (v/v) glutaraldehyde in PBS in the ratio of 10:10:1 (by volume) (Kusudo *et al.*, 1991). After one hour at room temperature, FCS (1 % v/v) was added to SRBCs and the mixture centrifuged at 400 x g for 5 minutes. Labelled SRBCs were resuspended to a working concentration of 1 % (v/v) in L-15, 10 % FCS, glutamine, pen/strep.

3.4.9 Macrophage enzyme assays

Macrophage monolayers were set up by the method described in Section 3.2.5 to assess the activity of respiratory burst enzymes from dietary trial 2 headkidney macrophages (Section 5.4.2.2).

(a) Detection of extracellular O_2^- by the reduction of ferricytochrome C.

Assays for the detection of extracellular O_2^- by the reduction of ferricytochrome C are described by Pick and Mizel (1981), and Secombes (1990). Solutions of ferricytochrome C (type III) were prepared by dissolving at a concentration of 2 mg ml^{-1} in phenol red-free HBSS and adding either 1 µg ml^{-1} phorbol myristate acetate (PMA), with or without, superoxide dismutase (SOD) which was used at a final concentration of 300 µg ml^{-1}. Optimal stimulation by PMA occurred between 0.5 - 5.0 µg ml^{-1} after a one hour incubation (Secombes *et al.*, 1988). 1 µg ml^{-1} was subsequently used for assays. The monolayer was washed twice with phenol red-free HBSS before adding 100

100 µl of either solution to the well. The assay was read immediately after being set up with an Enzyme-linked Immunosorbent Assay (ELISA) reader (Dynatec MR 5000 ELISA reader, coupled to a star LC-20 printer), at 550 nm, and read again at 30 and 60 minutes. A solution of ferricytochrome C with added PMA and SOD was used as a zero reference for the reader.

(b) Detection of intracellular O_2^- by the reduction of nitroblue tetrazolium

Assays for the detection of intracellular O_2^- by the reduction of nitroblue tetrazolium (NBT) are described by Rook *et al.* (1985) and Secombes (1990). Solutions of NBT were prepared at 1 mg ml^{-1} in L-15. PMA (1 µg ml^{-1}), or PMA (1 µg ml^{-1}) and SOD at a final concentration of 300 µg ml^{-1}, was added to the solution. The monolayer was washed twice with phenol red-free HBSS before the addition of 100 µl of either solution to the well. The plate was incubated at 15°C for 60 minutes, after which the supernatants were removed, and the cells fixed with methanol. The wells were washed several times with 70 % methanol and allowed to air dry. To dissolve the insoluble reduced NBT, formazan, which resulted, 120 µl of 2 M KOH and 140 µl dimethyl sulfoxide (DMSO) were added to each well, with gentle shaking and the plate was read with an ELISA reader at 610 nm. The reader had previously been blanked against 120 µl of 2 M KOH and 140 µl DMSO.

(c) The detection of hydrogen peroxide by the oxidation of phenol red

The following procedure is outlined by Pick and Mizel (1981) and Secombes (1990). A solution of 0.01 % horseradish peroxidase (HRP) (w/v) was prepared using phenol red-free HBSS, to which had been added 0.02 % (w/v) phenol red and 1 µg ml^{-1} PMA.

PMA, or PMA (1 μg ml^{-1}) and SOD at a final concentration of 300 μg ml^{-1}. Monolayers were washed twice with phenol red-free HBSS before the addition of 100 μl of the HRP solution to the wells. The plate was incubated at 15°C for 60 minutes, after which the reaction was stopped with 10 μl 1 N NaOH $well^{-1}$. The assay was read by a ELISA reader at 610 nm, against a blank of the HRP solution and 10 μl of NaOH.

(d) Determination of macrophage numbers in wells.

The number of macrophages present in the microtitre wells was measured by adding 100 μl of a lysis buffer to the well, and counting the number of liberated nuclei with a haemocytometer. The lysis buffer contained 0.1 M citric acid, 1 % (v/v) Tween 20 and 0.05 % crystal violet in distilled water (Secombes 1990). The absorbances from each of the above assays were adjusted to correspond to a cell number of 1 $x10^5$ cells $well^{-1}$.

3.4.10 Phagocytosis and killing of bacteria by macrophages

The colorimetric assay described by Secombes (1990) from a modification of Peck's method (1985), was used to assess the killing capacity of macrophages (see Section 5.4.2.1). A macrophage monolayer was prepared in flat bottom 96 well, microtitre plates. The wells were washed with L-15 medium before assaying. No bactericidal pen/strep was present in the washing medium.

A bacterial culture of *Aeromonas salmonicida* (strain MT 423), grown to log phase in tryptic soya broth (TSB), was serially diluted from 10^8 bacteria ml^{-1} down to 10^3 bacteria ml^{-1} with the broth (see Section 3.6.3 for bacterial growth). Aliquots of 20 μl from each dilution were added in triplicate to the macrophage monolayer. Sterile PBS

in place of bacteria was used as a negative control. The plates were centrifuged at 150 x g for 5 minutes and incubated at 15°C for 0 and 4 hours. The monolayers were washed three times with L-15 medium, warmed to 15°C. Macrophages were lysed with 50 µl of 0.2 % (v/v) Tween 20 in sterile distilled water. Remaining live bacteria were allowed to grow overnight at 15°C in 100 µl of broth. A control of bacteria in the absence of macrophages was included in the assay as a positive control at this point. The number of surviving bacteria were evaluated by adding 10 µl well^{-1} of a 5 mg ml^{-1} solution of 3(4.5-di-methylthiazoyl-2-yl)2.5 diphenyltetrazolium bromide (MTT) in distilled water. Plates were incubated for 15 minutes at room temperature, before reading them spectrophotometrically at 610 nm. The plate was blanked against broth and MTT.

$$\% \text{ surviving bacteria} = \frac{OD_{610} \text{ for 4 hours incubation}}{OD_{610} \text{ for 0 hours incubation}} \times 100$$

3.4.11 Engulfing of bacteria by macrophages

Headkidney macrophages were isolated as described in Section 3.2.3. The concentration of cells was adjusted to 2 x10^7 ml^{-1} in L-15, glutamine, pen/strep and 0.1% FCS. Monolayers of macrophages were prepared by placing 50 µl aliquots of the cell suspension on to sterile circular cover slips (13 mm diameter). For convenience, these were placed in 24 well tissue culture plates. Macrophages were allowed to adhere for 2 hours at 15°C in a humidified chamber before washing twice with HBSS, warmed to 15°C. Phagocytosis of *Aeromonas salmonicida* (strain MT 004) took place by adding 100 µl of a 1 x10^7 ml^{-1} bacterial suspension (in L-15, 5 % FCS v/v) onto the macrophage monolayer (see Section 3.6.3 for bacterial growth) and incubating at 15°C

in a humid atmosphere, for 3 hours (the bacteria had been previously opsonised with 1/100 of complement deactivated trout anti-*Aeromonas salmonicida*, strain MT 423, for 30 minutes). After the 3 hour period, the macrophages were washed 5 times with PBS, fixed with 90 % methanol, and stained overnight with Giemsa (10 % w/v). The cover slips were destained by immersing 3 times into Gurr's buffer, pH 6.8, then into 90 % ethanol for 30 seconds. Once dry, the cover slips were mounted and examined under oil immersion. Controls of macrophages without bacteria were prepared for a comparison. The number of bacteria engulfed in 200 macrophages was counted in triplicate, and the phagocytic index (Ph.I.) for each dietary group was compared by Student 't' test.

$$\text{Ph.I.} = \frac{\text{Total no. bacteria engulfed by the 200 cells examined}}{200}$$

3.4.12 Production of macrophage activation factor

Assaying for MAF activity was as described by Hardie *et al*, 1990. Suspensions of headkidney leucocytes from dietary trial 2 fish were prepared and leucocytes were isolated on a 51 % / 34 % Percoll gradient. One ml triplicates of cells, resuspended to a concentration of 5 $x10^6$ ml^{-1} in L-15, 50 µM 2-ME, 10 $\mu l ml^{-1}$ Con A and 5 $ng ml^{-1}$ PMA, were placed in the wells of a 24 well plate. The plates were incubated for 3 hours at 15°C. Cells were washed five times with HBSS, before culturing at 15°C in L-15, 10 % FCS, pen/strep and 50 µM 2-ME. After 48 hours, supernatants collected from the wells were added to macrophage monolayers from control fish (i.e. fish on a commercial diet). Supernatants were titrated 1/2 - 1/16 in L-15, 10 % FCS, P/S and 50 µM 2-ME. Monolayers were incubated for 48 hours before examining macrophage

function by the reduction of nitroblue tetrazolium during respiratory burst (see Section 3.4.9 (b)).

3.4.13 Measurement of macrophage inhibition factor

The inhibition of macrophage migration by sensitized leucocytes was measured by the method of Secombes (1986). Headkidney macrophages were isolated from dietary trial 2 salmon 6 weeks after vaccinating the fish with *Aeromonas salmonicida* (Section 3.6.4) and cell concentrations were adjusted to 5 $x10^7$ ml^{-1} in L-15 medium. Haematocrit tubes were filled with the suspension, and sealed with cristaseal. After centrifuging for 5 minutes at 300 x g, the tubes were split at the cell/fluid interface with a file, and fractions containing the cells were placed into wells of a 6 well tissue culture plate. Silicone grease was used to adhere the tube to the plastic. L-15 medium, containing FCS (10 %), pen/strep, glutamine (2mM) and $1x10^7$ ml^{-1} *Aeromonas salmonicida* (to which the fish had been previously exposed) was placed in the wells. Triplicate tubes of both test and controls containing no antigen, were set up for each fish. The radius of cell migration from the capillary tubes, was measured at 24 and 48 hours using a microscope graticule. Plates were inverted for 2 hours to show that the migrating cells were macrophages.

3.5 PREPARATION OF ANTI-SALMON IgM

3.5.1 Introduction

Anti-salmon IgM was prepared for use as a diagnostic agent to determine the antibody titres of fish, especially in the vaccinated groups of the dietary trials, and was also used in the detection of *in vitro* antibodies from the stimulation of leucocytes with antigen. Conjugating the antiserum to HRP removed an incubation step from the ELISA protocol (see Section 3.6.5). The disadvantage of polyclonal anti-salmon IgM, as discussed in Section 2.5.1, is its inability to separate T and B cell populations in fish, since the T-like cells have membrane determinants which will react with the polyclonal serum (Yamaga *et al.*, 1978).

3.5.2 Isolation of salmon IgM

Atlantic salmon serum (2 mls) was precipitated with 22 % (w/v) sodium sulphate. The precipitate was centrifuged at 13,000 rpm for 15 minutes, in a microcentrifuge. The pellet which formed was washed twice with 18 % (v/v) sodium sulphate (aq). The IgM precipitate, dissolved in PBS, was desalted by dialysis over 16 hrs at 4°C, against 2 x 2 l changes of PBS. It was further purified by chromatography using a DE 52 column (Johnstone and Thorpe, 1987). IgM was collected in 1 ml aliquots and the absorbance of each fraction measured at 280 nm. Figure 3.1 shows the elution profile at 280 nm, of purified salmon IgM from DE 52 column. Fractions 3 and 4 were pooled and used to immunize a rabbit. The total protein of the pooled fractions was 8.84 mg. This was based on 1 mg of IgM protein having an absorbance of 1.1 at 280 nm (Johnstone and Thorpe, 1987).

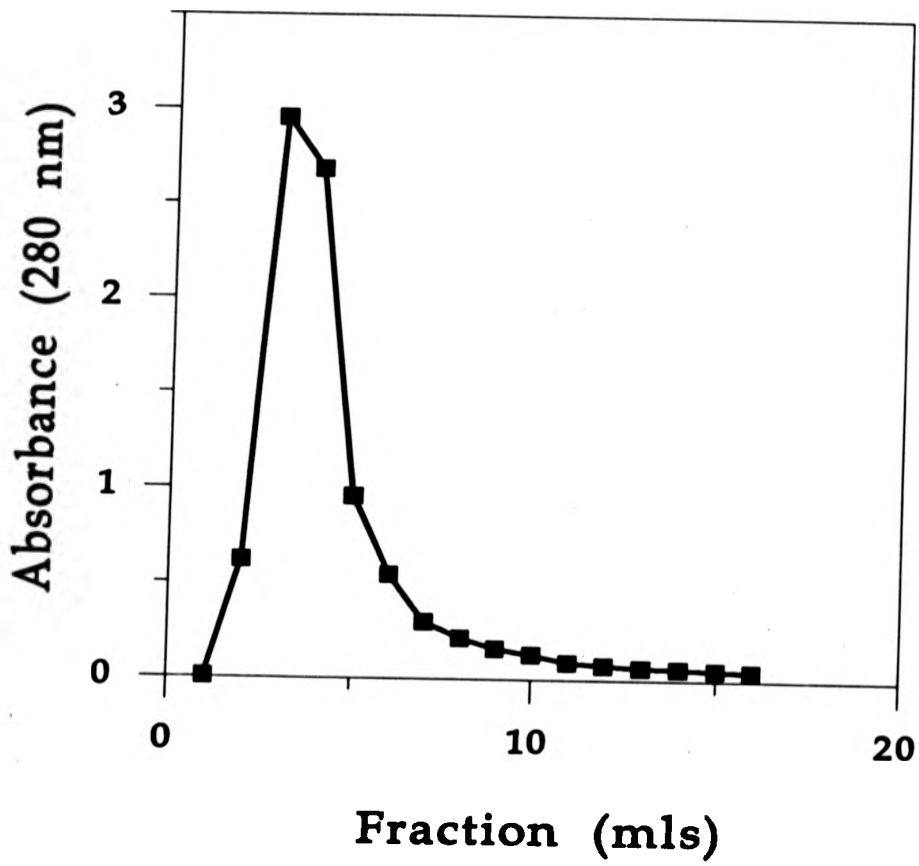


Figure 3.1 Elution profile of salmon IgM from DE 52 column. Fractions 3 and 4 were pooled and used as purified IgM.

3.5.3 Production of a rabbit anti-salmon IgM antibody

A New Zealand albino rabbit (male) was subcutaneously injected (s.c.) with 0.4 ml of a 1:1 (v/v) suspension of Freunds complete adjuvant and 0.68 mg of the purified IgM. Two weeks later, the rabbit was injected s.c. with 0.6 ml of a 1:1 (v/v) suspension of Freunds incomplete adjuvant and 1.02 mg of the purified IgM. A week later, the rabbit was test bled from the ear, and the serum titre evaluated by ELISA (see Section 3.5.4). The serum was found to have a titre of 1/10,000, which was sufficient to give the rabbit a final boost with an intravenous injection of 0.6 mg salmon IgM in 0.5 ml of sterile saline. The animal was bled out by cardiac puncture a week later.

3.5.4 ELISA determination of rabbit anti-salmon IgM antibody

ELISA plates (high protein binding) were coated with 10 $\mu g\ ml^{-1}$ of purified salmon IgM (see Section 3.5.2), which had been acid treated (see Appendix 1) and dissolved in coating buffer at pH 9.6 (100 $\mu l\ well^{-1}$). See Appendix 1 for ELISA buffers. Plates, having been incubated overnight at 4°C, were washed 3 times with PBS containing 0.38 M NaCl and 0.05 % v/v Tween 20. Nonspecific binding sites were blocked by incubating overnight at 4°C with 250 µl of a 3 % w/v casein solution in distilled water. Plates were warmed to room temperature before use and washed 3 times with the above wash buffer. A sample 100 $\mu l\ well^{-1}$ of ten fold serial dilutions of the test serum (diluted with PBS) were added to the wells. The plate was incubated for 60 minutes at room temperature, washed 5 times with wash buffer, and 100 µl of α-rabbit IgG-HRP (diluted to 1/1000 in wash buffer; 10% FCS) was added to the wells before incubating for a further 60 minutes at room temperature. After 5 washes with high salt wash buffer, 100 µl chromogen/substrate was added to each well and incubated for 10

minutes at room temperature. The reaction was stopped with 50 µl well^{-1} of 2 M H_2SO_4 and the plate was read spectrophometrically at 450 nm against a blank of chromogen/substrate and stop solution. Non immunized rabbit sera (1/100) was included on the plate as a negative control and neat monoclonal supernatant, specific for fish IgM (2G9) with anti-mouse IgG-HRP (1/1000) as the secondary antibody, was used as a positive control. The end point of the titre, taken as mean values of duplicate wells with an absorbance 3 times that of the background, was 1/1,000,000. Figure 3.2 shows the titration curve for rabbit α-salmon IgM.

3.5.5 Conjugation of anti-serum to horse radish peroxidase

The rabbit α-salmon IgM, prepared as above, was conjugated to HRP (Wilson and Nakane, 1978). Horse radish peroxidase which had been activated by dissolving 5 mg in 0.1 ml freshly prepared 0.3 M sodium bicarbonate ($NaHCO_3$) pH 8.1. A 0.1 ml aliquot of fluorodinitrobenzene, dissolved in ethanol (1 % v/v solution), was added to the HRP solution, and gently mixed on a magnetic stirrer, protected from the light. One ml of an 0.05 M solution of sodium periodate was added to the HRP solution, and the solution mixed for a further 30 minutes. One ml of ethylene glycol (0.16 M in distilled water) was then added and the solution gently mixed for a further hour. The HRP solution was dialysed at 4°C, against 3 x 2 l changes of 0.01 M $NaHCO_3$ buffer, pH 9.5. The antiserum was precipitated with sodium sulphate as described in Section 3.5.2, but IgG was precipitated with 14 % w/v Na_2SO_4 (Johnstone and Thorpe, 1987). The purified antibody was desalted by dialysing against 3 x 2 l changes of 0.01 M $NaHCO_3$ buffer, pH 9.5, at 4°C. The concentration of the antibody was determined spectrophotometrically at 280 nm, assuming 1 mg of IgG has an absorbance of 1.4. The

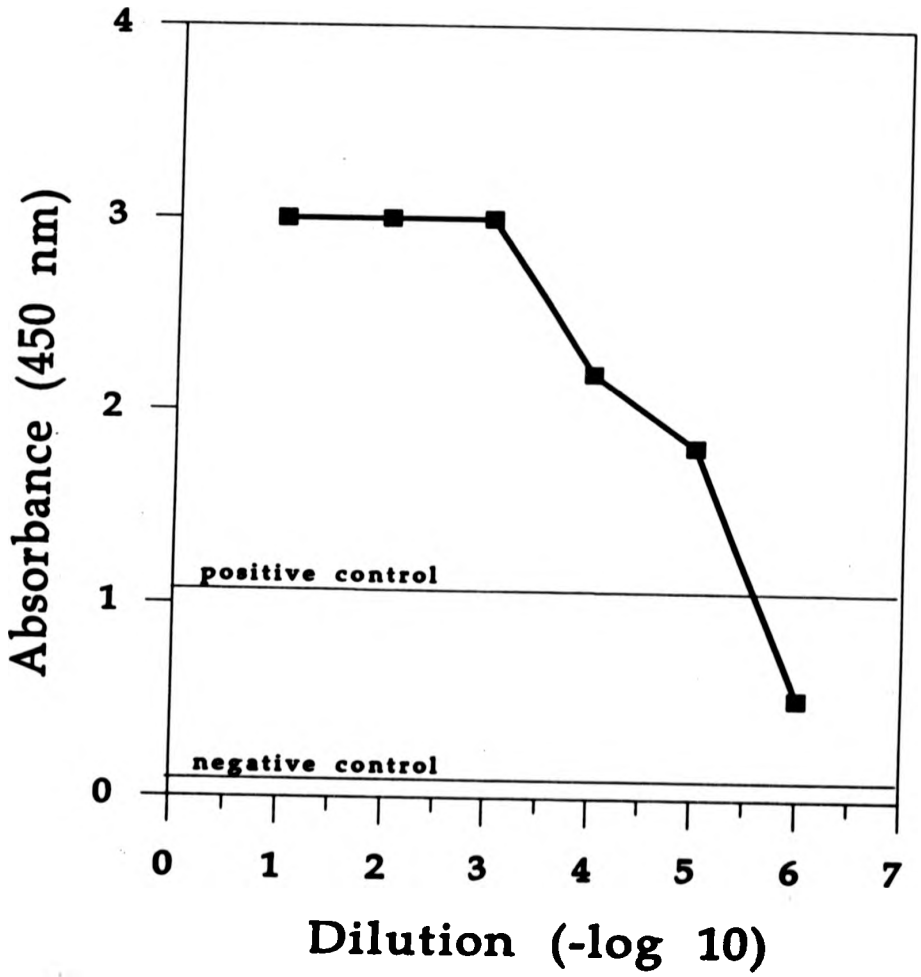


Figure 3.2

Titration curve for rabbit anti-salmon IgM by ELISA after final boost and bleed.

purified IgG was adjusted to 5 mg ml^{-1} in 0.01 M $NaHCO_3$ buffer pH 9.5 and 1 ml was added to 3 ml of activated HRP solution. Conjugation was brought about by gently mixing the HRP solution with the antibody for 3 hours at room temperature. Sodium borohydride (5 mg) was then added and the solution was incubated overnight at 4°C. The conjugate was dialysed against 3 x 2 l changes of PBS (0.02 M phosphate, 0.15 M NaCl) pH 7.0, at 4°C, aliquoted and stored at -20°C. The OD_{409} / OD_{280} ratio should be in the range of 0.3 - 0.7, and for the above conjugate, the value was 0.6. This ratio gives an indication of how much unbound HRP is present. High unbound HRP levels results in higher backgrounds.

The working dilution of the conjugate was determined by titration, in the ELISA described in Section 3.5.4. Ten fold serial dilutions were made in wash buffer/10 % FCS, and added at 100 µl $well^{-1}$. The conjugate was incubated for 1 hour in place of the primary and secondary antibodies mentioned in Section 3.5.4. The remainder of the assay followed the protocol in Section 3.5.4. Taking the end point as 3 times background, the working concentration of the conjugate was 1/300 (Figure 3.3).

3.5.6 SDS-polyacylamide gel electrophoresis (PAGE) of rabbit anti-salmon IgM, serum from the dietary fish and *Aeromonas salmonicida*

The purity of the rabbit anti-salmon IgM was assessed by sodium dodecyl sulfate electrophoresis (SDS-PAGE) under reduced conditions (Pilstrom and Petersson, 1991). In parallel, sera from the dietary fish, and *Aeromonas salmonicida* for use in a western blot (see Section 3.6.6) were also run on the same gel, and will be discussed elsewhere.

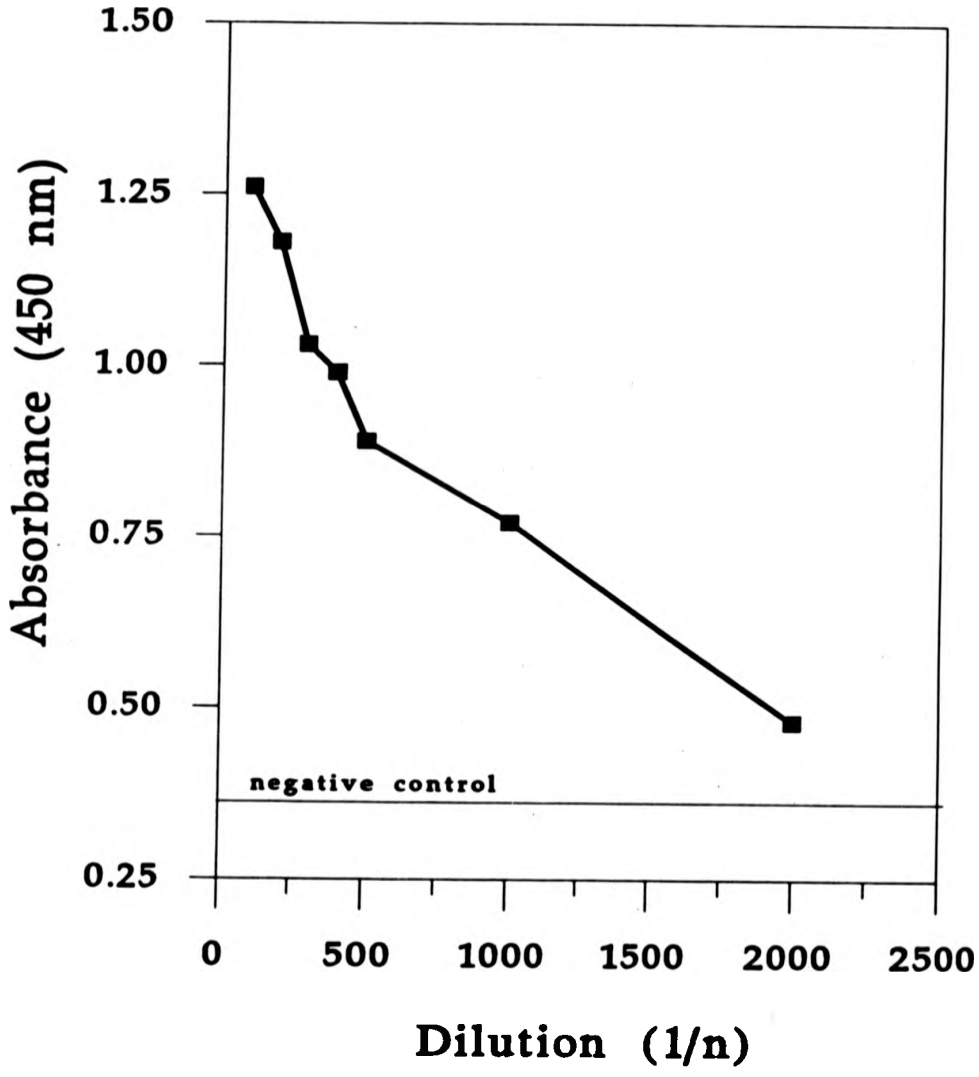


Figure 3.3 Titration curve for rabbit anti-salmon IgM-HRP by ELISA

Electrophoresis of antiserum was carried out with a mini-Protean II electrophoresis system (Biorad Labs. Ltd.). The gel holder was assembled following manufacturers instruction manual. Separating gel was de-gassed *in vacuo* for 15 minutes before adding SDS, ammonium persulphate (APS), and N,N,N,N,-Tetromethyl-ethylenediamine (TEMED)(see Appendix 1). The gel was quickly poured between the plates, taking care not to introduce bubbles, and 200 µl of 0.01 % SDS was layered onto the gel, before allowing it to polymerise overnight. Sample wells were prepared using stacking gel (see Appendix 1). This was also de-gassed for 15 minutes under vacuum. The separating gel was washed with distilled water to remove the 0.01 % SDS before layering on the stacking gel. Sample wells were formed by inserting a well-forming comb into the stacking gel. After polymerisation (60 minutes), samples were added to the wells (5.0 µl of 5.0 µg protein $well^{-1}$ in sample buffer) with a micro syringe. The samples had previously been heated to 98°C for 4 minutes in sample buffer at a concentration of 1 mg ml^{-1}, with the exception of the bacterial sample, which was boiled for 10 minutes in sample buffer at a concentration of 5 mg ml^{-1}. Low weight molecular markers were added to one of the wells. The gel was electrophoresed against gel buffer at 100 V until the dye front (visualized by the addition of bromophenol blue (0.2 % w/v) to sample buffer) was at the bottom of the gel. The gel was stained with coomassie blue (see plate 5.4, lane 5). The major protein bands for the purified salmon IgM (lane 5) under reduced conditions by SDS-page electrophoresis lie around 81 KD and 30-27 KD, which correspond to other quoted values (Pilstrom and Petersson, 1991) for fish immunoglobulin and these bands are of a greater concentration than in the corresponding unpurified antisera (lanes 2-4).

3.6 DIETARY TRIALS

3.6.1 Introduction

Three dietary trials were set up over the course of the study to examine the effects of diet modulation on the immune response of Atlantic salmon. Stocks of fish were allowed to acclimate for three weeks, during which time they were maintained on Ewos No.3 or No.4 crumb diet (Section 3.1.4), before starting them on the experimental diets. Fish were bulk weighed every 4 weeks and their food rations were adjusted according to Table 3.1. Formulation and preparation of diets used in the studies are shown later in Section 3.6.2 and the fatty acid profiles for each batch of diet is given in Table 5.6. The time interval of each study was taken from day zero (fish were weighed, measured, test bled and placed on experimental diets) to day final (when fish were vaccinated, and challenged (trial 1 & 2), or only challenged with bacteria (trial 3)). Automatic feeders were unavailable for the studies, so fish were fed twice daily (9 a.m. & 5 p.m.) to satiation.

Physiological parameters, such as growth, fish condition and haematology of the dietary fish are given in Section 5.2. The condition of cultured fish is often assessed by length-weight data and assumes that heavier fish for a given length are in better condition. In the subsequent work, fish condition was based on the following condition factor (C.F.) index.

$$C.F. = \text{Weight} / \text{Length}^3$$

A hepatosomatic index (Hp.I.) and splenomatic index (Sp.I.) were included in the study.

$$\text{Hp.I.} = \frac{\text{weight of liver (g)}}{\text{Body weight (g)}} \qquad \text{Sp.I.} = \frac{\text{weight of spleen (g)}}{\text{Body weight (g)}}$$

Trial 1:

The period of duration was from the end of May to the beginning of September 1990, lasting 105 days. Three populations of 100 potential S_2 Atlantic salmon parr of average weight (12 g) were placed in tanks. These fish were maintained on batch 1 diets, the lipid profiles of which are found in Table 5.6 and had (n-3)/(n-6) PUFA ratios of 5.4 ±0.8 (high (n-3) PUFA diet), 0.2 ±0.0 (high (n-6) PUFA diet), compared to a commercial diet (Ewos No.4, Table 5.5) with a (n-3)/(n-6) PUFA ratio of 5.4 ±0.4. The first trial examined the effects of dietary fatty acids on the lipid content of the immunocompetent tissues and on *in vivo* immune responses.

Trial 2:

The second trial ran from the end of January to the end of June 1991, lasting 150 days. The starting population of fish were Atlantic salmon parr S_2, with an average weight of 18 g. The fish were divided into 2 tanks each containing 100 fish, and were maintained on batch 2 diets, the lipid profiles of which are found in Table 5.6 with (n-3)/(n-6) PUFA ratios of 5.2 ±0.5 (high (n-3) PUFA diet) and 0.3 ±0.0 (high (n-6) PUFA diet). A commercial dietary group was excluded from this trial since the fatty acid profile and (n-3)/(n-6) PUFA ratio of the commercial diet in trial 1 resembled the high (n-3) PUFA diet.

Trial 3:-

Two populations containing 100 Atlantic salmon parr S_2 with an average weight of 20.1 g, were placed in tanks. Feeding commenced in mid February 1992 and was terminated 112 days from the onset, in mid June 1992. The lipid profiles of batch 3 diets are found in Table 5.6 and had (n-3)/(n-6) PUFA ratios of 5.5 ±1.6 (high (n-3) PUFA diet) and 0.3 ±0.0 (high (n-6) PUFA diet).

All three trials examined *in vivo* immune responses, but trial 1 and 2 additionally compared the effects of different dietary (n-3)/(n-6) PUFA ratios on the lipid composition of immunocompetent tissues and the leucocytes from these tissues. The second trial was also concerned with assessing the time necessary for different dietary (n-3)/(n-6) fatty acid ratios to have an effect on the lipid content of Atlantic salmon blood cells. Fish from trial 2 and trial 3 were also used to examine *in vitro* immune responses.

3.6.2 Preparation of diets

Diets were formulated to resemble a commercially available pelleted diet. The dry ingredients, presented in Table 3.3, were blended together with the oil and antioxidant before adding the choline chloride (10 ml kg^{-1} diet of a 40 % (aq)(w/v) and 5 % - 10 % water). The ingredients were pelleted through a 2.5 mm die of a California steam pelleter (steam was not used), and allowed to dry in air before storing at -20°C. Experimental diets contained 17 % lipid and 51 % protein.

Table 3.3

Ingredients of laboratory prepared diets

Ingredients	Ingredients (g kg^{-1} diet)	
	Trial 1 and 2	Trial 3
LT-Fishmeal[1]	700.0	700.0
Oil[2]	100.0	100.0
Starch[3]	-	138.5
Dextrin[4]	40.0	-
Vitamin mix[5]	10.0	10.0
Mineral mix[6]	47.1	47.1
α-Cellulose[7]	98.5	-
Antioxidant mix[8]	0.4	0.4
Choline chloride	4.0	4.0

[1]Ewos Ltd., Westfield, West Lothian, U.K.
[2]Fosol, Seven Seas, Hull, U.K. Sunflower oil, or Fish oil containing 16% EPA, 10% DHA for batch 1 and 2 and 18% EPA, 12% DHA for batch 3, 2I.U. Vitamin E g^{-1} oil were supplied with each fish oil.
[3]Passelli WA4, Tunnel Avebe Starches, Gillingham U.K.
[4]Sigma Chemical Co.
[5]Vitamin mix contained the following (g/kg mix): α-tocopherol acetate 20; menadoine 1; ascorbic acid 100; thiamine hydrochloride 1; riboflavin 2; pyridoxine hydrochloride 1.2; calcium pantothenate 4.4; nicotinic acid 15; biotin 0.1; folic acid 0.5; cyanocobalamin 0.002; myo-inositol 40; retinyl acetate 0.5; cholecalciferol 0.48; α-cellulose 804.8; N.B. (All vitamins were supplied by Sigma Chemical Co.)
[6]Mineral mix contained the following (g/kg mix): $CaCO_3$ 17.75(Fisons); $CaH_4(PO_4)_2H_2O$ 416.6(BDH); K_2HPO_4 206.0(BDH); $NaH_2PO_4.2H_2O$ 130.0(Fisons); NaCl 66.4(Fisons); KCl 50.0(Sigma); $MgCO_3$ 91.0(Sigma); $FeSO_4.7H_2O$ 30(Fisons); $ZnSO_4.7H_2O$ 4.0(BDH); $CuSO_4.5H_2O$ 1.0(Sigma); $MnSO_4.4H_2O$ 3.6(Sigma); KI 0.2(BDH); $CoSO_4.7H_2O$ 1.0(Sigma).
[7]ICN Biomedicals Inc., High Wycombe, Great Britain.
[8]Antioxidant mixture contained the following: butylated hydroxyanisole 200g (Sigma); n-propyl gallate 60g(Sigma); citric acid 40g(Sigma) dissolved in 1000 ml 11-propylene glycol(Sigma).

3.6.3 Bacteria preparation

Both *Vibrio anguillarum* (strain 7875) and *Aeromonas salmonicida* (strain MT 423) were maintained on tryptic soy agar (TSA) plates at 14°C by subculturing the plates every 3 weeks. Bacterial suspensions were prepared for use as antigen in ELISA and stimulation assays by seeding bacteria into TSB and growing at 14°C until log phase growth was reached, approximately 24 hours later. The cultures were centrifuged at 2,000 g for 10 minutes and the resultant pellets were washed twice with sterile PBS. Bacteria were killed by resuspending the pellet in 10 ml of sterile PBS, in a sterile glass bottle and heating it in a water bath at 60°C for 60 minutes. Sterility was determined by smearing 100 µl of the suspension onto a TSA plate and culturing for 48 hours at 14°C. The concentration of bacteria was measured spectrophotometrically at 610 nm, according to Campbell (unpublished data) (see Figure 3.4).

3.6.4 Vaccination and challenge

Before challenging fish as described below, a preliminary challenge was carried out to determine the bacterial concentrations necessary to produce 80 % mortalities in trial 1 and 2, and 50 % mortalities in trial 3. Fish were starved for 24 hours before anaesthetising and handling for injections and sampling of blood, which were carried out with the minimum of disturbance to the fish.

Effectiveness of vaccination in trial 1 and 2 was assessed by relative percentage survival (RPS).

$$RPS = 1 - \frac{\text{\% specific mortalities in vaccinated group}}{\text{\% specific mortalities in unvaccinated group}} \times 100$$

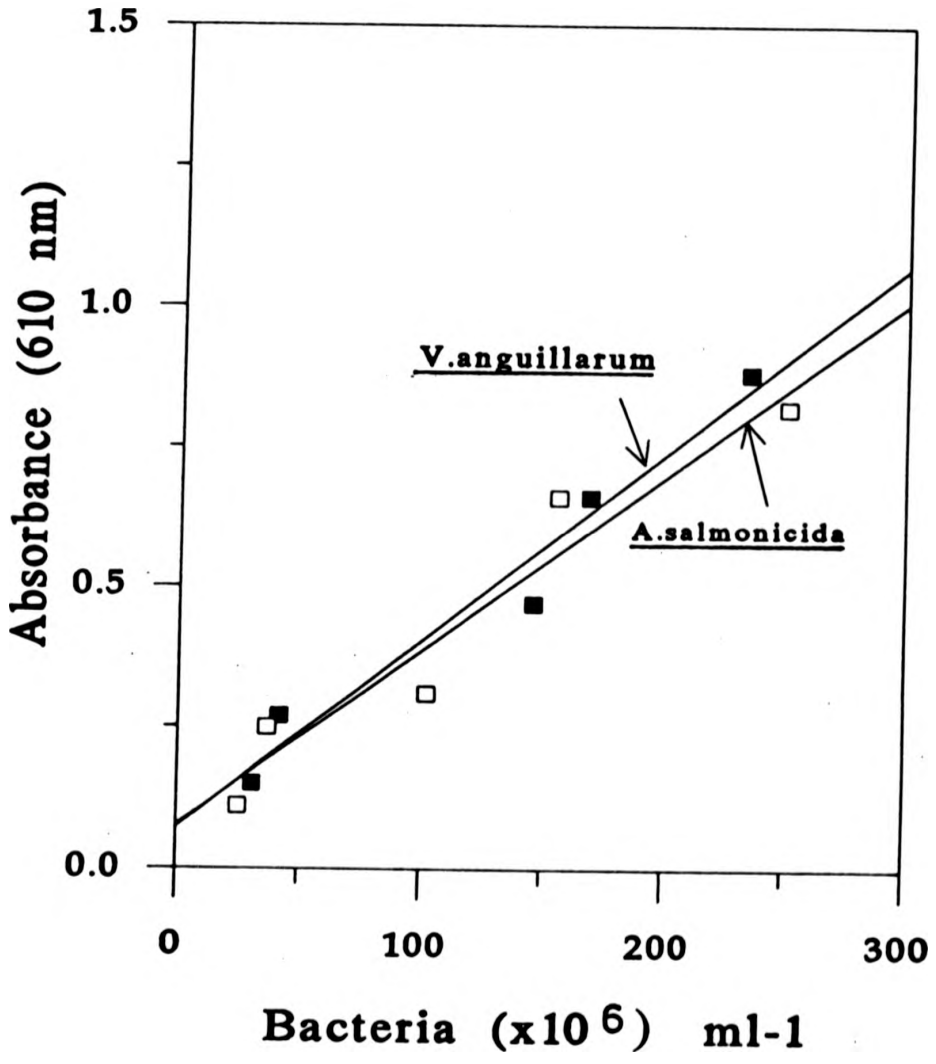


Figure 3.4

Concentration of *Vibrio anguillarum* and *Aeromonas salmonicida* (bacteria ml-1) at 610 nm. (Courtesy of Dr. R. Campbell)

Certain standards are necessary for the vaccination to be considered effective; (a) that at least 25 fish are used in duplicate for each test population, (b) that at least 60 % of the control fish die specifically as a result of the challenge, (c) non-specific death must not exceed 10 % of the test population and (d) the mortality rate of the vaccinated fish must be below 24 % for the test to be considered positive (Amend, 1981). Cumulative mortalities between dietary groups for each trial were compared by a logrank test (Peto, *et al.*, 1977).

Specific mortalities were confirmed by streaking kidney swabs onto TSA plates which were incubated at 14°C for 7 days. *Aeromonas salmonicida* produced a characteristic brown pigment on the TSA plate (see Plate 3.3), but specificity was confirmed by ELISA. Using the protocol described in Section 3.6.5, 1 $x10^8$ ml^{-1} of heat killed bacteria, obtained from the kidney swab, were attached to ELISA plates. Primary antibody for the first dietary trial was a 1/100 dilution of rabbit anti-*Vibrio anguillarum* and for the second dietary trial, a 1/100 dilution of rabbit anti-*Aeromonas salmonicida* was used (both antibodies had been prepared by M.F. Tatner, University of Stirling). The secondary antibody (anti-rabbit IgG-HRP), incubation times and temperatures are as described below. Specific deaths were taken as mean values of duplicate wells which were 3 times the absorbance of background. Plates from trials 1 and 3, and trial 2 contained positive controls of *V. anguillarum* and *A. salmonicida* respectively (coated to the plate), and negative controls were nonimmunized rabbit sera.

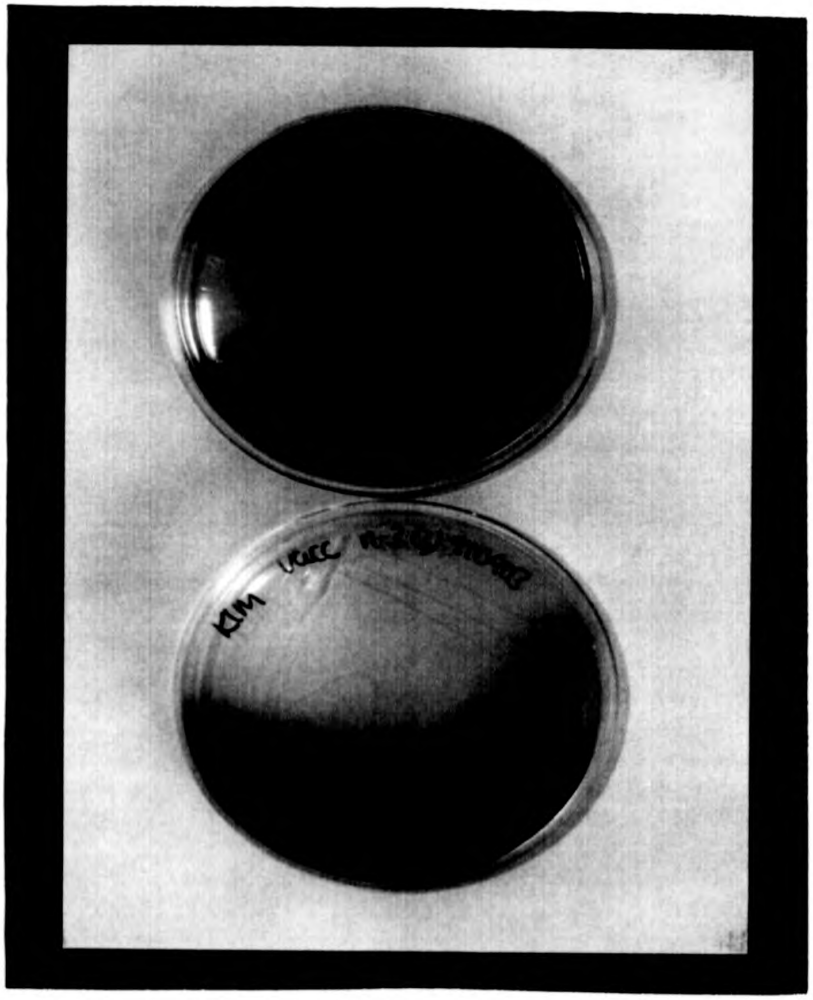


Plate 3.3 Cultures of *Aeromonas salmonicida*

(A) Cultured strain (B) Kidney swab from infected fish

Trial 1:-

Vibrio anguillarum (strain 7875) was prepared, as described in Section 3.6.3. Bacteria were killed by resuspending the pellet in 10 ml 0.6 % (v/v) formalin in sterile PBS, in a sterile glass bottle and incubating overnight at 4°C. The pellet was then washed 5 times with sterile PBS and centrifugated at 2,000 g. Sterility was tested by streaking out 100 µl of bacterial suspension onto a TSA plate and culturing for 48 hours at 14°C. The bacterial concentration was adjusted to 2 mg ml^{-1} in sterile PBS.

Fish from the high (n-3) and (n-6) PUFA dietary groups and half of the commercial group were vaccinated with 0.2 ml of bacterial suspension by intraperitoneal injection (i.p.). Water temperature at this time was 13.7°C. At week 4, half of the vaccinated fish were challenged with 1 $x10^6$ live bacteria in 0.1 ml sterile saline by an i.p. injection. A group of unvaccinated fish on the commercial diet was also challenged as a control group. The fish were moved to black bins containing two water filters per 100 l of water, aerated with compressed air. Fish were not fed throughout the challenge. The remaining fish were revaccinated, and rechallenged by i.p. injection at week 8. The RPS value for vaccinated fish from the three groups was compared to the unvaccinated fish on a commercial diet.

Trial 2:-

At the end of the trial, fish received a primary vaccination against *Aeromonas salmonicida* on day 150. Half of the fish population from each dietary group were vaccinated with 0.1 ml i.p. of an *Aeromonas salmonicida* vaccine [batch no. mlC batch V/ST3] obtained from Ian Bricknell (SOAFD, Aberdeen). At week seven, vaccinated

and non-vaccinated fish were challenged with live *Aeromonas salmonicida*, strain MT 423, by bath challenge. Water was turned off for 24 hours, during which time fish were exposed to 2.5 x 10^5 bacteria ml^{-1} of water. Feeding was resumed 24 hours after challenging. Water temperature was 12.0°C.

Trial 3:-

Unvaccinated salmon were challenged with *Vibrio anguillarum* to which they had no previous exposure. They were challenged with 1 x 10^5 live *Vibrio anguillarum* (strain 7875) i.p in 0.1 ml of saline, 112 days from the onset of the trial. Feeding was resumed 24 hours after challenge. Water temperature was 14.2°C.

3.6.5 Determination of antibody titres by agglutination and ELISA

In dietary trial 1, fish were bled at two week intervals after vaccination. In dietary trial 2, the fish were bled similarly, but a pre-immune bleed was taken prior to vaccination. Blood sampling and serum preparation was as outlined in Section 3.2.3. Antibody titres were tested by ELISA and agglutination (see Table 5.39).

(a) Measurement of antibody titres from vaccinated groups by ELISA.

ELISA plates were coated with 0.05 µg ml^{-1} poly-l-lysine dissolved in coating buffer pH 9.6 (50 µl $well^{-1}$). Plates were incubated for 60 minutes at room temperature and then washed twice with PBS. Bacteria were added to each well (1 x 10^8 ml^{-1} of heat killed bacteria), and incubated overnight at 4°C. Glutaraldehyde (0.05 % v/v) was added at 50 µl $well^{-1}$ and the plates incubated at room temperature for 20 minutes. The plates were then washed 3 times with PBS containing 0.38 M NaCl and 0.05 % (v/v) Tween-

20 before postcoating wells with 250 µl of a 3 % (w/v) casein solution, and incubating plates overnight at 4°C. After warming to room temperature the plates were washed 3 times with wash buffer and two-fold serial dilutions of sera samples (diluted with PBS) were added to wells (100 µl $well^{-1}$). The plates were then incubated for 6 hours at room temperature, or overnight at 4°C. They were washed 5 times with wash buffer before adding 100 µl $well^{-1}$ of rabbit α-salmon IgM-HRP (diluted to 1/300 in wash buffer with 10% v/v FCS) (see Section 3.5.5 for conjugate preparation and titration). The plates were incubated for 60 minutes at room temperature before washing with high salt wash buffer. Chromogen/substrate was added to each well and incubated for 10 minutes at room temperature. The reaction was stopped with 50 µl $well^{-1}$ of 2 M H_2SO_4. Plates were read at 450 nm with an ELISA reader, blanking against chromogen/substrate and stop solution. Positive controls, consisting of trout α-Vibrio or trout α-furunculosis (both prepared by M.F. Tatner, University of Stirling), and negative controls of normal fish serum for the first assay and normal rabbit serum for the second, were included in the ELISA. The end point of the titre was taken as mean values of duplicate well which had an absorbance 3 times that of the background.

(b) Measurement of antibody titres from vaccinated groups by agglutination.

Doubling dilutions of test serum using saline (0.88 % w/v) with 0.1 % w/v gelatin, were made in a round bottomed microtitre plate. The volume of the dilution was 25 µl $well^{-1}$, discarding the excess 25 µl from the final row. A bacterial suspension of 1 $x10^9$ ml^{-1} in PBS, was added to the wells (25 µl $well^{-1}$). As with the ELISA, positive controls, consisting of trout α-Vibrio for trial 1 or trout α-furunculosis trial 2 (both prepared by M.F. Tatner, University of Stirling), and negative controls of unvaccinated fish serum

for the first assay and normal rabbit serum for the second, were included. The plate was incubated overnight at 4°C. The titre end point was taken to be the last well with visible agglutination. A comparison of sensitivity of the ELISA compared to agglutination was made using the positive control as an internal standard.

3.6.6 Western blot

Western blots were used to examine the specificity of pre-immune and post vaccinated sera against the immunizing antigen. Bacterial samples were run on a Mini-Protean 11 electrophoresis system as described in Section 3.5.6. Transblotting onto nitrocellulose paper was carried out according to Wiens *et al.* (1990) (see Appendix 1 for buffer preparation). The gel was gently removed from the electrophoresis plate into transfer buffer for 10 minutes. This, and the transblotting materials (filter paper and nitrocellulose paper), were equilibrated in transblot buffer. Three filter papers were placed on the cathode side of the transblotter (Biorad), and air bubbles were removed with a Pasteur pipette. The gel, and then the nitrocellulose paper, were positioned on the assembly, with a further three filter papers and the anode on top of these. The gel was blotted onto the nitrocellulose paper by applying 20 volts for 20 minutes.

Non-specific binding sites, present on the nitrocellulose paper, were blocked with 1 % v/v BSA in Tris-buffered saline (TBS) with 0.1 % (v/v) Tween-20 for 60 minutes at 37°C. The paper was washed 3 times with 5-minute washes of TBS and placed in a mini protean II multiscreen (Biorad). Each lane was incubated with a 1/10 dilution of test sera in TBS overnight at 4°C. The blot was washed as before and incubated with rabbit anti-salmon IgM (1/100) for 60°C at room temperature. After washing as

described above, the paper was incubated with a 1/100 dilution of anti-rabbit IgG-HRP for 60 minutes at room temperature. The plate was again washed, but included a fourth rinse of 1 minute using TBS with no Tween-20. Colour was developed with 2 ml of 4-chloro-naphthol solution, with 10 ml of PBS and 10 µl of H_2O_2 at 37°C until bands materialised. The reaction was stopped by soaking the paper in distilled water for 10 minutes.

3.6.7 Indirect fluorescent antibody technique (IFAT)

Slides were cleaned with acetone and a smear of bacteria was fixed within a marked circle on the slide with methanol for 5 minutes. Test antisera was added to the bacteria (100 µl), and the slide incubated for 5 minutes at room temperature. The slides were then washed with PBS and placed in a PBS bath for a further 5 minutes. Rabbit α-salmon IgM (100 µl of 1/1,000 in PBS) was added to the slide which was then incubated for 5 minutes at room temperature. The slide was washed as before. Fluorescein isothiocyanate (FITC) -labelled anti-rabbit serum (goat) diluted 1:100 in PBS (100 µl) was added to the slides, which were incubated and washed as before. Slides were mounted in hydromount, and coverslips sealed with nail varnish. The slides were stored in the dark at 4°C, and were examined for fluorescing bacteria under oil immersion with a fluorescent microscope, as soon as possible after preparation. Positive controls consisted of trout α-furunculosis (prepared by M.F. Tatner, University of Stirling) and negative controls were normal rabbit serum.

3.6.8 Stress testing

Fish were injected i.p. with 0.1 ml of prednisolone (100 mg kg-1 body weight) and maintained at 18°C for 10 days. Water temperature was increased from 12°C to 18°C at a rate of one degree every four hours.

3.7 HISTOLOGY

Examinations of cell populations were carried out using stained cell smears and cytospins. Geimsa/ May-Grunwald stains were used for differential counts of blood smears. B-like cell were counted by using FITC labelling.

3.7.1 Preparation of blood smears

A small drop of blood or cell suspension (in serum) was placed at one end of a microscope slide (precleaned in methanol (MeOH)). A smaller slide was placed next to the drop and the fluid was allowed to flow along the edge of the slide. It was then pushed in a quick, smooth action along the lower slide. Only a small volume of suspension was required or else the smear produced was too dense to stain. The slide was quickly air dried, and fixed in 95 % methanol for 1-2 minutes.

3.7.2 Preparation of cytospins

Cytosmears were prepared using a Shandon Cytospin 3, and prepared in accordance with the manufacturers suggested method. Cells were resuspended to 1 $x10^6$ ml^{-1} in medium with 50 % FCS. A volume of 250 µl was placed in the bucket adjacent to the slide. The slide was centrifuged at 1,000 rpm for 5 minutes. Cytospins were rapidly air dried and either fixed with 95 % methanol for 1-2 minutes, or stored at -70°C for FITC

labelling.

3.7.3 Geimsa/ May-Grunwald staining

Slides were stained with filtered May-Grunwald [in absolute ethanol (1:1 v/v)] stain for 6-7 minutes. The stain was removed, and the Geimsa stain [15% in Sorensen's buffer (v/v)] was added directly to the slides without washing. This was left on for a further 10 minutes before washing the slides with Sorensen's buffer, air drying, and mounting.

3.7.4 Fluorescein isothiocyanate (FITC)-labelling of cells

A 50 µl aliquot of 1/100 monoclonal ascites dilution (clone 2G9), was placed on the cytosmear (Section 3.7.2) and incubated for 5 minutes at room temperature in a wet box. As negative control, 50 µl of normal mouse serum was used in parallel for each slide. The slides were rinsed twice with PBS (pH 7.3) and soaked in a PBS bath for 5 minutes. Anti-mouse IgG-FITC (1/100 in PBS) was placed on the slide (50 µl) for 5 minutes. The slides were washed as before. The cytosmears were mounted with hydromount and the edges of the coverslip were sealed with clear nail varnish. The slides were stored at 4°C until examined under a fluorescent microscope (Anderson, 1990).

3.8 LIPID ANALYSIS

3.8.1 Introduction

Most of the lipid analytical techniques employed are based on those described by Christie (1982) who discusses the methodology necessary to avert contamination of lipid

samples, procedures to prevent degeneration of samples by autoxidation and lipases, and the potential hazards associated with lipid analysis.

3.8.2 Lipid extraction

The extraction of lipid from cells, serum, tissues or diets employed the method of Folch *et al.*, (1957). Butylated hydroxytoluene (BHT) was added to extractions and storage solvents at 0.01 % (w/v) to prevent auto-oxidation. Prior to lipid extraction, cell pellets were resuspended in 1 ml of PBS, and serum was diluted in 1 ml of PBS. The samples were then placed in glass stoppered tubes and 8 ml of chloroform / methanol (2:1 v/v) were added. The tubes were shaken vigorously, and allowed to stand on ice for 60 minutes. One ml of 0.88 % (w/v) was then added to each tube.

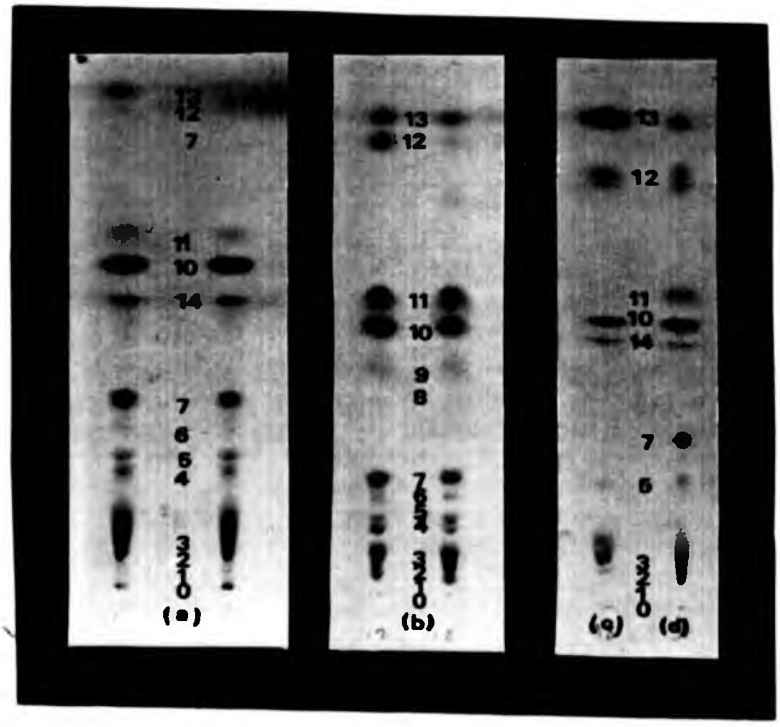
For the extraction of lipids from diets and tissues, diets were first ground with a mortar and pestle, and then either these, or tissue samples were placed in 2 ml of distilled water along with 16 ml of chloroform / methanol (2:1 v/v). Samples were homogenized for 2 minutes on ice using a Teflon-in glass homogenizer (TriR model K43). Two ml of 0.88 % KCl (aq) were then added to the homogenates. After addition of the KCl solution, all extractions were shaken vigorously and the tubes were then centrifuged for 1 minute at 1,000 g to separate the organic and aqueous phases. Upper aqueous layers were discarded and the lower organic phases were filtered through pre-washed (in chloroform : methanol 2:1) Whatman No.1 filter papers into pre-weighed test tubes. Solvent was evaporated off under a stream of oxygen-free nitrogen; for larger amounts of solvent, a rotary evaporator was used. Samples were desiccated overnight under vacuum, and lipid yields were determined gravimetrically. Extracted lipid samples

dissolved in chloroform : methanol (2:1 v/v) containing 0.01 % BHT, were stored under oxygen-free nitrogen, at -20°C in storage vials.

3.8.3 Lipid class analysis

Lipid class analysis was carried out by high performance thin-layer chromatography (HPTLC), using 10 x 10 cm glass plates coated with silica gel 60, in layers of 0.1 mm thickness. Methodology for HPTLC is reviewed elsewhere by Henderson and Tocher (1992). HPTLC has the advantage over other chromatographic techniques in being rapid, with high sensitivity and resolution. It also requires little sample to carry out the procedure. For use in this study, plates were first cleaned by pre-developing with chloroform / methanol (2:1 v/v). Having desiccated the plates under vacuum for 30 minutes, a 1 cm band of adsorbent was scraped off from the top edge. With the scraped edge turned to the right-hand side of the plate, samples were applied along the plate, in 3 mm streaks, 1 cm from the bottom edge (10 µg of total extracted lipid was applied per streak). Lipid classes were separated by a single dimension, double development system. Polar lipids were separated on the lower half of the plate using first, a solvent system of methyl acetate / chloroform / propan-2-ol / methanol / 0.25 % KCl (aq) (25:25:25:10:9 by vol.) (Vitiello & Zanetta, 1978). The plate was desiccated for 60 minutes under vacuum, before placing in a second solvent system of hexane / diethyl ether / glacial acetic acid (40:10:1 v/v/v), (Christie 1982) and fully developed. Plate 3.4 illustrates the separation of total lipid from Atlantic salmon erythrocytes, leucocytes, serum and a standard of cod roe lipid classes by the method outlined above.

The two solvent development system generally produced reliable separation of the lipid



14-solvent front
13-sterol esters
12-triacylglycerol
11-free fatty acids
10-cholesterol
9-cerebrosides
8-sulphatides
7-phosphatidylethanolamine
6-cardiolipin
5-phosphatidylinositol
4-phosphatidylserine
3-phosphatidylcholine
2-sphingomyelin
1-lysophosphatidylcholine
0-origin

Plate 3.4

Lipid class separation of Atlantic salmon (a) RBCs, (b) WBCs, (c) serum and (d) a standard of cod roe lipid, using 10 x 10 cm HPTLC plate, developed in a single dimension, double development system of (1) methyl acetate / chloroform / propan-2-ol / methanol / 0.25% KCl (aq) (25:25:25:10:9 by vol.) (Vitiello & Zanetta (1978)) and (2) hexane / diethyl ether / glacial acetic acid (40:10:1 v/v/v) (Christie 1982).

classes. Occasional problems occurred in resolving phosphatidylinositol from phosphatidylserine, and triacylglycerols from sterol esters with the solvent systems described above. The latter separation was improved by decreasing the diethyl ether / hexane ratio. The R_f values (i.e. the position of the lipid class from the origin, relative to the solvent front from the origin) were found to vary within and between batches of plates. The addition of cod roe standard to the plate facilitated the identification of component lipid classes.

Separated lipid classes were visualised by either saturating the plate with copper acetate 3 % (w/v) in 8 % H_3PO_4 (v/v) (Fewster et al. 1969), and then charring it at 160°C for 20 minutes, or spraying the plate with 2',7'-dichlorofluorescin, 0.1 % (w/v) in 95 % MeOH containing 0.01 % (w/v) BHT and viewing under a U.V. light. Solvent was removed from the plates by desiccation under vacuum, prior to spraying with stains. The first method of visualisation which is also more sensitive, benefits from being able to obtain a permanent record from the plate. On charring, the bands of the lipid classes became brown / black in colour. The cholesterol and sterol esters initially appeared blue, a fact which can be used diagnostically, but these darken in hue as charring proceeded. The main disadvantage with this method is that the lipid is destroyed and no further lipid analysis is possible. Staining with 2',7'-dichlorofluorescin does not have this problem and was therefore used when further lipid analysis was required. The bands appeared as yellow fluorescent spots under UV light, when this stain was used.

Lipid classes on HPTLC plates sprayed with copper acetate 3 % (w/v) in 8 % H_3PO_4 (v/v) were quantified by photodensitometry using a Shimadzu dual wavelength flying spotscanner Model CS-9000 (Shimadzu Corp. Kyoto, Japan) linked to a Shimadzu DR-13 recorder and a GDU-10C display. Lanes were scanned in a ziz-zag mode, correcting for background, at 370 nm. The response of the detector was only proportional to weight of lipid class spots in the range of 0.5 µg to 5 µg per lipid class (Hodgson, 1990). Care was therefore taken not to overload the plates, but with insufficient loading, minor lipid classes were not detectable. The densitometer expressed lipid classes as percentages, proportional to weight of peak area. Figure 3.5 is a typical densitometer trace of cod roe standard.

Autoradiography was preformed by separating ^{14}C-labelled lipid classes from extracted lipid, by the HPTLC method outlined above followed by exposure of the developed chromatogram to X-ray film in a light proof cassette. The procedure was carried out in a darkroom with the aid of a safelight. The cassette was stored at -80°C for one week. The film was then developed using LX 24 developer (393 ml in 878 ml distilled water) for 5 minutes, then washed and transferred to FX 40 fixer (400 ml in 1600 ml distilled water) for five minutes. Radioactive ink, placed at the corners of the HPTLC plate, marked the plates position relative to the X ray film. The bands of radioactive lipid classes, on the HPTLC plate, were marked, scraped and placed in a scintillation vial with 2 ml of Ecoscint scintillation fluid. Radioactivity was counted by liquid scintillation using a TRiCarb 2000CA liquid scintillation counter (Canberra-Packard Instruments).

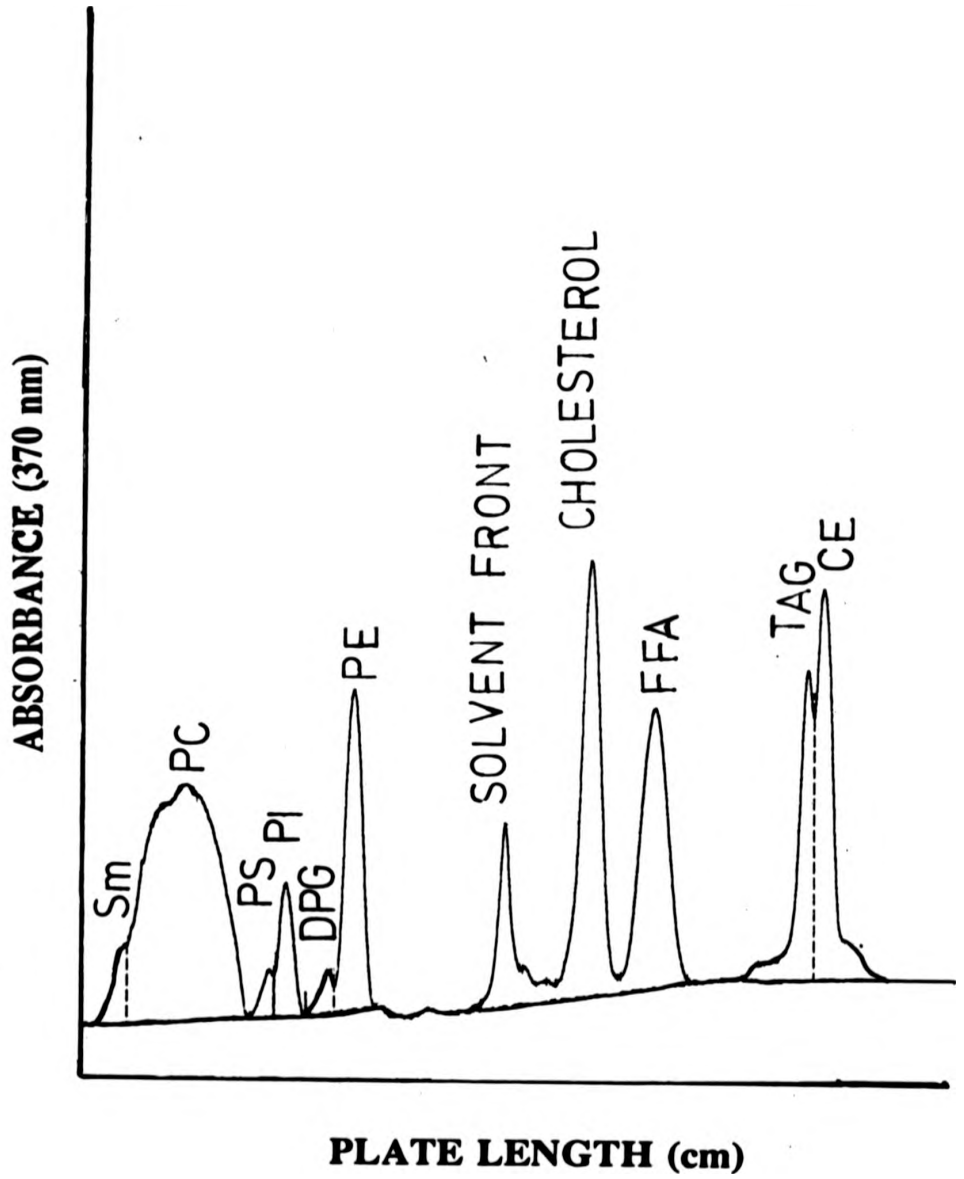


Figure 3.5 Photodensitometric scan of cod roe standard lipid classes.

Abbreviations: Sm-Sphingomyelin, PC-phosphatidylcholine, PS-phosphatidylserine, PI-phosphatidylinositol, PE-phosphatidylethanolamine, DPG-diphosphatidylglycerol (cardiolipin), FFA-free fatty acids, TAG-triacylglycerols, CE-cholesterol ester (sterol ester).

3.8.4 Transesterification of fatty acids and purification of fatty acid methylesters

Fatty acids, in the lipid samples, were converted to methyl esters by acid-catalysed transesterification, as described by Christie (1982). Solvent-free lipid samples were dissolved in 1 ml of toluene and 2 ml of 1 % (v/v) H_2SO_4 in MeOH added. The tubes were stoppered after flushing with nitrogen, and incubated overnight on a hot block at 50°C. On cooling to room temperature, 5 ml of distilled water was added to each tube. Fatty acid methyl esters (FAMEs) were extracted with 2 x 5 ml hexane / diethyl ether 1:1 (v/v), and 3 ml of 2 % $KHCO_3$ (aq) (w/v) added to the combined extracts. The organic / aqueous phases were separated by centrifuging for 2 minutes at 1,000 g. After retaining the upper organic phase, solvent was removed from it by a stream of oxygen free nitrogen and the FAMEs obtained were dried by desiccating under vacuum.

Purification of FAMEs, was carried out by HPTLC, using the solvent system hexane / diethyl ether / glacial acetic acid (40:5:1 by volume) on 10 x 10 cm HPTLC plates. FAMEs were identified on the plate by spraying the plate with 2',7'-dichlorofluorescin, examining it under U.V light (see Section 3.8.3) and matching the FAMEs' R_f values to those of a FAME standard. The silica containing the FAMEs was scraped from the plate and extracted with 2 x 5 ml hexane / diethyl ether 1:1 (v/v). Particulate silica and 2',7'-dichlorofluorescin were removed from the organic phase by shaking the extract with 3 ml of 2 % $KHCO_3$ (aq) (w/v) and centrifuging for 2 minutes at 1,000 g. The upper organic fraction was retained and solvent was removed under a stream of oxygen-free nitrogen. The purified FAMEs were then resuspended to a concentration of 5 mg ml^{-1} in hexane containing 0.01 % BHT, and stored at -20°C until analyzed further.

To prepare FAMEs from individual lipid classes, lipid samples were separated into classes by HPTLC, and visualised as described in Section 3.8.3, using 2,7-dichlorofluorescin. Bands of adsorbent containing lipid classes were marked, and scraped from the plate into test tubes. Two ml of 1 % (v/v) H_2SO_4 in MeOH were added and the samples treated as described above. The resultant FAMEs were resuspended to a concentration of 5 mg ml^{-1} in hexane / 0.01 % BHT and stored at -20°C.

3.8.5 Gas-Liquid Chromatography (GLC) analysis of FAMEs

FAMEs were separated using a Packard 436 gas chromatograph (Packard Instruments Ltd., Caversham, U.K.) equipped with an open fused silica capillary column, 50 m in length and 0.32 mm i.d., coated with CP Wax 51 liquid phase (Chrompack, Middelburg, The Netherlands). Hydrogen was used as the carrier gas and sample application was by direct on column injection of 0.6 µl of a 5 mg ml^{-1} FAME sample in hexane containing 0.01 % BHT. The temperature of the flame ionisation detector was 250°C. During sample analysis the temperature of the oven started from 50°C for 0.1 minute post injection increasing to 190°C at rate 39°C min^{-1}. The temperature was then raised at a rate of 1.5°C min^{-1} to a final temperature of 225°C. The run was terminated after 45 minutes. Separated components, quantified by a Shimadzu C-R3A recording integrator attached to the chromatograph, were identified by comparing peaks retention times with those of a known fish oil standard. (See Figure 3.6)

Samples were prepared with the addition of 23:0 fatty acid as an internal standard. The amount added was a tenth of the total extracted lipid weight and was added after lipid

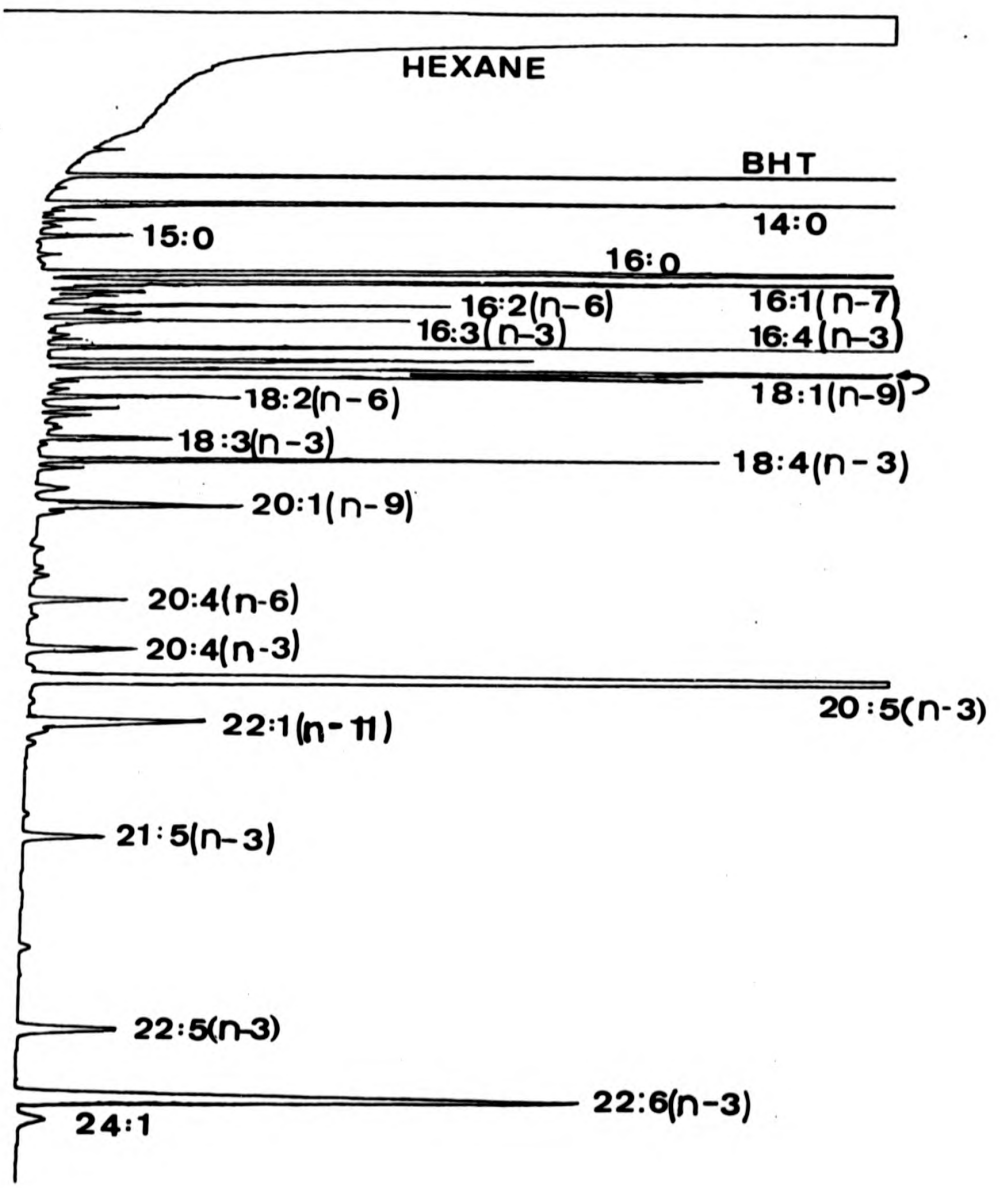


Figure 3.6 Gas chromatographic trace of fish oil standard.

class analysis, but prior to transmethylation. The addition of an internal standard, of known concentration, allowed the quantification of fatty acids as an absolute amount by the direct proportion of the internal standard peak area with that of the fatty acid. Table 3.4 compares three different internal standards (17:0, 21:0 and 23:0) used to calculate fatty acid concentrations in Atlantic salmon erythrocytes. The internal fatty acid standard chosen was 23:0, since its chromatographic peak did not interfere with other peaks present in fish tissue.

3.9 DATA EVALUATION

Significance of differences between experimental groups were determined by either Student 't' test or ANOVA, depending on the number of test groups. Analyses of data was performed using Statgraphics statistical package (Statistical Graphics Corp.). Non-homogenous data was arcsine square root transformed before analysis, and the difference between the means of the groups was determined by Tukeys' test. Results were considered significant at * = $0.05 > p > 0.01$, ** = $0.01 > p > 0.001$, *** = $p < 0.001$ for the Student 't' test, and $p \leq 0.05$ for ANOVA. Significant differences in values are indicated by a different superscript letter. Details of additional analysis can be found with the relevant methodology.

Table 3.4

A comparison of three different internal standards (17:0, 21:0 and 23:0) used to calculate fatty acid weights (ng/10^6 cells) in Atlantic salmon erythrocytes.

Fatty acids	ng/10^6 cells		
	17:0	21:0	23:0
14:0	6.5 ±0.9	10.4 ±3.3	17.1 ±4.7
15:0	1.8 ±0.1	1.6 ±0.2	2.5 ±0.2
16:0	84.3 ±10.4	88.7 ±2.4	106.7 ±4.5
18:0	19.8 ±1.6	21.1 ±0.9	26.8 ±2.2
20:0	0.6 ±0.2	0.6 ±0.1	1.2 ±0.1
22:0	1.3 ±0.4	0.6 ±0.1	2.3 ±0.4
Σ saturates	**114.3 ±2.3**	**123.0 ±1.2**	**156.6 ±2.0**
14:1	2.7 ±0.8	2.4 ±0.4	2.3 ±0.1
16:1	5.0 ±0.8	3.9 ±0.6	3.1 ±1.2
18:1	24.7 ±2.7	27.1 ±2.5	37.6 ±5.6
20:1	10.9 ±0.9	11.2 ±1.3	11.9 ±0.6
22:1	9.1 ±0.8	8.7 ±2.6	10.1 ±1.4
24:1	6.9 ±0.5	8.6 ±0.2	8.6 ±1.0
Σ monoenes	**59.3 ±1.1**	**61.9 ±1.3**	**73.6 ±1.6**
18:2	3.8 ±0.3	4.7 ±0.3	6.7 ±1.1
20:4	10.8 ±0.4	11.0 ±0.2	12.4 ±0.7
22:2	1.0 ±0.6	0.8 ±0.5	2.0 ±0.8
22:5	1.7 ±0.3	1.7 ±0.1	2.4 ±0.4
Σ (n-6)	**20.0 ±0.3**	**19.6 ±0.3**	**26.1 ±0.4**
18:3	0.8 ±0.1	0.7 ±0.6	1.3 ±0.4
18:4	0.4 ±0.2	0.6 ±0.3	0.8 ±0.4
20:4	2.1 ±0.1	2.2 ±0.2	2.4 ±0.3
20:5	30.5 ±2.4	32.1 ±0.3	34.3 ±2.6
22:5	8.2 ±1.0	7.9 ±1.3	9.7 ±0.9
22:6	116.0 ±0.9	122.0 ±2.2	130.9 ±8.8
Σ (n-3)	**159.1 ±0.6**	**168.2 ±0.8**	**182.4 ±1.8**
(n-3)/(n-6)	**7.8 ±0.4**	**8.6 ±0.6**	**7.0 ±1.1**

In addition to the above fatty acids, Σ (n-6) includes 16:2, 18:3, 20:2, 20:3 and Σ (n-3) includes 16:3, 16:4.

Values are expressed as mean of rbc lipids from three fish (μg 10^6 $cell^{-1}$) ±SD

CHAPTER 4 LIPID COMPOSITION OF IMMUNOCOMPETENT CELLS OF ATLANTIC SALMON

4.1 INTRODUCTION

The lipid class and fatty acid composition of peripheral blood erythrocytes, leucocytes and serum, headkidney T and B cells and macrophages from Atlantic salmon parr are presented in this Chapter. While the literature offers little information on the lipid composition of immunocompetent cells and tissues of teleost fish, some data are available on the lipid composition of their erythrocytes. An analysis of the lipid structure of peripheral blood cells from commercially fed Atlantic salmon *(Salmo salar)*, was a prerequisite for the dietary study which follows in Chapter 5, and was necessary for monitoring any dietary modulation of the cells. Blood cells are a convenient model for examining modulation of lipids by diet as they are easy to isolate without having to sacrifice the animal.

A comparison between the lipid composition of blood cells from freshwater Atlantic salmon parr and another cold freshwater teleost, the rainbow trout (*Oncorhynchus mykiss)*, was made. The reason for the inclusion of the analysis of rainbow trout blood cells was that they were used in some of the leucocyte cultures in Chapter 6, as well as being used to optimise the lipid methodology.

The extent to which lipids vary between individual fish depends on environmental influences, for example whether the fish is a freshwater or marine species, whether it is wild or reared in captivity, or on the water temperature due to latitude or seasonal

fluctuations. The physiological state of the fish also has a relevance on the lipid content (Castell, 1979; Sheridan *et al.*, 1985a; Henderson and Tocher, 1987 and van Vliet and Katan, 1990) and variations in temperature and salinity formed the basis of the following analyses.

As explained in Chapter 2, coldwater fish generally have an essential fatty acid requirement for (n-3) PUFAs. The essentiality of (n-3) PUFAs has not been fully determined in warmwater teleosts, such as the African catfish, but they appear to grow satisfactorily on (n-6) PUFA. The lipid compositions of peripheral blood erythrocytes and leucocytes were compared between coldwater Atlantic salmon, warmwater African catfish (*Clarias gariepinus*) and rabbit, as a source of mammalian blood cells. Rabbit blood cells were included in the comparison, because firstly, their diets tend to be rich in (n-6) PUFA, and secondly, the extent of elongation of (n-3) PUFA is not as great as in fish. The influence of dietary fatty acids on the fatty acid profiles of these cells was also examined in these three species.

Changes in the lipid composition of cell membranes of poikilotherms in response to environmental temperatures were discussed earlier in section 2.4. The mechanism of homeoviscous adaptation is generally reflected by changes in the proportions of lipid classes and the degree of unsaturation of the component fatty acids (Cossins and Prosser 1982; Hazel 1984). Alterations to peripheral blood cells of channel catfish (*Ictalurus punctatus),* resulting in an increased unsaturation of phospholipids with decreasing temperature, has been shown to occur (Bly *et al*, 1986b; Bly and Clem, 1988). The present study was undertaken to examine whether similar adaptations to decreased

environmental temperature occur in the lipids of the blood cells of Atlantic salmon.

The amount of total lipid in smolts has been shown to decrease during smoltification, while the relative amount of (n-3) PUFAs increase (Vanstone and Markert 1968; Ota and Yamada 1971; Sheridan 1989). A comparison was therefore made between the lipid composition of leucocytes from freshwater Atlantic salmon parr and their marine smolt counterparts. The fatty acids profiles of blood cells were also compared with their dietary fatty acids. The relevance of this part of the study was to determine if the lipid patterns of Atlantic salmon parr peripheral blood leucocytes change on smoltification, as occurs with their total body lipids.

4.2 LIPID COMPOSITION OF ATLANTIC SALMON ERYTHROCYTE, LEUCOCYTES AND SERUM

4.2.1 Lipid content

Cells and serum were isolated from the peripheral blood of six Atlantic salmon parr (mean weight 30 g) which had been maintained on a commercial diet (Ewos No 4 crumb). The lipid content of cells and serum can be found in Table 4.1, along with their protein content. The mean values presented include results from other extractions, using fish taken from a variety of temperatures. The lipid content of an erythrocyte (0.92 ±0.6 μg $1x10^6$ $cells^{-1}$) was less than that of an individual leucocyte (40.0 ±30.0 μg $1x10^6$ $cells^{-1}$).

4.2.2 Lipid class composition

The lipid class composition of erythrocytes, leucocytes and serum, extracted in section 4.2, were determined for three of the six samples. Values are presented in Table 4.2 and are summarised in Figure 4.1. A high PUFA content in the fish cells and serum led to problems initially with auto-oxidation. This occurred even when the lipid was protected from atmospheric oxygen. One of the consequences of this auto-oxidation was poor resolution of the lipid class bands by HPTLC, with bands streaking into one another. This was particularly excessive in the RBC extracts, presumably due to the presence of the pro-oxidant iron in the haemoglobin of these cells. The problem was overcome by adding BHT to the extraction solvents. The amount of BHT present in the lipid extracts was shown by TLC to be very small and was therefore excluded when determining the lipid yield. BHT ran just behind the solvent front and could be

Table 4.1

The lipid and protein content of Atlantic salmon erythrocytes, peripheral blood leucocytes and serum

		Lipid	Protein
Erythrocytes	$\mu g\ (10^6\ cells)^{-1}$	0.92 ±0.6	20.7 ±2.4
Leucocytes	$\mu g\ (10^6\ cells)^{-1}$	40.0 ±30.0	41.0 ±7.0
Serum	$mg\ ml^{-1}$	21.8 ±7.4	51.6 ±3.3

Values are expressed as the mean ±SD for 25 fish for lipid content, and for 5 fish for protein content.

Table 4.2

The percentage lipid class composition of Atlantic salmon erythrocytes, peripheral blood leucocytes and serum

Lipid class (% Total lipid)	Erythrocytes	Leucocytes	Serum
Total polar lipid	76.0 ±1.6	36.2 ±5.4	46.7 ±5.7
Total neutral lipid	24.0 ±1.6	63.8 ±5.4	53.5 ±5.7
Polar lipid:			
LPC	-	1.3 ±0.1	0.2 ±0.1
SM	3.9 ±0.5	3.5 ±0.3	2.9 ±0.3
PC	39.2 ±2.2	15.4 ±3.1	35.7 ±1.7
PS	6.6 ±1.0	2.6 ±0.9	-
PI	5.1 ±0.6	1.9 ±0.5	3.8 ±1.0
Cardiolipin	1.8 ±0.2	0.7 ±0.5	1.7 ±2.5
PE	16.6 ±1.0	3.6 ±1.7	3.8 ±1.0
Sulphatides	0.6 ±0.6	1.5 ±0.5	-
Cerebrosides	2.0 ±0.2	5.8 ±1.2	-
Neutral lipid:			
Cholesterol	15.8 ±1.3	16.3 ±1.4	12.1 ±1.8
Free fatty acids	1.6 ±0.7	10.0 ±1.7	2.5 ±0.7
Triacylglycerols	4.9 ±4.3	9.9 ±2.6	16.6 ±2.3
Sterol esters	1.4 ±0.2	25.5 ±3.8	22.3 ±2.1
Unknowns	0.4 ±0.3	2.0 ±1.4	0.8 ±0.8

Abbreviations: LPC-lysophosphatidylcholine, SM-sphingomyelin, PC-phosphatidylcholine, PS-phosphatidylserine, PI-phosphatidylinositol, PE-phosphatidylethanolamine.

Values are expressed as the mean percent of total lipid ±SD for three fish.

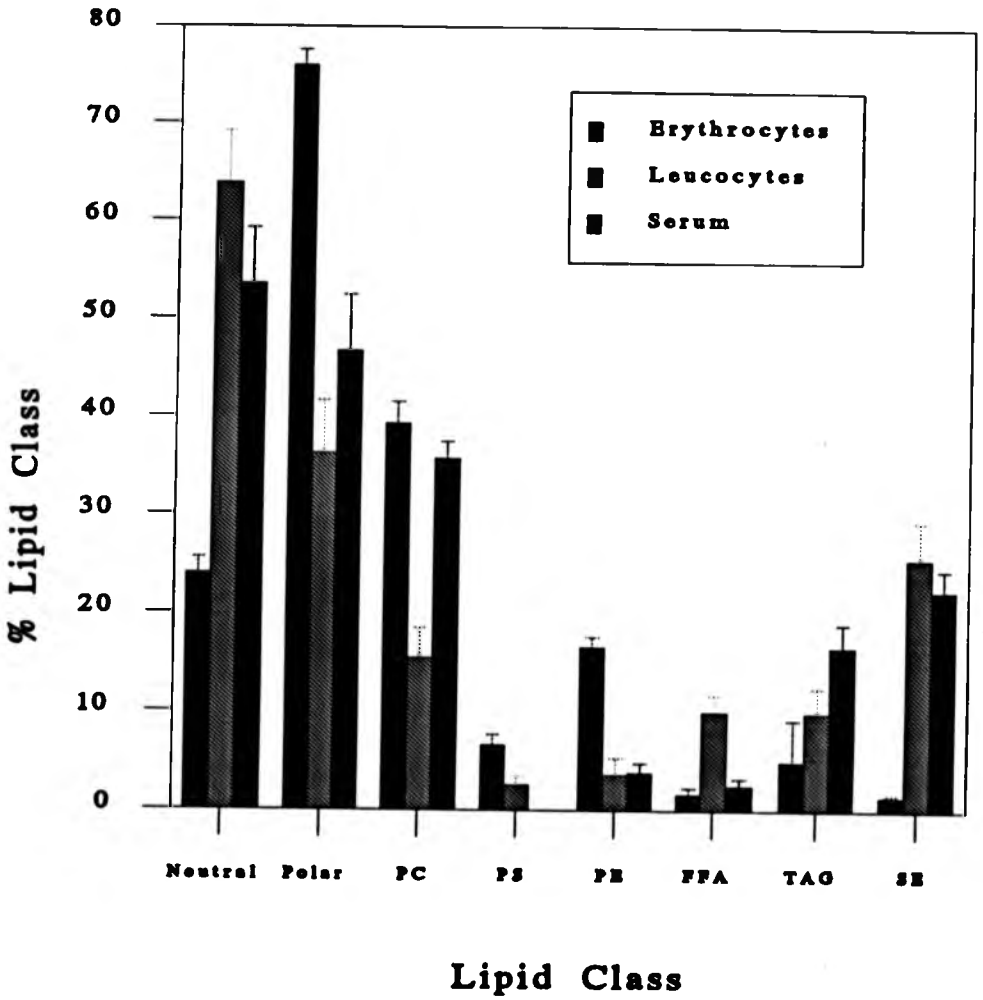


Figure 4.1 The percentage of lipid classes in lipid of Atlantic salmon erythrocytes, leucocytes and serum.

Abbreviations: Neutral- total neutral lipid, Polar- total polar lipid, PC-phosphatidylcholine, PS-phosphatidylserine, PE-phosphatidylethanolamine, FFA-free fatty acids, TAG-triacylglycerols, SE-sterol esters. Values are expressed as the mean percent of total lipid ±SD for three fish.

distinguished from the sterol esters, which stained a blue/black hue after 5 minutes of charring.

The polar lipid content of the Atlantic salmon erythrocytes was greater than that of the neutral lipid, as a result of high PC and PE levels. The erythrocyte lipid also contained higher proportions of phospholipid than that of leucocytes and serum. Leucocyte lipid, by comparison, contained higher levels of neutral lipid, reflected in the high proportions of free fatty acids, triacylglycerols and sterol esters. Any contribution to the TAG portions of the leucocyte lipid by serum contamination was excluded, since washing the leucocytes several times before lipid analysis did not alter the lipid class profile. The polar lipids of these cells also contained more sulphatides and cerebrosides than those of the erythrocytes. A characteristic lipid class profile was obtained for serum with high amounts of PC, triacylglycerols and sterol esters, and low proportions of PS and PE.

4.2.3 Fatty acid composition of total lipid

Fatty acid analysis of the lipid extracts from section 4.2 was as described above. It was decided to quantify the total amount of fatty acids in the cells, rather than the amount of extracted lipid, because of inaccuracies associated with weighing such small samples, especially with the leucocyte lipid. Fatty acid quantification was carried out by the addition of an internal standard, explained in section 3.8.4. The total fatty acid content was 0.22 ±0.1 $\mu g/1x10^6$ cells for the erythrocytes, 2.2 ±0.4 $\mu g/1x10^6$ cells for the leucocytes, and 4.8 ±0.6 mg/ml for the serum.

Table 4.3

The percentage fatty acid composition of Atlantic salmon erythrocytes, peripheral blood leucocytes and serum

Fatty acids (% Total FA)	Erythrocytes	Leucocytes	Serum
da 16:0	-	-	0.5 ±0.1
14:0	1.3 ±0.8	1.7 ±0.4	1.2 ±0.1
15:0	0.3 ±0.1	2.1 ±0.6	0.6 ±0.1
16:0	21.0 ±0.6	26.7 ±0.7	21.6 ±2.7
18:0	6.3 ±0.6	8.9 ±0.3	5.7 ±0.8
22:0	-	0.7 ±0.4	0.4 ±0.1
Σ saturates	**29.3 ±0.8**	**41.2 ±1.0**	**31.1 ±3.4**
16:1	2.1 ±0.8	6.5 ±0.6	4.7 ±0.3
18:1	5.9 ±0.8	19.9 ±0.8	14.8 ±1.4
20:1	-	2.2 ±0.3	2.8 ±0.6
22:1	1.1 ±0.1	1.6 ±0.4	2.3 ±0.6
24:1	1.1 ±0.0	1.4 ±0.6	1.8 ±1.0
Σ monoenes	**12.4 ±1.7**	**31.7 ±2.5**	**27.0 ±2.8**
18:2	0.5 ±0.0	1.7 ±0.2	2.5 ±0.6
18:3	0.2 ±0.0	1.4 ±0.5	0.1 ±0.2
20:2	0.1 ±0.0	0.5 ±0.1	0.1 ±0.2
20:4	2.9 ±0.3	2.5 ±0.5	1.8 ±0.3
22:2	0.2 ±0.1	0.7 ±0.1	0.3 ±0.2
Σ (n-6) PUFA	**4.1 ±0.1**	**7.4 ±0.4**	**5.4 ±0.8**
16:3	0.4 ±0.0	0.9 ±0.1	0.7 ±0.2
18:3	0.2 ±0.1	0.7 ±0.0	0.8 ±0.6
18:4	0.4 ±0.4	0.5 ±0.1	0.4 ±0.3
20:4	0.5 ±0.1	0.2 ±0.0	0.5 ±0.1
20:5	7.5 ±1.9	3.6 ±0.7	7.3 ±0.7
22:5	1.5 ±0.0	0.6 ±0.0	1.4 ±0.1
22:6	43.5 ±4.3	12.7 ±2.2	24.8 ±4.9
Σ (n-3)	**54.3 ±2.0**	**19.7 ±2.8**	**36.4 ±5.7**
(n-3)/(n-6)	**13.2 ±0.7**	**2.6 ±0.5**	**6.7 ±2.1**

Abbreviations: da - dimethyl acetals. Values are expressed as the mean percent of total fatty acids for three fish ±SD. In addition to fatty acids shown, Σ saturates include 17:0, 20:0; Σ monoenes include 14:1; Σ (n-6) PUFA includes 16:2, 20:3, 22:5; Σ (n-3) PUFA include 16:4; where one or more of the three values is < 0.5

The total lipid of erythrocytes of Atlantic salmon contained lower percentages of saturated fatty acids, monoenes and (n-6) PUFA, than the leucocytes (Table 4.3), but contained substantially more (n-3) PUFA, especially 20:5(n-3) and 22:6(n-3). Consequently, the lipid of erythrocytes had a very high (n-3)/(n-6) PUFA ratio (13.2), whereas that of leucocytes was much lower (2.6). The SFA profile of serum resembled those of the erythrocytes, whereas the pattern of monoenoic fatty acids in serum was closer to that of the leucocytes. The (n-6) and (n-3) PUFA content of the serum lipid resulted in a final (n-3)/(n-6) PUFA ratio which lay between that of the erythrocytes and the leucocytes. The fatty acid values obtained for the erythrocytes, leucocytes and serum are summarised in Figure 4.2.

4.2.4 Fatty acid composition of lipid classes

Fatty acid profiles of PC, PS, PI and PE from erythrocytes and leucocytes were analysed following the methodology in sections 3.8.3 - 3.8.5 by pooling the three remaining erythrocyte and leucocyte lipid extracts from section 4.2.1. Very small amounts of FAMEs were obtained from individual leucocyte phospholipid classes, in keeping with the fact that phospholipids make up only 36 % of the total lipid of these cells. The fatty acid components of erythrocyte and leucocyte lipid classes are shown in Tables 4.4 and 4.5 respectively. The fatty acid patterns of both erythrocyte and leucocyte PC, PS, PI and PE were typical of a cold water fish, (i.e. contain high levels of (n-3) PUFA (Henderson and Tocher, 1987)). Of the erythrocyte lipid classes, PE had the highest level of (n-3) PUFA (67.4 %) and the lowest level of saturated fatty acids (17.2 %). PC contained high levels of 16:0, while PS and PI contained high levels of 18:0. PI also had a high 20:4(n-6) content (13.6 %), resulting in an overall high (n-6) PUFA

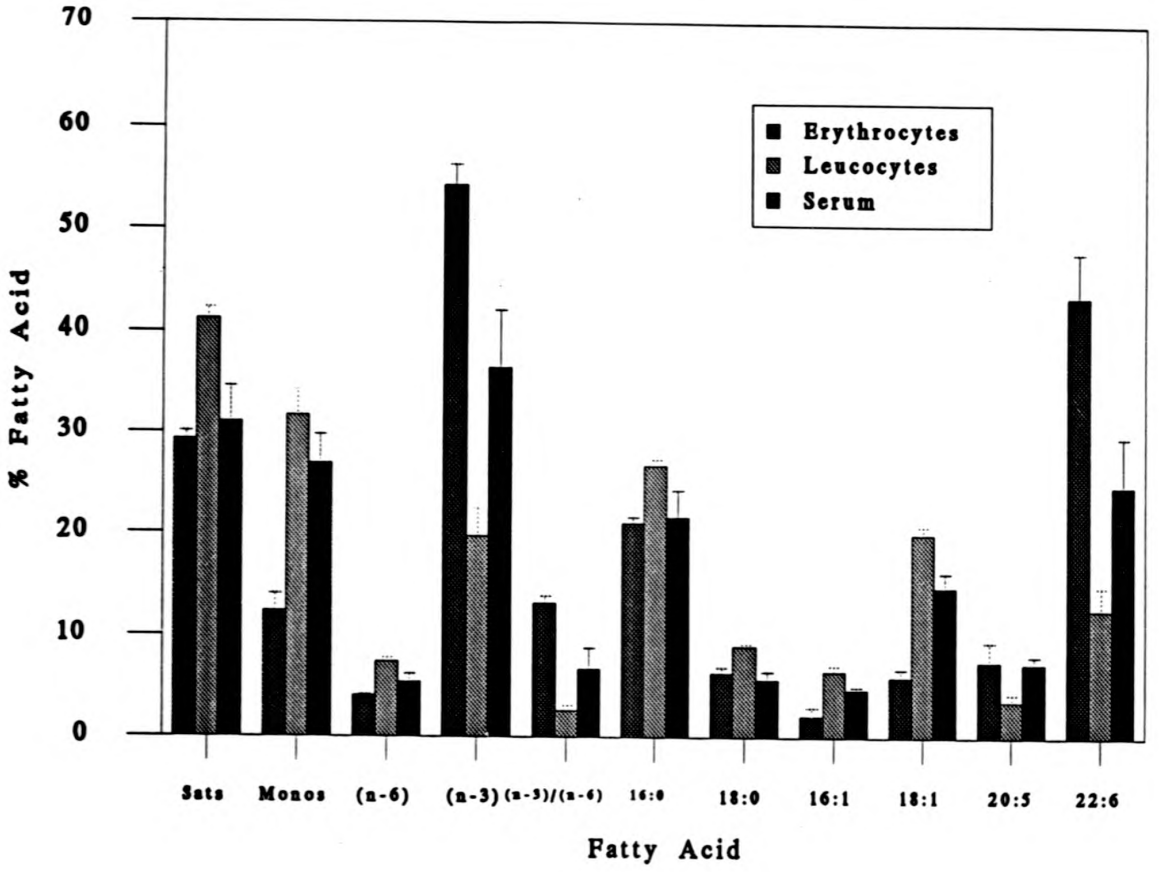


Figure 4.2 The percentage fatty acid composition of major fatty acids in the erythrocytes, leucocytes and serum of Atlantic salmon.

Abbreviations: Sats- total saturated fatty acids, Monos- total monoenoic fatty acids, (n-6)- total (n-6), (n-3)- total (n-3) PUFA, 20:4(n-6), 20:5(n-3), 22:6(n-3). Values are expressed as the mean percent of total fatty acids for three fish ±SD.

Table 4.4

The percentage fatty acid composition of the phospholipid classes of Atlantic salmon erythrocytes

Fatty	Phospholipid Class			
Acids (%)	PC	PS	PI	PE
14:0	0.8	0.2	0.2	0.2
16:0	31.0	14.0	14.1	8.0
17:0	0.2	0.6	0.6	-
18:0	3.2	17.6	19.0	8.6
24:0	1.6	0.3	0.4	-
Σ saturates	**37.3**	**33.2**	**34.3**	**17.2**
14:1	0.6	0.2	0.8	0.8
16:1	1.6	1.5	1.9	0.8
18:1	5.7	5.7	6.9	4.1
20:1	1.4	0.2	1.6	3.4
22:1	0.8	0.8	0.6	2.0
24:1	0.2	0.5	0.5	-
Σ monoenes	**10.2**	**8.8**	**12.3**	**11.1**
18:2	0.4	0.8	1.0	0.6
20:4	1.4	7.3	13.6	1.8
22:2	0.2	0.5	1.0	0.4
Σ (n-6)	**2.4**	**9.9**	**16.5**	**3.6**
16:3	0.4	0.6	0.6	0.4
18:4	0.2	0.5	0.5	0.2
20:4	0.2	0.4	0.4	1.2
20:5	6.6	5.4	4.1	6.0
22:5	1.1	1.5	1.3	2.6
22:6	41.0	37.3	26.9	56.6
Σ (n-3) PUFA	**49.5**	**46.4**	**34.4**	**67.4**
(n-3)/(n-6)	**20.7**	**4.7**	**2.1**	**18.7**

Values are for a pooled sample of three fish.

Abbreviations: PC-phosphatidylcholine, PS-phosphatidylserine, PI-phosphatidylinositol, PE-phosphatidylethanolamine. In addition to fatty acids shown, Σ saturates includes 15:0, 20:0; Σ (n-6) PUFA includes 16:2, 20:2, 20:3, 22:5; and Σ (n-3) includes 16:4, 18:3 where one or more of the four values is < 0.5

Table 4.5

The percentage fatty acid composition of the phospholipid classes of Atlantic salmon peripheral blood leucocytes

Fatty acids	Phospholipid Class			
(% Total FA)	PC	PS	PI	PE
14:0	0.7	1.0	0.6	1.6
15:0	0.5	0.3	0.4	2.0
16:0	28.8	15.9	9.2	14.3
18:0	3.9	18.1	24.5	9.3
22:0	-	0.2	-	1.3
Σ saturates	**34.3**	**35.8**	**34.7**	**28.4**
16:1	4.8	1.7	0.5	1.6
18:1	20.0	18.1	6.9	11.8
20:1	1.1	1.5	1.3	4.4
22:1	0.6	0.2	0.3	2.0
24:1	1.5	4.4	1.9	1.3
Σ monoenes	**28.0**	**25.9**	**10.9**	**21.0**
18:2	1.9	2.4	1.7	4.2
18:3	0.5	0.6	1.0	-
20:2	-	0.7	0.5	2.7
20:4	3.1	0.9	13.9	1.3
22:2	0.2	0.7	-	-
22:5	0.5	-	-	1.3
Σ (n-6) PUFA	**6.8**	**6.0**	**17.3**	**9.4**
16:3	0.5	0.8	0.3	-
20:4	0.5	0.3	0.2	1.3
20:5	7.3	3.6	6.9	2.7
22:5	2.5	2.9	2.3	1.6
22:6	12.3	24.1	26.8	35.6
Σ (n-3) PUFA	**23.6**	**32.3**	**37.1**	**41.2**
(n-3)/(n-6)	**3.5**	**5.3**	**2.1**	**4.4**

Values are for a pooled sample of three fish.

Abbreviations: PC-phosphatidylcholine, PS-phosphatidylserine, PI-phosphatidylinositol, PE-phosphatidylethanolamine. In addition to fatty acids shown, Σ saturates includes 20:0; Σ (n-6) PUFA includes 16:2, 20:3; Σ (n-3) includes 18:3, 18:4; where one or more of the four values is < 0.5

level.

Similarly, in leucocytes PE had the highest level of (n-3) PUFA unsaturation, and the lowest level of SFA, but the content of (n-3) PUFA (41.2 %) was notably less than that observed in erythrocyte PE (67.4 %). Levels of 16:0 were highest in PC, while PS and PI contained high levels of 18:0. As in erythrocytes, PI contained an elevated (n-6) PUFA level due to a high content of 20:4(n-6). With the exception of PI, the level of (n-3) PUFA was notably lower in all phospholipids of leucocytes than in those of erythrocytes. The major fatty acids found in the erythrocytes and leucocytes of Atlantic salmon are summarised in Figures 4.3 and 4.4 respectively.

4.3 LIPID COMPOSITION OF HEADKIDNEY MACROPHAGES AND T AND B CELLS

Macrophages were isolated from the headkidney of six Atlantic salmon parr (mean weight 35 g) which had been maintained at a water temperature of 14 °C, on a commercial diet (Ewos No 4 crumb). Headkidney from the same fish population was used for T and B cell isolation using the relevant methodology described in section 3.2. Cell suspensions were adhered to plastic for 2 hours before panning, to remove resident macrophages. Although 1.1 $\pm 0.05 \times 10^6$ adhering cells were obtained (mean value over the six fish), the purity of the macrophages was not assessed. After panning, the purity of the adherent and non adherent fractions were assessed by FITC-labelling of cells with a monoclonal against sIg^+ B-like cells. Further details of the method are found in section 3.7.4. The adherent fraction (B cell population) contained 84.3 % ±4.3

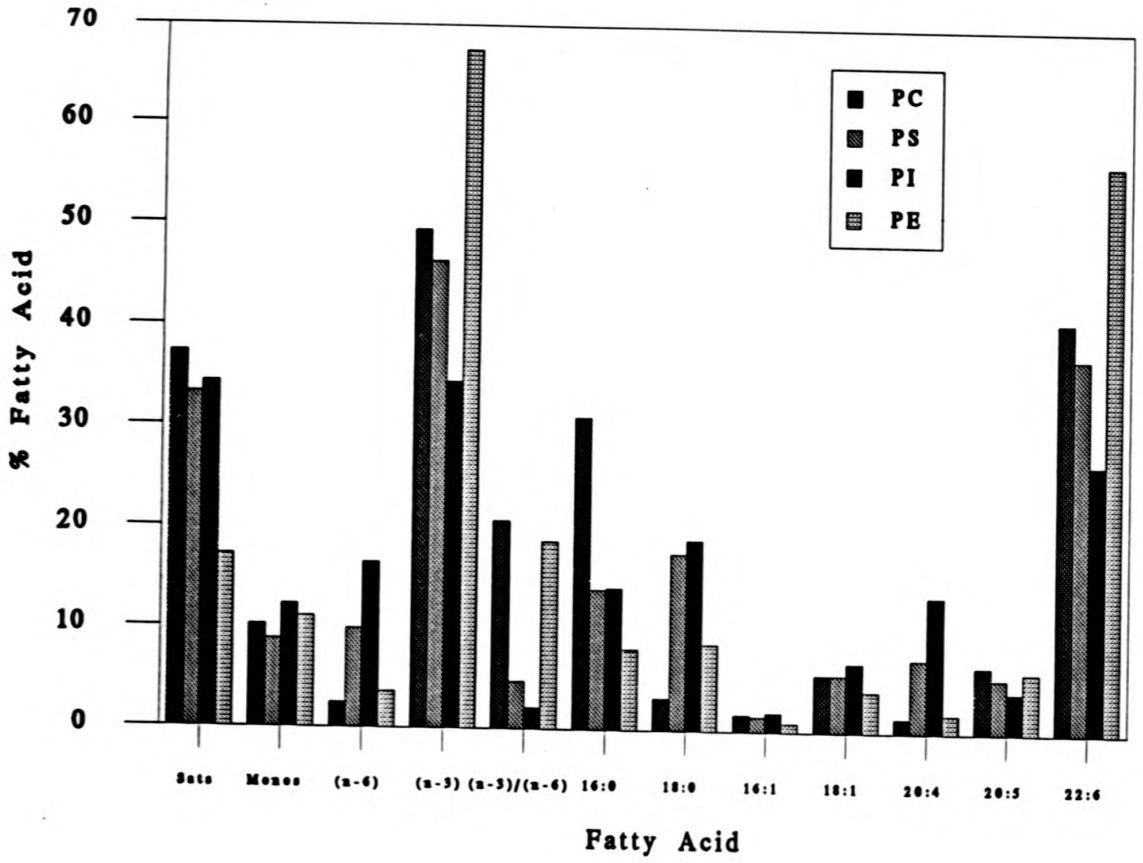


Figure 4.3 The percentage fatty acid composition of the lipid classes of Atlantic salmon erythrocytes.

Abbreviations: Sats- total saturated fatty acids, Monos- total monoenoic fatty acids, (n-6)- total (n-6), (n-3)- total (n-3) PUFA, 20:4(n-6), 20:5(n-3), 22:6(n-3). Values are for a pooled sample of three fish.

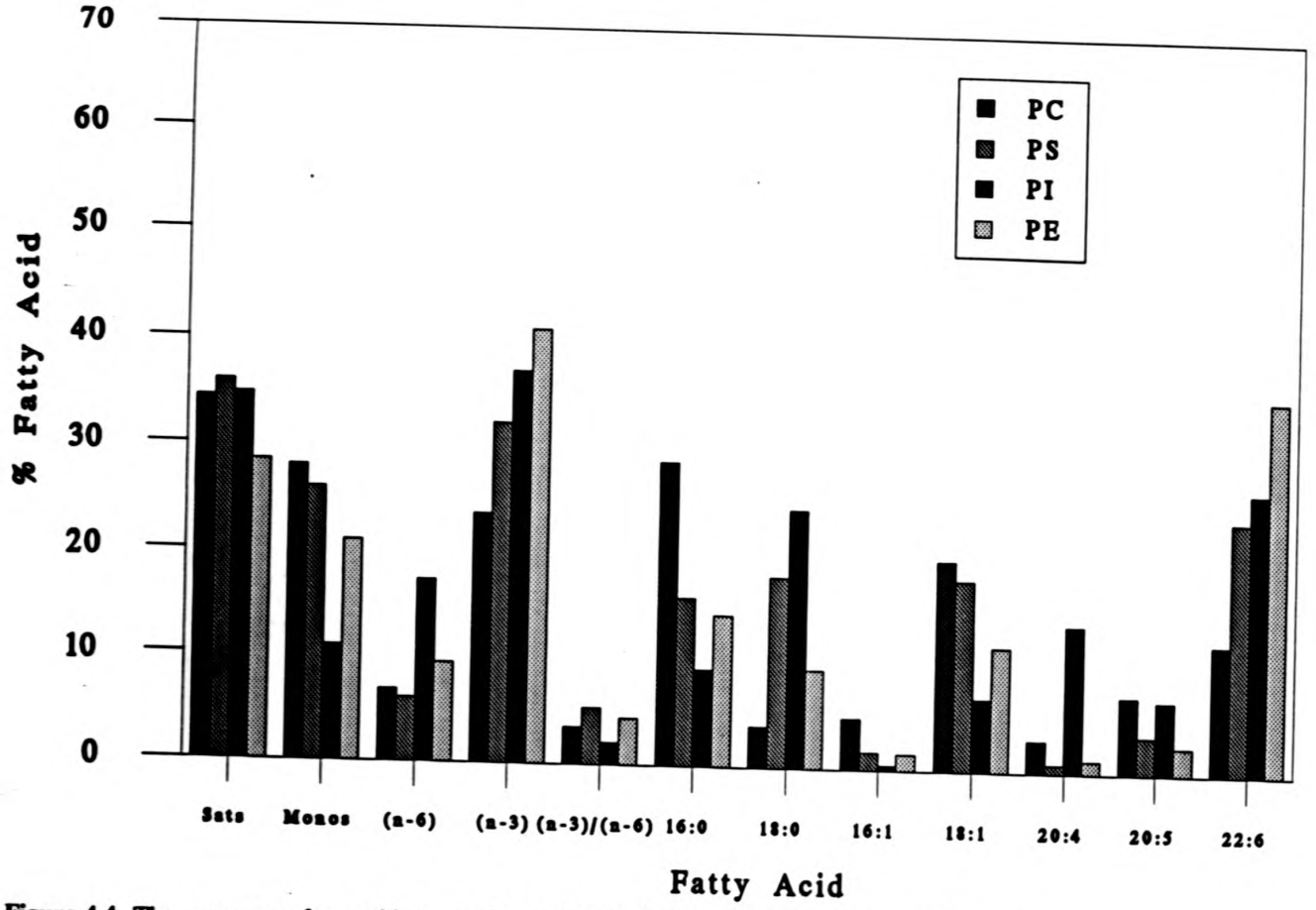


Figure 4.4 The percentage fatty acid composition of the lipid classes of Atlantic salmon leucocytes.

Abbreviations: Sats- total saturated fatty acids, Monos- total monoenoic fatty acids, (n-6)- total (n-6), (n-3)- total (n-3) PUFA, 20:4(n-6), 20:5(n-3), 22:6(n-3). Values are for a pooled sample of three fish.

(for 3 samples) Ig positive cells, while the nonadherent fraction (T cell population) contained 21.9 % ±6.4 (for 3 samples) Ig positive cells. Cell numbers obtained were 2.5 ±0.4 $x10^6$ adhering cells and 6.7 ±0.2 $x10^6$ nonadhering cells. Macrophage and granulocyte contamination was not determined before lipid analysis.

4.3.1 Lipid class composition

The lipid class compositions of the headkidney macrophages and T and B cells were determined by HPTLC for three samples, each consisting of pooled extracted lipid from two fish. Percentages of the lipid classes are presented in Table 4.6. Unpurified headkidney leucocytes, containing a mixed population of lymphocytes and macrophages, were also included in the study.

Macrophages contained a higher percentage of polar lipid in their cellular lipid than the unpurified leucocytes and the T and B lymphocytes, due mainly to high proportions of PC and PE, and a lower cholesterol content. T and B cells had notably higher proportions of cerebrosides in their lipid than macrophages and leucocytes. The high standard deviations which were incurred, especially with the T cells, were attributed to the small lipid samples used for the analysis.

4.3.2 Fatty acid composition

Lipid extracts remaining after lipid class analysis in section 4.3.1 were pooled and used for fatty acid analysis. Values are expressed in Table 4.7 as the mean percent of total fatty acids for pooled samples from six fish. Although insufficient lipid samples meant sample variation could not be determined, there were no marked differences between

Table 4.6

The percentage lipid class composition of Atlantic salmon head kidney leucocytes, macrophages and T and B cells

Lipid class	Leucocytes	Macrophages	T cells	B cells
Total polar lipid	33.0 ±5.7	55.1 ±5.3	39.8 ±14.1	30.2 ±2.3
Total neutral lipid	67.0 ±5.7	44.9 ±5.3	60.2 ±14.1	69.8 ±2.3
Polar lipid:				
LPC	-	0.3 ±0.3	-	-
SM	2.1 ±0.0	1.7 ±0.7	3.5 ±2.5	1.6 ±0.8
PC	13.1 ±0.8	26.2 ±1.3	17.7 ±7.0	14.6 ±1.8
PS	3.1 ±0.9	3.2 ±1.4	2.7 ±1.0	2.1 ±0.3
PI	1.5 ±0.6	2.2 ±0.9	3.1 ±1.6	1.8 ±0.0
Cardiolipin	3.4 ±1.5	4.2 ±1.2	3.5 ±1.4	2.2 ±1.0
PE	8.5 ±1.2	14.4 ±2.6	4.6 ±1.7	3.7 ±0.6
Sulphatides	0.7 ±0.4	2.4 ±0.7	1.5 ±0.3	1.1 ±0.3
Cerebrosides	0.6 ±0.2	0.6 ±3.7	3.3 ±0.4	3.1 ±0.8
Neutral lipid:				
Cholesterol	23.1 ±1.3	15.1 ±1.7	24.5 ±2.3	25.4 ±3.2
Free fatty acids	15.9 ±1.1	6.6 ±0.7	5.0 ±1.5	10.8 ±0.9
Triacylglycerols	19.1 ±2.6	16.1 ±2.0	13.8 ±7.7	17.4 ±0.6
Sterol esters	8.9 ±1.2	6.3 ±1.8	16.2 ±8.3	16.1 ±5.6
Unknowns	0.5 ±0.4	0.8 ±0.2	0.6 ±0.4	0.1 ±0.2

Abbreviations: LPC-lysophosphatidylcholine, SM-sphingomyelin, PC-phosphatidylcholine, PS-phosphatidylserine, PI-phosphatidylinositol, PE-phosphatidylethanolamine.

Values are expressed as the mean percent of total lipid ±SD for three fish.

Table 4.7

The fatty acid composition (wt %) of total lipid from Atlantic salmon head kidney leucocytes, macrophages and T and B cells

Fatty acids (% Total FA)	Leucocytes	Macrophages	T cells	B cells
14:0	2.6	2.7	2.7	3.0
15:0	4.0	0.7	1.6	1.6
16:0	27.6	26.3	30.2	30.1
18:0	6.7	9.5	10.1	10.8
22:0	0.3	0.7	0.2	1.1
24:0	0.3	1.4	0.3	1.2
Σ saturates	**41.7**	**42.7**	**45.4**	**49.2**
16:1	4.4	11.5	8.0	9.8
18:1	16.5	17.7	18.8	15.8
20:1	2.1	1.5	0.8	2.8
22:1	1.3	0.9	1.2	1.2
24:1	1.7	1.9	1.2	2.8
Σ monoenes	**26.0**	**33.4**	**29.9**	**32.3**
16:2	0.8	0.4	0.5	0.0
18:2	2.4	4.4	4.1	5.3
18:3	0.3	0.5	0.2	0.6
20:2	0.3	1.0	0.6	1.7
20:3	0.2	0.0	1.4	0.0
20:4	3.1	0.7	1.5	0.6
Σ (n-6) PUFA	**7.5**	**7.0**	**8.8**	**8.3**
16:3	0.6	0.4	0.3	0.0
18:3	0.4	0.4	0.3	0.7
20:4	0.6	0.0	0.4	0.0
20:5	6.8	2.8	3.4	2.0
22:5	1.5	0.8	0.6	0.4
22:6	13.7	12.5	10.8	7.0
Σ (n-3)	**24.0**	**16.9**	**15.9**	**10.2**
(n-3)/(n-6)	**3.2**	**2.4**	**1.8**	**1.2**

Values are for a pooled sample of three fish except for kidney leucocytes which are the mean percentage for three fish. In addition to the above fatty acids, Σ saturates includes 17:0, 20:0; Σ monoenes includes 14:1; Σ (n-6) PUFA includes 22:2, 22:5; Σ (n-3) includes 18:4; where one or more of the values is < 0.5

the fatty acid composition of the various leucocyte populations. The total lipids of headkidney T and B cells contained greater levels of saturation than those of leucocytes and macrophages, while headkidney leucocytes contained lower monoenoic fatty acid levels and higher (n-3) PUFA levels. The total lipid of T and B cells contained similar levels of (n-6) PUFA, which were slightly higher than those of leucocytes and macrophages.

4.4 COMPARISON OF THE LIPID COMPOSITIONS OF ERYTHROCYTES AND LEUCOCYTES FROM ATLANTIC SALMON AND RAINBOW TROUT.

Erythrocytes and leucocytes were isolated from the peripheral blood of Atlantic salmon parr (potential S2) (mean weight 30 g), and rainbow trout (mean weight 30 g, 150 g or 500 g). Three fish were randomly sampled from each population. Fish had been maintained on commercial diets, Ewos No 4 crumb for salmon, and Ewos 2.5 mm, 4.0 mm and 6.0 mm for rainbow trout (30 g, 150 g, 500 g) respectively. The water temperature was 15 °C for both species.

4.4.1 Lipid class composition

Lipid class analysis, carried out on erythrocyte and leucocyte lipid extract, is presented in Table 4.8. Only the values for the 30 g trout have been included since values for the 150 g and 500 g trout were very similar to those shown. No difference in the lipid class composition of erythrocytes was observed between salmon and trout. Phospholipids, particularly PC, predominated in the erythrocyte lipid of both species. The leucocyte lipid of the salmon, on the other hand, had a notably lower percentage of phospholipid, than the trout. This was attributable to the lower proportions of PE

Table 4.8

A comparison of the percentage lipid class composition between the erythrocytes of Atlantic salmon (30 g) and rainbow trout (30 g)

Lipid class	Erythrocytes		Leucocytes	
% Total lipid	Salmon	Trout	Salmon	Trout
Total polar lipid	76.0 ±4.3	71.2 ±1.6	36.2 ±5.4	51.5 ±9.7
Total neutral lipid	24.0 ±4.3	28.8 ±1.6	63.8 ±5.4	48.5 ±9.7
Polar lipid:				
LPC	-	0.1 ±0.2	1.3 ±0.1	-
SM	3.9 ±0.5	1.6 ±0.1	3.5 ±0.3	2.9 ±0.7
PC	39.2 ±2.2	36.8 ±0.8	15.4 ±3.1	13.2 ±2.6
PS	6.6 ±1.0	6.7 ±1.2	2.6 ±0.9	3.2 ±1.8
PI	5.1 ±0.6	3.4 ±0.3	1.9 ±0.5	1.6 ±0.5
Cardiolipin	1.8 ±0.2	0.1 ±0.1	0.7 ±0.5	-
PE	16.6 ±1.0	19.3 ±1.4	3.6 ±1.7	15.9 ±4.2
Sulphatides	0.6 ±0.6	-	1.3 ±0.5	0.6 ±0.5
Cerebrosides	2.0 ±0.2	3.2 ±0.6	5.8 ±1.2	14.0 ±2.8
Neutral lipid:				
Cholesterol	15.8 ±1.3	19.6 ±1.2	16.3 ±1.4	21.0 ±4.7
Free fatty acids	1.6 ±0.7	2.2 ±0.4	10.0 ±1.7	6.6 ±3.4
Triacylglycerols	4.9 ±4.3	3.7 ±1.7	9.9 ±2.6	8.7 ±4.9
Sterol esters	1.4 ±0.2	3.3 ±1.3	25.5 ±3.8	11.7 ±6.1
Unknowns	0.4 ±0.3	-	2.0 ±1.4	0.5 ±0.5

Abbreviations: LPC-lysophosphatidylcholine, SM-sphingomyelin, PC-phosphatidylcholine, PS-phosphatidylserine, PI-phosphatidylinositol, PE-phosphatidylethanolamine.

Values are expressed as the mean percentage of total lipid ±SD for three fish

(3.6 vs. 15.9 %) and higher proportions of sterol esters (25.5 vs 11.7 %) in the lipid of salmon leucocytes, than in the leucocytes of rainbow trout.

4.4.2 Fatty acid composition

The fatty acid content for the salmon and trout (30 g) erythrocytes was 0.21 ±0.05 $\mu g/1x10^6$ cells and 0.19 ±0.03 $\mu g/1x10^6$ cells, and 2.2 ±0.4 $\mu g/1x10^6$ cells and 1.8 ±0.7 $\mu g/1x10^6$ cells for the salmon and trout leucocytes. Values for the fatty acid composition of total lipid from erythrocytes, leucocytes and diets can be found in Table 4.9.

The fatty acid compositions of total lipid from the erythrocytes of the two fish species were generally similar, whereas the lipid of salmon leucocytes contained more SFA (41.2 %) than those of the trout (29.2 %), which in turn contained levels of higher monoenoic fatty acids than the salmon (40.0 % compared to 31.7 %). However, no significant difference was observed in the (n-3)/(n-6) PUFA ratios of the leucocyte lipids from the two species whereas this ratio was higher in lipid of erythrocytes from salmon than that from trout. The diet of salmon had a higher (n-3)/(n-6) PUFA ratio than the trout diet, since the latter contained more 18-2(n-6) and less 20:5(n-3) and 22:6(n-3). The lipid of erythrocytes of both species had lower levels of monoenoic fatty acids than their dietary lipid, but had higher levels of 22:6(n-3). The lipid of leucocytes from both species was enriched in 18:0 and 18:1 compared with their dietary lipids.

Table 4.9

A comparison of the fatty acid composition between the erythrocytes, leucocytes and diets of Atlantic salmon (30 g) and rainbow trout (30 g)

Fatty acids	Erythrocytes		Leucocytes		Diets	
(% Total FA)	Salmon	Trout	Salmon	Trout	Salmon	Trout
da 16:0	-	0.3 ±0.0	-	2.2 ±0.8	-	-
14:0	1.3 ±0.8	0.5 ±0.1	1.7 ±0.4	0.1 ±0.1	5.6 ±0.1	5.7 ±0.2
15:0	0.3 ±0.1	0.4 ±0.0	2.1 ±0.6	-	0.5 ±0.0	0.5 ±0.1
16:0	21.0 ±0.6	22.0 ±0.6	26.7 ±0.7	9.3 ±1.1	16.5 ±0.1	14.9 ±0.1
17:0	0.2 ±0.1	0.2 ±0.0	0.8 ±0.1	0.5 ±0.3	-	-
18:0	6.3 ±0.6	6.4 ±0.1	8.9 ±0.3	16.0 ±1.6	3.6 ±0.0	3.1 ±0.1
Σ saturates	**29.3 ±0.8**	**30.0 ±0.3**	**41.2 ±0.4**	**29.2 ±0.9**	**26.6 ±0.1**	**25.9 ±1.0**
da18:1	-	0.2 ±0.0	-	3.3 ±2.0	-	-
16:1	2.1 ±0.8	1.7 ±0.3	6.5 ±0.6	4.2 ±2.7	5.8 ±0.0	5.9 ±0.3
18:1	5.9 ±0.8	8.0 ±1.0	19.9 ±0.8	23.3 ±1.4	14.7 ±0.2	15.3 ±0.7
20:1	1.8 ±0.0	2.9 ±0.1	2.2 ±0.3	4.4 ±0.4	6.8 ±0.1	8.7 ±0.3
22:1	1.1 ±0.1	2.2 ±0.2	1.6 ±0.4	1.2 ±0.6	9.7 ±0.3	12.7 ±0.2
24:1	1.5 ±0.0	1.1 ±0.2	1.4 ±0.6	3.6 ±1.0	1.0 ±0.2	1.1 ±0.2
Σ monoenes	**12.4 ±1.7**	**16.0 ±1.1**	**31.7 ±2.5**	**40.0 ±3.6**	**38.2 ±0.6**	**43.7 ±1.1**
18:2	0.5 ±0.0	1.1 ±0.2	1.7 ±0.2	1.9 ±1.1	2.3 ±0.1	4.5 ±0.6
18:3	-	-	1.4 ±0.5	0.3 ±0.2	-	-
20:4	2.9 ±0.3	3.1 ±0.2	2.5 ±0.5	3.8 ±0.7	0.9 ±0.0	0.7 ±0.1
Σ (n-6) PUFA	**4.1 ±0.1**	**5.4 ±0.1**	**7.4 ±0.4**	**8.5 ±0.6**	**5.5 ±0.2**	**7.0 ±0.6**
16:3	-	-	0.9 ±0.1	0.5 ±0.3	-	-
16:4	-	-	-	-	0.9 ±0.1	0.4 ±0.0
18:3	-	-	0.7 ±0.0	0.6 ±0.3	1.1 ±0.0	0.6 ±0.1
18:4	-	-	-	-	2.9 ±0.0	1.4 ±0.1
20:4	0.5 ±0.1	0.6 ±0.1	-	-	0.9 ±0.0	2.8 ±0.1
20:5	7.5 ±1.9	6.3 ±0 7	3.6 ±0.7	3.2 ±0.6	9.9 ±0.1	8.5 ±0.2
22:5	1.5 ±0.0	1.3 ±0.1	0.6 ±0.0	0.8 ±0.2	1.6 ±0.1	1.4 ±0.1
22:6	43.5 ±4.3	39.7 ±1.6	12.7 ±2.2	16.3 ±3.8	12.0 ±0.4	9.8 ±1.0
Σ (n-3) PUFA	**54.3 ±2.0**	**48.5 ±0.9**	**19.7 ±2.8**	**22.4 ±3.9**	**29.9 ±0.6**	**24.5 ±1.1**
(n-3)/(n-6)	**13.2 ±0.7**	**8.9 ±0.3**	**2.6 ±0.5**	**2.6 ±0.6**	**5.4 ±0.4**	**3.5 ±1.1**

Abbreviations: da - dimethyl acetals. Values are expressed as the mean percent of total fatty acids for three fish ±SD. In addition to the fatty acids shown above, Σ saturates include 20:0, 22:0; Σ (n-6) PUFA includes 16:2, 20:2, 20:3, 22:2; were one or more of the six values is < 0.8

4.5 COMPARISON OF THE LIPID COMPOSITIONS OF ERYTHROCYTES AND LEUCOCYTES FROM ATLANTIC SALMON, AFRICAN CATFISH AND RABBIT

Erythrocytes and leucocytes were isolated from the peripheral blood of Atlantic salmon parr (potential S2), African catfish and rabbit. Three parr salmon (mean weight 18 g) were randomly sampled from a stock of fish maintained in freshwater at a temperature of 9 °C and which had been fed a commercial diet (Ewos No. 3 crumb for salmon). African catfish, maintained in freshwater at a temperature of 27 °C, were fed on a commercial diet (Ewos No. 6 mm for trout). Three fish, weighing approximately 300 g, were randomly sampled. Blood was sampled from the ear veins of three rabbits, which had been maintained on a commercial diet. The amount of lipid extracted from salmon, catfish and rabbit erythrocytes was 0.64 ±0.21, 0.52 ±0.1 and 0.42 ±0.1 μg $(10^6$ cells$)^{-1}$ respectively. The lipid extracted from leucocytes was too small to measure accurately.

4.5.1 Lipid class composition

Profiles of lipid classes extracted from erythrocytes and leucocytes are presented in Tables 4.10 and 4.11, respectively. The major lipid classes of the two cell types are presented in Figure 4.5 and Figure 4.6.

The erythrocytes from salmon, catfish and rabbit all contained more polar than neutral lipid, with the erythrocytes of catfish containing the highest levels and rabbit the least. Rabbit erythrocytes contained higher levels of cholesterol and SM (and/or LPC since SM and LPC were unresolved) than the fish RBCs. Although lysophosphatidylcholine

Table 4.10

A comparison of the lipid class composition between the erythrocytes of Atlantic salmon, African catfish, and rabbit.

Lipid class (% Total lipid)	Atlantic Salmon	African Catfish	Rabbit
Total polar lipid	56.4 ±2.6	60.6 ±0.6	51.5 ±1.7
Total neutral lipid	43.6 ±2.6	39.4 ±0.6	48.5 ±1.7
Polar lipid:			
LPC	0.5 ±0.3	0.9 ±0.6	10.0 ±0.4]
SM	2.6 ±0.3	2.0 ±0.5	]
PC	21.6 ±0.2	21.7 ±1.5	12.0 ±1.4
PS	7.6 ±0.5	8.2 ±1.0	6.3 ±0.4
PI	5.5 ±0.8	5.6 ±0.2	2.7 ±0.2
Cardiolipin	-	0.3 ±0.4	1.8 ±0.3
PE	18.6 ±1.6	18.7 ±1.6	12.9 ±0.7
Sulphatides	-	0.9 ±0.2	2.3 ±0.3
Cerebrosides	-	2.1 ±0.6	3.5 ±1.3
Neutral lipid:			
Cholesterol	20.4 ±0.8	31.6 ±1.2	36.2 ±0.7
Free fatty acids	7.9 ±0.1	4.6 ±0.5	3.0 ±0.3
Triacylglycerols	3.3 ±0.7	0.7 ±0.0	0.6 ±0.4
Sterol esters	10.8 ±1.0	2.3 ±0.1	8.8 ±2.1
Unknowns	1.2 ±0.2	0.3 ±0.3	-

Abbreviations: LPC-lysophosphatidylcholine, SM-sphingomyelin, PC-phosphatidylcholine, PS-phosphatidylserine, PI-phosphatidylinositol, PE-phosphatidylethanolamine.

Values are expressed as the mean % lipid class ±SD of total lipid for three fish or rabbits. Lysophosphatidylcholine and sphingomyelin were unresolved by HPTLC for rabbit erythrocytes.

Table 4.11

A comparison of the lipid class composition between the leucocytes of Atlantic salmon, African catfish, and rabbit

Lipid class (% Total lipid)	Atlantic Salmon	African Catfish	Rabbit
Total polar lipid	39.7 ±8.4	40.3 ±2.8	45.8 ±2.9
Total neutral lipid	57.7 ±8.4	59.7 ±2.8	54.2 ±2.9
Polar lipid:			
LPC	-	0.7 ±0.5	1.5 ±0.1
SM	1.0 ±0.1	2.0 ±0.6	2.6 ±0.4
PC	15.8 ±2.3	14.6 ±1.1	16.5 ±1.9
PS	2.5 ±1.1	3.7 ±0.2	6.8 ±0.4
PI	1.8 ±0.4	2.4 ±0.5	1.6 ±0.2
Cardiolipin	0.4 ±0.5	2.5 ±0.6	1.3 ±0.9
PE	10.3 ±2.9	9.2 ±1.2	14.0 ±0.4
Sulphatides	2.7 ±0.1	1.8 ±1.3	0.5 ±0.2
Cerebrosides	5.3 ±0.9	3.4 ±2.7	0.9 ±0.0
Neutral lipid:			
Cholesterol	17.4 ±1.7	25.7 ±4.6	47.3 ±1.0
Free fatty acids	11.1 ±2.1	11.3 ±3.3	3.6 ±0.1
Triacylglycerols	9.9 ±2.6	1.5 ±0.6	3.0 ±1.6
Sterol esters	19.3 ±6.0	20.4 ±2.5	3.0 ±1.6
Unknowns	2.5 ±0.2	0.9 ±1.2	0.2 ±0.3

Values are expressed as the mean % lipid class ±SD of total lipid for three fish or rabbits. Abbreviations: LPC-lysophosphatidylcholine, SM-sphingomyelin, PC-phosphatidylcholine, PS-phosphatidylserine, PI-phosphatidylinositol, PE-phosphatidylethanolamine.

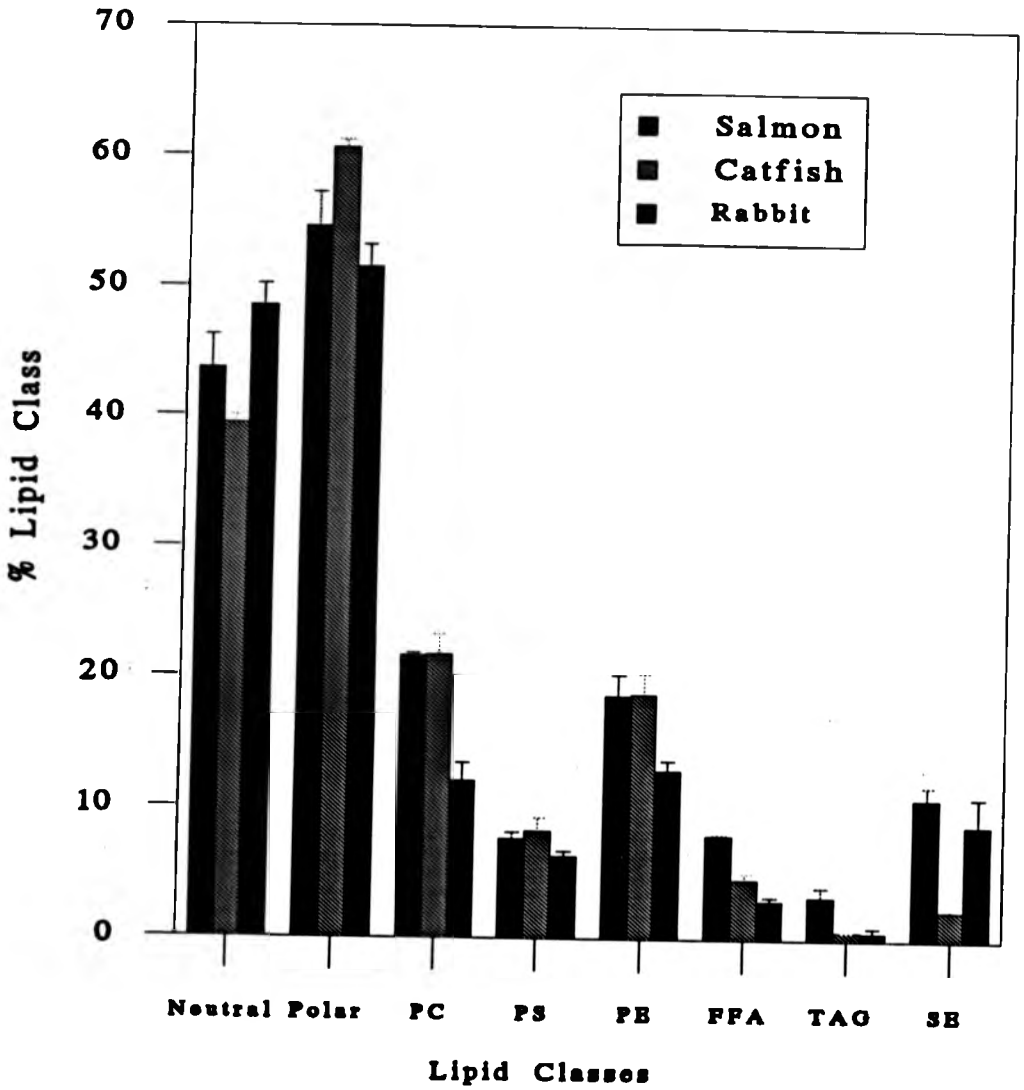


Figure 4.5 Differences in the percentage lipid class composition between the erythrocytes of Atlantic salmon, African catfish, and rabbit.

Abbreviations: Neutral- total neutral lipid, Polar- total polar lipid, PC-phosphatidylcholine, PS-phosphatidylserine, PE-phosphatidylethanolamine, FFA-free fatty acids, TAG-triacylglycerols, SE-sterol esters. Values are expressed as the mean % lipid class ±SD of total lipid for three fish or rabbits.

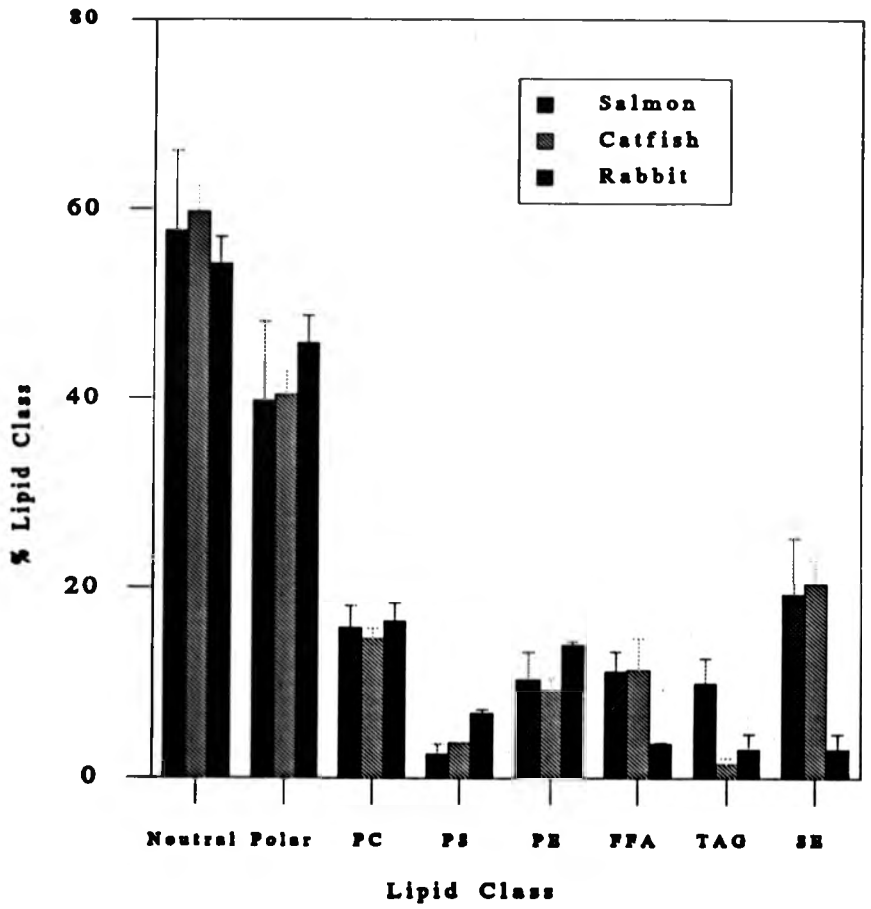


Figure 4.6 Differences in the percentage lipid class composition between the leucocytes of Atlantic salmon, African catfish, and rabbit.

Abbreviations: Neutral- total neutral lipid, Polar- total polar lipid, PC-phosphatidylcholine, PS-phosphatidylserine, PE-phosphatidylethanolamine, FFA-free fatty acids, TAG-triacylglycerols, SE-sterol esters. Values are expressed as the mean % lipid class ±SD of total lipid for three fish or rabbits.

and sphingomyelin in rabbit erythrocytes were unresolved by densitometry, they were distinguishable by eye and the LPC fraction contributed only a small part of the overall value. The lipid of erythrocytes of salmon contained a higher proportion of TAG and a lower proportion of cholesterol than the lipid of catfish and rabbit erythrocytes. The lipid from both the rabbit and salmon erythrocytes contained higher fractions of cholesterol esters than that of the catfish erythrocytes.

With all 3 species, the lipid class composition of leucocyte lipid differed from that of erythrocyte lipid. Most obviously, the lipid of leucocytes of salmon, catfish and rabbit all contained greater levels of neutral lipid than phospholipid. However, while all three species had similar overall levels of total neutral lipid in leucocyte lipid, the fish leucocytes had higher TAG, FFA and SE levels than that of the rabbit which had higher cholesterol levels (47.3 %). The lipid of salmon leucocytes contained higher percentages of TAG than those of the catfish (9.9 vs. 1.5 %). The phospholipid patterns of the leucocytes from the three species were similar.

4.5.2 Fatty acid composition

Lipid extract remaining from section 4.5.1 was used to determine the fatty acid components of lipid from salmon, catfish and rabbit erythrocytes and leucocytes and these are presented in Table 4.12 and Table 4.13, respectively. The fatty acid content of the erythrocytes and leucocytes, determined using an internal standard (23:0), were 0.4 ±0.1, 0.2 ±0.02 and 0.14 ±0.1 $\mu g/1x10^6$ cells for the erythrocytes of salmon, catfish and rabbit respectively, and 1.9 ±0.1, 2.5 ±0.5 and 7.8 ±1.3 $\mu g/1x10^6$ cells for their corresponding leucocytes. Thus, the erythrocyte of all three species had similar fatty

Table 4.12

A comparison of the fatty acid composition of lipid from the erythrocytes of Atlantic salmon, African catfish, and rabbit.

Fatty acids (% Total FA)	Atlantic Salmon	African Catfish	Rabbit
da16:0	-	0.8 ±0.2	0.4 ±0.3
14:0	0.6 ±0.2	1.5 ±0.1	0.4 ±0.1
16:0	20.8 ±3.5	20.4 ±0.2	25.8 ±0.6
18:0	4.2 ±1.1	12.4 ± 0.3	16.7 ±0.3
24:0	1.2 ±0.7	0.8 ±0.2	0.4 ±0.3
Σ saturates	**28.2 ±3.2**	**36.0 ±0.6**	**44.3 ±0.9**
14:1	0.7 ±0.5	0.4 ±0.1	1.4 ±0.3
16:1	2.1 ±0.6	2.0 ±0.2	1.3 ±0.3
18:1	6.9 ±2.5	21.8 ±0.5	14.4 ±0.4
20:1	1.4 ±0.3	2.9 ±0.3	0.9 ±0.2
22:1	0.8 ±0.2	0.9 ±0.7	0.6 ±0.2
24:1	1.3 ±0.2	0.5 ±0.1	1.3 ±0.8
Σ monoenes	**13.2 ±3.0**	**28.6 ±0.9**	**20.0 ±1.4**
16:2	1.5 ±0.6	0.4 ±0.0	1.1 ±0.0
18:2	0.2 ±0.6	2.4 ±0.2	25.2 ±1.1
20:2	0.2 ±0.2	0.2 ±0.0	0.8 ±0.1
20:4	2.2 ±0.7	5.0 ±0.4	4.1 ±0.2
Σ (n-6) PUFA	**5.2 ±1.7**	**9.0 ±0.5**	**32.3 ±1.5**
18:3	0.2 ±0.2	0.3 ±0.0	0.7 ±0.1
20:4	0.5 ±0.0	0.5 ±0.2	-
20:5	7.7 ±0.9	14.6 ±0.9	0.6 ±0.2
22:5	2.7 ±1.2	1.0 ±0.0	0.6 ±0.1
22:6	41.4 ±2.4	9.2 ±0.1	0.8 ±0.2
Σ (n-3) PUFA	**53.5 ±1.2**	**26.5 ±0.8**	**3.4 ±0.2**
(n-3)/(n-6)	**10.4 ±3.6**	**3.0 ±0.3**	**0.1 ±0.0**

Abbreviations: da - dimethyl acetals.

Values are expressed as the mean % of total fatty acids ±SD for 3 fish or rabbits. In addition to the above fatty acids, Σ saturates include 15:0, 20:0, 22:0, Σ (n-6) PUFA includes 18:3, 20:3, 22:2, 22:5, Σ (n-3) include 16:3, 16:4, 18:4 where one or more of the three values is < 0.5

Table 4.13

A comparison of the fatty acid composition of lipid from the leucocytes of Atlantic salmon, African catfish, and rabbit.

Fatty acids (% Total FA)	Atlantic Salmon	African Catfish	Rabbit
da16:0	-	0.5 ±0.5	-
14:0	1.7 ±0.8	0.3 ±0.2	0.7 ±0.2
15:0	1.3 ±0.2	0.7 ±0.2	0.9 ±0.4
16:0	20.6 ±2.6	19.1 ±1.8	17.7 ±0.4
17:0	2.0 ±1.0	-	-
18:0	11.3 ±0.6	12.0 ±1.2	19.3 ±0.5
20:0	0.8 ±0.1	0.6 ±0.2	0.4 ±0.0
22:0	1.0 ±0.1	1.0 ±0.2	0.8 ±0.1
24:0	1.1 ±1.5	-	0.8 ±0.1
Σ saturates	**39.7 ±3.5**	**34.2 ±3.2**	**40.6 ±0.1**
da18:1	-	0.6 ±0.5	-
14:1	-	-	0.8 ±1.2
16:1	5.9 ±1.0	5.6 ±1.7	4.7 ±2.2
18:1	17.2 ±0.5	16.6 ±2.0	12.3 ±0.5
20:1	2.5 ±0.5	3.7 ±1.4	1.1 ±0.1
22:1	2.2 ±0.5	4.0 ±2.8	0.8 ±0.1
24:1	1.6 ±0.4	1.2 ±0.4	1.4 ±0.2
Σ monoenes	**29.5 ±2.1**	**31.8 ±5.5**	**21.0 ±0.3**
16:2	1.0 ±0.4	0.5 ±0.2	0.9 ±0.0
18:2	4.6 ±1.0	3.6 ±0.5	19.4 ±1.8
18:3	0.3 ±0.4	0.2 ±0.0	0.5 ±0.1
20:2	0.3 ±0.2	0.3 ±0.1	0.7 ±0.1
20:3	0.3 ±0.3	0.3 ±0.1	1.0 ±0.1
20:4	2.0 ±0.7	3.7 ±0.7	9.9 ±0.8
22:2	-	0.7 ±0.2	0.2 ±0.0
22:4	-	0.1 ±0.1	0.7 ±0.1
22:5	0.2 ±0.0	0.4 ±0.0	0.4 ±0.0
Σ (n-6) PUFA	**8.7 ±1.9**	**9.9 ±1.1**	**33.7 ±1.1**

Table 4.13 (cont.)

A comparison of the fatty acid composition of lipid from the leucocytes of Atlantic salmon, African catfish, and rabbit.

Fatty acids (% Total FA)	Atlantic salmon	African catfish	Rabbit
16:3	1.6 ±0.5	0.7 ±0.2	0.6 ±0.2
16:4	1.5 ±0.8	1.1 ±0.8	-
18:3	1.4 ±0.5	0.5 ±0.0	0.5 ±0.0
18:4	0.1 ±0.2	0.2 ±0.2	-
20:4	0.2 ±0.1	1.8 ±2.0	-
20:5	3.9 ±0.9	7.7 ±2.3	1.3 ±0.5
22:5	0.9 ±0.2	1.2 ±0.2	1.0 ±0.0
22:6	12.7 ±2.1	10.9 ±1.7	1.3 ±0.2
Σ (n-3) PUFA	**21.1 ±4.4**	**24.1 ±5.8**	**4.7 ±0.9**
(n-3)/(n-6)	**2.5 ±1.1**	**2.4 ±0.8**	**0.1 ±0.0**

Abbreviations: da - dimethyl acetals. Values are expressed as the mean % of total fatty acids ±SD for 3 fish or rabbits.

acid contents but rabbit leucocytes had a higher content than those from the two fish species. The total lipid of rabbit erythrocytes had a higher content of saturated fatty acids than those of the fish erythrocytes. The lipid of catfish and rabbit erythrocytes had higher proportions of monounsaturated fatty acids than that of salmon erythrocytes, due mainly to their 18:1 content. No great difference was observed between the SFA of the three leucocytes populations, but the rabbit leucocytes contained lower levels of monoenoic fatty acids in their lipid. Both the erythrocytes and leucocytes from salmon, catfish and rabbit contained 16:0 as their main SFA, with the exception of the lipid from rabbit leucocytes, which contained higher levels of 18:0. The erythrocytes of the catfish contained substantially higher percentages of 18:0 and 18:1 in their lipid than those of salmon.

Lipid from triplicate samples of diets, used to maintain the salmon (Ewos No. 3), catfish (Ewos 6 mm for trout) and rabbits (4 mm pellet, SDS, Witham, U.K.) diets extracted and their total fatty acids analysed (Table 4.14). The overall percentage SFA in each diet was similar. However, whereas the monoenoic fatty acids of the fish diets were composed of 16:1, 18:1, 20:1 and 22:1(n-11), 18:1 predominated in the rabbit diet. The influence of diet on the fatty acid composition of erythrocytes and leucocytes can be seen in the fatty acid profiles of these cells. The rabbit diet had high proportions of 18:2(n-6) and very little (n-3) PUFA in its lipid content, while the lipids of fish diets were rich in 20:5(n-3) and 22:6(n-3). The fatty acids of rabbit erythrocytes resembled those of its diet by having a high percentage (3.2 %) of (n-6) PUFA. Likewise, the erythrocytes of fish had higher levels of (n-3) PUFA, reflected in their (n-3)/(n-6) PUFA ratio of 13.2 ±0.7 for salmon erythrocytes, 3.0 ±0.3 for catfish RBCs, compared with

Table 4.14

The percentage fatty acid composition of the diets of Atlantic salmon, African catfish, and rabbit

Fatty acids (% Total FA)	Atlantic Salmon Diet	African Catfish Diet	Rabbit Diet
14:0	5.6 ±0.1	5.7 ±0.1	0.6 ±0.0
15:0	0.5 ±0.0	0.5 ±0.1	-
16:0	16.5 ±0.1	14.9 ±0.1	19.5 ±0.7
18:0	3.6 ±0.0	3.1 ±0.1	2.0 ±0.1
22:0	0.1 ±0.1	0.2 ±0.2	0.3 ±0.0
Σ saturates	**26.6 ±0.1**	**24.8 ±0.6**	**22.8 ±1.0**
16:1	5.8 ±0.0	5.9 ±0.3	0.5 ±0.4
18:1	14.7 ±0.2	15.3 ±0.7	21.3 ±0.1
20:1	6.8 ±0.1	8.7 ±0.3	0.6 ±0.1
22:1	9.7 ±0.3	12.7 ±0.2	0.2 ±0.1
24:1	1.0 ±0.2	1.1 ±0.0	-
Σ monoenes	**38.2 ±0.6**	**43.7 ±1.1**	**23.5 ±0.2**
16:2	0.8 ±0.3	0.4 ±0.1	0.2 ±0.2
18:2	2.3 ±0.1	4.5 ±0.6	46.4 ±1.1
20:4	0.9 ±0.0	0.7 ±0.1	-
22:2	0.5 ±0.1	0.5 ±0.2	-
22:5	0.4 ±0.1	-	-
Σ (n-6) PUFA	**5.5 ±0.2**	**7.0 ±0.6**	**46.6 ±1.1**
16:4	0.9 ±0.1	0.4 ±0.0	-
18:3	1.1 ±0.0	0.6 ±0.1	7.0 ±0.2
18:4	2.9 ±0.0	1.4 ±0.1	-
20:4	0.9 ±0.0	2.8 ±0.1	-
20:5	9.9 ±0.1	8.5 ±0.2	0.2 ±0.1
22:5	1.6 ±0.1	1.4 ±0.1	-
22:6	12.0 ±0.4	9.8 ±1.0	-
Σ (n-3) PUFA	**29.9 ±0.6**	**24.5 ±1.1**	**7.2 ±0.2**
(n-3)/(n-6)	**5.4 ±0.4**	**3.5 ±0.4**	**0.15 ±0.0**

Values are expressed as the means of three samples ±SD. In addition to the fatty acids shown, Σ saturates include 20:0, 22:0, Σ (n-6) PUFA includes 18:3, 20:2, Σ (n-3) include 16:3 where one or more of the values is < 0.5

0.1 ±0.0 for those of the rabbit. The low ratio in rabbit erythrocytes was due to a pronounced 18:2(n-6) level, whereas the high ratio content in the salmon and catfish erythrocytes was due to 22:6(n-3) and 20:5(n-3). The percentage of 22:6(n-3) was higher than the level of 20:5(n-3) in the salmon erythrocytes, but vice versa in the catfish erythrocytes. Rabbit erythrocytes had very little long chain (n-3) PUFA in their lipid (3.4 %).

The lipids of leucocytes followed a similar pattern of unsaturation to those found in the erythrocytes. Thus, the lipid of rabbit leucocytes were enriched in 18:2(n-6), and it's elongation product 20:4(n-6). In contrast, the lipids of fish leucocytes were high in 20:5(n-3) and 22:6(n-3). As previously shown, the proportion of total (n-3) PUFA was much greater in the lipids of erythrocytes of salmon than found in their leucocytes. The levels of 22:6(n-3) were again observed to be slightly higher in salmon leucocytes than the catfish leucocytes, with the latter having higher levels of 20:5(n-3). The lipids of catfish erythrocytes and leucocytes contained similar percentages of total (n-3) PUFA (26.5 % and 26.1 % respectively) . Rabbit leucocyte lipid contained little long chain (n-3) PUFA, and 20:4(n-6) was consequently the most predominant long chain PUFA.

4.6 COMPARISON OF THE LIPID COMPOSITION OF ERYTHROCYTES AND LEUCOCYTES IN ATLANTIC SALMON: EFFECT OF TEMPERATURE

Atlantic salmon (potential S2) (mean weight 25 g) were maintained on a commercial diet, in tanks containing 150 l water, with a flow rate of 1.5 l min^{-1}. Fish from stock maintained at a water temperature of 2°C, were divided equally into two groups. One group was maintained at 2°C and the other was acclimated to 12°C over a 2 day period, using 2 tank heaters. Fish in both groups were killed and analysed after 35 days. Methods used for blood collection, cell separation (i.e erythrocytes and peripheral blood leucocytes) and lipid analysis were as described previously.

4.6.1 Lipid class composition

Lipid from erythrocytes had a higher phospholipid content than leucocytes, being particularly rich in PC and PE (Table 4.15). The proportion of PE in erythrocytes was significantly higher at 2°C than 12°C (13.8 % vs. 12.3 %), but no statistically significant difference in the lipid composition of leucocytes was observed in relation to temperature.

4.6.2 Fatty acid composition

Total fatty acids content for each group of erythrocytes and leucocytes, determined from an internal standard (23:0), were 0.19 ±0.1 and 0.13 ±0.04 $\mu g/1x10^6$ cells for erythrocytes at 2°C and 12°C respectively, and 4.4 ±0.6 and 3.4 ±0.5 $\mu g/1x10^6$ cells for leucocytes at 2°C and 12°C respectively.

Differences existed between erythrocytes and leucocytes, in the fatty acid composition

Table 4.15

A comparison of the lipid class composition of erythrocytes and leucocytes from Atlantic salmon maintained at 2°C and 12°C.

Lipid class	Erythrocytes		Leucocytes	
(% Total lipid)	2°C	12°C	2°C	12°C
Total polar lipid	60.0 ±1.8	55.2 ±4.2	32.4 ±10.8	30.7 ±5.5
Total neutral lipid	40.0 ±1.8	44.8 ±4.2	67.6 ±10.8	69.3 ±5.5
Polar lipid:				
LPC	0.5 ±0.2	0.5 ±0.0	1.1 ±0.6	-
SM	1.1 ±1.1	0.3 ±0.1	4.0 ±1.2	8.1 ±1.2
PC	29.9 ±2.5	33.4 ±2.0	10.2 ±4.9	8.7 ±1.8
PS	6.0 ±2.6	3.3 ±0.8	2.3 ±1.3	2.4 ±0.6
PI	4.4 ±1.9	2.3 ±0.7	1.4 ±0.8	1.6 ±0.7
Cardiolipin	2.7 ±0.4	1.7 ±0.4	1.6 ±1.1	1.3 ±0.7
PE	13.8 ±0.2*	12.3 ±0.4*	3.5 ±2.3	2.4 ±1.1
Sulphatides	0.5 ±0.3	0.5 ±0.0	2.0 ±0.4	2.1 ±0.2
Cerebrosides	1.3 ±0.9	1.0 ±0.4	6.2 ±1.6	4.0 ±0.7
Neutral lipid:				
Cholesterol	19.9 ±1.8	20.8 ±0.7	16.6 ±1.9	14.7 ±2.0
Free fatty acids	5.0 ±0.7	6.4 ±0.4	8.3 ±2.2	10.3 ±0.6
Triacylglycerols	13.9 ±1.3]	17.5 ±4.4]	13.5 ±3.0	9.2 ±6.9
Sterol esters	]	]	28.3 ±7.1	34.0 ±3.3
Unknowns	1.3 ±0.6	0.1 ±0.2	0.8 ±1.2	1.1 ±0.7

Abbreviations: LPC-lysophosphatidylcholine, PC-phosphatidylcholine, PS-phosphatidylserine, PI-phosphatidylinositol, PE-phosphatidylethanolamine. Values are expressed as the mean % lipid class of total lipid ±SD for three fish. Triacylglycerols and sterol esters, from the erythrocytes, were unresolved by TLC. Data was analysed by Student 't' test * $0.05 \leq p \geq 0.01$

of total lipid (Table 4.16), as previously observed, whereby the lipid of erythrocytes had a higher degree of unsaturation than that of the leucocytes, due to its high content of 22:6(n-3) and 20:5(n-3). Conversely, leucocyte lipid possessed higher proportions of 18:0 and 18:1, than that of erythrocytes at both temperatures. However, no significant effect on the degree of unsaturation of total lipid within either cell population was observed in relation to temperature.

Table 4.16

A comparison of the fatty acid composition of erythrocytes and leucocytes from Atlantic salmon maintained at 2°C and 12°C

Fatty acids	Erythrocytes		Leucocytes	
(% Total FA)	2°C	12°C	2°C	12°C
14:0	1.1 ±0.3	0.5 ±0.2	1.7 ±0.5	2.5 ±0.2
15:0	0.6 ±0.0	0.3 ±0.0	1.4 ±0.3	0.9 ±0.6
16:0	26.2 ±0.4	24.5 ±0.9	24.8 ±2.5	26.3 ±1.2
17:0	0.3 ±0.2	0.2 ±0.2	0.9 ±0.3	1.2 ±0.2
18:0	5.4 ±0.5	6.1 ±0.7	11.1 ±0.1	11.4 ±0.8
20:0	0.1 ±0.1	0.0 ±0.1	0.9 ±0.1	0.2 ±0.3
22:0	0.1 ±0.1	-	1.0 ±0.1	1.1 ±0.2
Σ saturates	**33.9 ±1.1**	**31.7 ±1.4**	**41.8 ±1.8**	**43.6 ±0.2**
16:1	2.8 ±0.4	1.9 ±0.2	6.6 ±1.4	9.3 ±3.4
18:1	6.0 ±0.4	6.0 ±0.4	21.2 ±2.5	21.3 ±0.8
20:1	1.1 ±0.7	1.7 ±0.1	2.6 ±0.4	2.1 ±0.3
22:1	1.1 ±0.2	0.9 ±0.1	1.6 ±0.1	1.6 ±0.4
24:1	2.3 ±0.2	2.0 ±0.1	3.0 ±0.9	1.0 ±1.5
Σ monoenes	**13.4 ±0.8**	**12.5 ±0.3**	**35.0 ±2.2**	**35.3 ±2.1**
16:2	0.2 ±0.2	0.2 ±0.2	0.9 ±0.3	2.0 ±0.9
18:2	0.9 ±0.0	1.2 ±0.4	3.6 ±1.4	2.5 ±0.1
18:3	0.0 ±0.1	0.1 ±0.1	0.6 ±0.3	1.9 ±0.9
20:2	0.2 ±0.0	0.2 ±0.1	1.0 ±0.7	0.4 ± 0.6
20:4	1.8 ±0.4	1.9 ±0.4	0.7 ±0.1	0.3 ±0.5
22:2	0.4 ±0.3	0.4 ±0.0	2.9 ±1.0	2.6 ±0.8
Σ (n-6) PUFA	**3.7 ±0.3**	**4.3 ±0.4**	**10.7 ±0.7**	**10.0 ±0.4**
16:3	0.3 ±0.2	0.3 ±0.2	1.1 ±0.3	1.3 ±0.0
18:3	0.2 ±0.0	0.3 ±0.2	1.0 ± 0.9	1.8 ±1.3
20:5	6.8 ±0.2	6.9 ±0.6	2.0 ±0.3	1.5 ±0.4
22:5	1.3 ±0.1	1.4 ±0.1	1.7 ±0.8	1.4 ±0.3
22:6	39.7 ±0.6	42.4 ±1.8	5.4 ±0.9	4.9 ±2.5
Σ (n-3) PUFA	**48.9 ±0.5**	**51.5 ±1.7**	**12.5 ±1.6**	**11.1 ±1.5**
(n-3)/(n-6)	**13.1 ±1.2**	**12.0 ±1.5**	**1.2 ±0.2**	**1.1 ±0.2**

Values are expressed as the mean % of total fatty acids ±SD for 3 fish. In addition to the fatty acids shown, Σ saturates include 24:0, Σ (n-6) PUFA includes 20:3, 22:5; Σ (n-3) include 16:4, 18:4, 20:4, where one or more of the four values is < 0.8

4.7 A COMPARISON OF THE LIPID COMPOSITION OF LEUCOCYTES FROM ATLANTIC SALMON FRESHWATER PARR AND MARINE SMOLTS

Three parr salmon (mean weight 18 g) were randomly sampled from a stock of fish, which had been maintained in freshwater at a temperature of 9 °C and fed on a commercial diet (Ewos No. 3 crumb). Marine Atlantic salmon smolts were provided courtesy of J.G. Bell (NERC unit, University of Stirling) and the culture conditions and diet preparations of these fish are described elsewhere (Bell *et al.*, in press). Diets fed to the smolts contained either fish oil (Fosol, Seven Seas Ltd.) (diet 1), sunflower oil (Tesco, Cheshunt, U.K.) (diet 2) or linseed oil (ICN Biomedical Ltd., High Wycombe, U.K.) (diet 3) at 100g oil / kg diet. Each diet contained 47 % protein and 16 % lipid, and the fatty acid profiles of the diets are shown in Table 4.17. Three smolts (≈ 250 g) taken from a water temperature of 10°C, were sampled from each dietary group.

4.7.1 Lipid class composition

The lipid class composition of the total leucocyte lipid is shown in Table 4.18. Leucocyte lipid from the freshwater salmon parr contained significantly lower proportions of phospholipid than the corresponding lipid from marine smolt leucocytes. This was reflected by the lowers levels of PC and PE in lipid from freshwater leucocytes. TAG and sterol esters proportions, on the other hand, were higher in the leucocyte lipid of the freshwater parr than smolts, although cholesterol was more abundant in the lipid of the latter fish.

4.7.2 Fatty acid composition

The total lipid of leucocytes from the fish characterised in the above section, were

Table 4.17

The percentage fatty acid composition of Atlantic salmon diets.

Fatty acids	Freshwater	Marine Salmon		
(% Total FA)	Salmon	Diet 1	Diet 2	Diet 3
14:0	5.6	6.6	1.8	1.8
15:0	0.5	-	-	-
16:0	16.5	14.8	9.5	9.1
18:0	3.6	2.4	4.3	3.5
Σ saturates	**26.6**	**24.6**	**15.9**	**14.9**
16:1	5.8	5.1	2.0	2.0
18:1	14.7	11.7	18.8	18.6
20:1	6.8	10.5	3.1	3.1
22:1	9.7	16.6	4.8	4.4
24:1	1.0	1.0	0.6	0.5
Σ monoenes	**38.2**	**45.2**	**29.5**	**28.7**
16:2	0.8	-	-	-
18:2	2.3	1.4	40.3	12.2
20:4	0.9	0.6	0.5	0.5
22:2	0.5	-	-	-
Σ (n-6) PUFA	**5.5**	**2.8**	**41.1**	**12.9**
16:4	0.9	-	-	-
18:3	1.1	1.3	0.5	31.8
18:4	2.9	3.1	1.0	0.9
20:4	0.9	0.7	0.2	0.2
20:5	9.9	6.5	3.6	3.4
22:5	1.6	0.9	0.5	0.5
22:6	12.0	8.8	4.9	4.5
Σ (n-3) PUFA	**29.9**	**21.3**	**10.7**	**41.3**
(n-3)/(n-6)	**5.4**	**7.6**	**0.3**	**3.2**

Values are expressed as means % of total fatty acids for 3 diet samples.
In addition to the fatty acids shown, Σ saturates include 20:0, 22:0, 24:0; Σ (n-6) PUFA includes 18:3, 20:2, 20:3; Σ (n-3) include 16:4, 18:3; where one or more of the four values is < 0.5. Freshwater salmon diet was Ewos No.3 crumb. Diet 1 - fish oil, diet 2 - sunflower oil, diet 3 - linseed oil.

Table 4.18

A comparison of the lipid class composition between the leucocytes of marine and freshwater Atlantic salmon

Lipid class	Freshwater	Marine salmon		
(% Total lipid)	Salmon	Diet 1	Diet 2	Diet 3
Total polar lipid	39.8 ±8.4[a]	57.1 ±3.5[b]	58.8 ±2.9[b]	64.9 ±2.8[c]
Total neutral lipid	57.7 ±8.4[a]	42.9 ±3.5[b]	41.2 ±2.9[b]	35.1 ±2.8[c]
Polar lipid:				
LPC	-	-	-	-
SM	1.0 ±0.2	2.9 ±0.6	2.4 ±0.2	4.5 ±2.2
PC	15.8 ±2.3	24.8 ±0.6	22.5 ±0.2	24.5 ±2.0
PS	2.5 ±1.1	4.8 ±0.1	5.4 ±0.6	6.4 ±0.5
PI	1.8 ±0.4	4.1 ±0.1	3.7 ±0.4	5.1 ±0.4
Cardiolipin	0.4 ±0.5	3.4 ±0.2	3.4 ±0.3	3.3 ±0.2
PE	10.3 ±2.9	12.8 ±1.2	15.7 ±0.6	16.7 ±0.6
Sulphatides	2.7 ±0.1	1.1 ±0.7	1.5 ±0.3	0.8 ±0.2
Cerebrosides	5.3 ±0.9	3.2 ±1.1	4.3 ±2.2	3.6 ±0.4
Neutral lipid:				
Cholesterol	17.4 ±1.7	22.1 ±1.6	23.5 ±1.0	24.5 ±1.2
Free fatty acids	11.1 ±2.1	5.5 ±2.3	7.7 ±2.2	4.9 ±1.4
Triacylglycerols	9.9 ±2.6	-	0.3 ±0.4	0.7 ±0.3
Sterol esters	19.3 ±6.0	15.3 ±4.2	9.7 ±2.2	5.1 ±2.5
Unknowns	2.6 ±0.2	-	-	-

Abbreviations: LPC-lysophosphatidylcholine, PC-phosphatidylcholine, PS-phosphatidylserine, PI-phosphatidylinositol, PE-phosphatidylethanolamine. Values are expressed as the mean % lipid class of total lipid ±SD for three fish. Data was analysed by ANOVA ($p \geq 0.05$). Values showing different superscript letters are significantly different.

analysed for their fatty acid profile (Table 4.19). Total fatty acids content for each group of leucocytes were 1.9 ±0.1, 1.8 ±0.3, 1.7 ±0.4, 2.0 ±0.9 $\mu g/1x10^6$ cells for the parr and the smolts fed on diets 1, 2, and 3, respectively (see Table 4.17). Although there were no significant differences between the groups in terms of leucocyte fatty acid content, the fatty acid composition differed slightly.

The lipid of leucocytes from freshwater parr contained significantly higher proportions of saturates than the marine smolts, but contained lower levels of both (n-3) and (n-6) PUFA, due to low proportions of 20:4(n-6), 20:5(n-3) and 22:6(n-3). The levels of these fatty acids were in fact even lower than in the marine fish maintained on the sunflower (diet 2) and linseed (diet 3) oil diets. The percentage of total (n-6) PUFA in the lipids of leucocytes of the parr were lower than the smolts which had been maintained on a comparable fish oil diet (diet 1) (8.7 % vs 12.1 %) (diet 1 actually contained less (n-6) PUFA than the parr diet).

The influences of the fatty acid composition of dietary lipid on the fatty acid composition of leucocytes from freshwater parr and marine smolts were evident. Thus, the leucocytes from fish fed on diet 2, high in 18:2(n-6), had elevated levels of this fatty acid and it's elongation products 20:2(n-6) and 20:4(n-6), while leucocytes from fish on diet 3, high in 18:3(n-3), had elevated levels of 18:3(n-3) and it's elongation products 20:5(n-3) and 22:6(n-3). The levels of 22:6(n-3) in the leucocyte lipid of the latter group were actually higher than in the lipids of leucocytes from fish fed diet 1 containing fish oil. Of the smolts examined, the fish on the linseed diet had the lowest total (n-6) PUFA levels in their leucocyte lipid, but the highest total (n-3) PUFA levels,

Table 4.19

A comparison of the fatty acid composition between the leucocytes of marine and freshwater Atlantic salmon

Fatty acids	Freshwater	Marine Salmon		
(% Total FA)	Salmon	Diet 1	Diet 2	Diet 3
14:0	1.7 ±0.8	1.2 ±0.2	0.8 ±0.1	0.8 ±0.1
15:0	1.3 ±0.2	0.7 ±0.4	0.5 ±0.2	0.3 ±0.0
16:0	20.6 ±2.6	22.0 ±1.6	20.0 ±1.0	19.2 ±0.6
17:0	2.0 ±1.0	-	-	-
18:0	11.3 ±0.6	8.8 ±1.9	10.0 ±0.6	9.5 ±0.5
20:0	0.8 ±0.1	0.3 ±0.1	0.5 ±0.4	0.5 ±0.5
22:0	1.0 ±0.1	-	-	-
24:0	1.1 ±1.5	-	-	-
Σ saturates	**39.7 ±3.5**	**34.2 ±1.5**	**33.1 ±0.6**	**30.5 ±0.3**
da 18:1	-	0.6 ±0.5	1.4 ±0.0	0.7 ±0.8
16:1	5.9 ±1.0	3.0 ±1.0	3.2 ±1.1	2.6 ±1.0
18:1	17.2 ±0.5	19.7 ±1.9	17.0 ±0.1	18.7 ±0.9
20:1	2.5 ±0.5	2.9 ±0.8	0.7 ±0.4	1.4 ±0.3
22:1	2.2 ±0.4	1.7 ±1.3	0.3 ±0.0	0.5 ±0.2
24:1	1.6 ±0.4	1.3 ±0.3	1.3 ±0.3	1.5 ±0.4
Σ monoenes	**29.5 ±2.1**	**29.2 ±1.4**	**23.9 ±1.8**	**25.3 ±1.3**
16:2	1.0 ±0.4	-	-	-
18:2	4.6 ±1.0	4.0 ±0.6	9.7 ±1.3	3.4 ±0.3
18:3	0.3 ±0.4	1.9 ±2.4	0.2 ±0.1	0.0 ±0.1
20:2	0.3 ±0.3	0.6 ±0.5	1.5 ±0.1	0.5 ±0.0
20:3	0.3 ±0.3	0.4 ±0.0	1.4 ±0.2	0.5 ±0.0
20:4	2.0 ±0.7	4.0 ±0.7	5.0 ±0.2	4.4 ±0.3
22:2	-	0.4 ±0.2	0.2 ±0.2	0.0 ±0.1
Σ (n-6) PUFA	**8.7 ±1.9**	**12.1 ±2.6**	**18.7 ±1.5**	**9.2 ±0.2**

Table 4.19 (cont.)

A comparison of the fatty acid composition between the leucocytes of marine and freshwater Atlantic salmon

Fatty acids (% Total FA)	Freshwater salmon	Marine Salmon Diet 1	Diet 2	Diet 3
16:3	1.6 ±0.5	0.5 ±0.1	0.5 ±0.2	0.4 ±0.2
16:4	1.5 ±0.8	-	-	-
18:3	1.4 ±0.5	0.6 ±0.2	0.4 ±0.0	2.6 ±0.9
20:4	0.2 ±0.1	0.5 ±0.1	0.1 ±0.1	0.7 ±0.1
20:5	3.9 ±0.9	7.3 ±3.6	3.7 ±0.4	7.3 ±1.2
22:5	0.9 ±0.2	0.9 ±0.2	0.8 ±0.0	1.2 ±0.1
22:6	12.7 ±2.1	14.6 ±2.2	18.8 ±0.8	22.6 ±1.1
Σ (n-3)	**21.1 ±4.4**	**24.5 ±2.7**	**24.3 ±0.9**	**34.9 ±1.3**
(n-3)/(n-6)	**2.6 ±0.5**	**2.0 ±0.6**	**1.3 ±0.2**	**3.8 ±0.2**

Values are expressed as the mean % of total fatty acids ±SD for 3 fish. In addition to the fatty acids shown, Σ (n-6) PUFA includes 22:5; Σ (n-3) includes 18:4; where one or more of the four values is < 0.5.

resulting consequently in the highest (n-3)/(n-6) PUFA ratio.

4.8 CHAPTER SUMMARY

In summary the data obtained in this section showed that:-

(a) There were marked differences between the erythrocytes and peripheral blood leucocytes of Atlantic salmon in terms of lipid content, lipid class composition and fatty acid composition. Leucocytes had a higher lipid content and contained higher levels of neutral lipid, particularly TAG. The total fatty acids of the erythrocytes were high in (n-3) PUFA, especially 22:6(n-3) and the overall (n-6) PUFA unsaturation was higher in the leucocytes than the erythrocytes. The PC and PE fractions of both cell types contained the highest degree of (n-3) unsaturation. PC was rich in 16:0, PS had high levels of both 16:0 and 18:0, as did PI phospholipid. The latter fraction was also very rich in 20:4(n-6) fatty acids in both cell types.

(b) The lipid of headkidney macrophages from Atlantic salmon was higher in phospholipids than leucocytes and T and B cells from the same tissue, but there were no differences between the lipid class composition of the latter cell types. The fatty acid patterns of the lipid from different headkidney cells were similar.

(c) The lipid compositions of Atlantic salmon and rainbow trout erythrocytes were similar, while the leucocytes of salmon contained higher proportions of neutral lipid than the trout leucocytes. The lipid of salmon leucocytes also had greater saturated fatty acid levels than those of the trout.

(d) The lipids of erythrocytes of Atlantic salmon and African catfish contained higher phospholipid levels than the rabbit erythrocytes, with the cells of the rabbit having proportionally greater sphingomyelin levels. The lipids of leucocytes from these animals contained similar levels of the various lipid classes, except for the cholesterol levels, which were much higher in the leucocytes of the rabbit. Fatty acid profiles of the erythrocyte and leucocyte lipid differed greatly between the fish and the rabbit. Rabbit cell fatty acids reflected their dietary 18:2(n-6) intake, whereas the fish had high levels of (n-3) PUFA. The erythrocytes and leucocytes of African catfish had similar fatty acid compositions, while the Atlantic salmon erythrocytes contained a higher degree of 22:6(n-3) fatty acid, which resulted in an overall higher (n-3) PUFA level than found in leucocytes of the catfish. The leucocyte lipid of salmon, by comparison had a higher content of saturated and monomeric fatty acids.

(e) The lipid class and fatty acid composition of both the erythrocytes and leucocytes of Atlantic salmon were largely unaffected by water temperature, except for a higher PE level in the erythrocytes of cold water acclimated fish.

(f) The lipid of leucocytes of Atlantic salmon parr contained higher neutral lipid levels than those found in the smolt, on account of higher TAG levels in the former. Similar fatty acid patterns were observed in the lipid of leucocytes of Atlantic salmon parr and smolts maintained on fish oil diets. The influence of dietary 18:2(n-6) or 18:3(n-3) fatty acids could be seen in the leucocytes of fish maintained on sunflower oil or linseed oil diets.

CHAPTER 5 DIETARY STUDIES WITH ATLANTIC SALMON MAINTAINED ON HIGH (N-3) AND (N-6) PUFA DIETS

5.1 INTRODUCTION

Potential pathogens, ubiquitous in the fish farming environment, can be responsible for massive mortalities and may bring significant financial losses to fish farmers. Indications are that diet modification may help to prevent disease (Blazer and Wolke, 1984a; 1984b; Li and Lovell, 1985; Blazer *et al.*,1989; Landolt, 1989; Hardie *et al.*, 1990; 1991; Sheldon and Blazer, 1991). PUFAs, as explained in Chapter 2, are important components in the mammalian diet for sustaining health, and research has indicated eicosapentaenoic acid and docosahexaenoic acid as two of the most active PUFAs of the (n-3) series, and arachidonic acid of the (n-6) series, in terms of eicosanoid synthesis. Large quantities of 20:5(n-3) and 22:6(n-3) are found in Atlantic salmon, albeit cultured fish receive less (n-3) in their diets than their wild counterparts (van Vliet and Katan, 1990).

It is important that the fish farmer maintains his stock on an economical diet which provides a profitable rate of fish growth and yet is nutritionally balanced, especially since nutritionally compromised fish succumb more easily to infectious diseases and exhibit symptoms of nutritional deficiencies discussed in Chapter 2.6.4. Salmon can efficiently utilise dietary proteins and lipids, but are less able to metabolise carbohydrates (Sargent *et al.*, 1989). Dietary lipids provide a cheaper source of energy than the carbohydrates and proteins when the amount of metabolizable energy they contain is considered. The complete oxidation of 1 g of fatty acid gives 9 kcal of

energy in contrast to 4 kcal for 1g of carbohydrate or protein. Rainbow trout have a dietary requirement of 1-2 % (n-3) fatty acid which is necessary to avert essential fatty acid deficiency symptoms (Castell 1979). The diet formulation and preparation used in this study met the nutritional requirements of salmon laid down by the National Research Council (N.R.C. 1981) and diets were manufactured to resemble a commercially available diet. The fish meal component of the experimental diets supplied sufficient (n-3) fatty acid to meet the (n-3) PUFA requirement of the salmon. Takeuchi and Watanabe (1977c), established that 0.25 % of both dietary 20:5(n-3) and 22:6(n-3) fatty acids could satisfy the EFA requirement of rainbow trout, proving them to be a more efficient source of EFA than 18:3(n-3). These, however, can be expensive while the addition of vegetable oils in conjunction with (n-3) enriched marine oils as provided in the high (n-6) PUFA experimental diet, could reduce diet production costs.

The physical properties of pellets are important in enticing fish to eat them. The final product, held together by natural or added binders, must be stable in water and ideally sink slowly through the water. Pellets have superseded wet and semi-moist feeds in the salmon farming industry, and are produced by compressing the raw ingredients through dies of the appropriate size. In the following work, pelleted diets were made in preferences to expanded diets simply for convenience.

The objectives of this chapter were firstly, to examine the effects of different (n-3)/(n-6) fatty acid ratios in the diets of Atlantic salmon parr, on the lipid composition of their immunocompetent tissues and cells, and secondly, to investigate these effects on both their specific and nonspecific immune function. Data on the specific immune responses

of Atlantic salmon in relation to dietary PUFA are presented later in Section 5.4. Firstly, a variety of nonspecific immune defence mechanisms in fish maintained on different dietary (n-3)/(n-6) fatty acid ratios was examined *in vitro*, and then the innate immune response of these fish subsequently investigated by *in vivo* challenge with *Vibrio anguillarum*. Similarly, an examination of adaptive immunity was carried out by challenging fish maintained on different dietary (n-3)/(n-6) fatty acid ratios with bacteria against which they had been previously vaccinated, and also by looking at *in vitro* antigen stimulation of their leucocytes and ability to produce plaque forming cells.

5.2 PHYSIOLOGICAL PARAMETERS

5.2.1 Fish growth

The growth performances of the Atlantic salmon in dietary trials 1, 2 and 3 are shown in Figures 5.1, 5.2 and 5.3 respectively and in Table 5.1. The figures show the weight gained by the fish over the course of the three dietary trials, and the ambient water temperature during these periods. An increased feeding rate was observed at elevated water temperatures and was adjusted for in the daily diet rations (see Table 3.1). The final weights and lengths of the fish for each trial, as well as their condition factors are given in Table 5.1. In the first trial, the final weights and lengths of fish fed the commercial diet were significantly higher ($P < 0.05$) than those fed the experimental diets, although this was not reflected by a higher C.F. value in this fish group. The H.I. value was significantly lower in this population ($P < 0.05$) than in the experimental populations. There was no significant difference in growth between the experimental groups of trial 1 or 3, but in trial 2 the (n-3) PUFA group had significantly higher

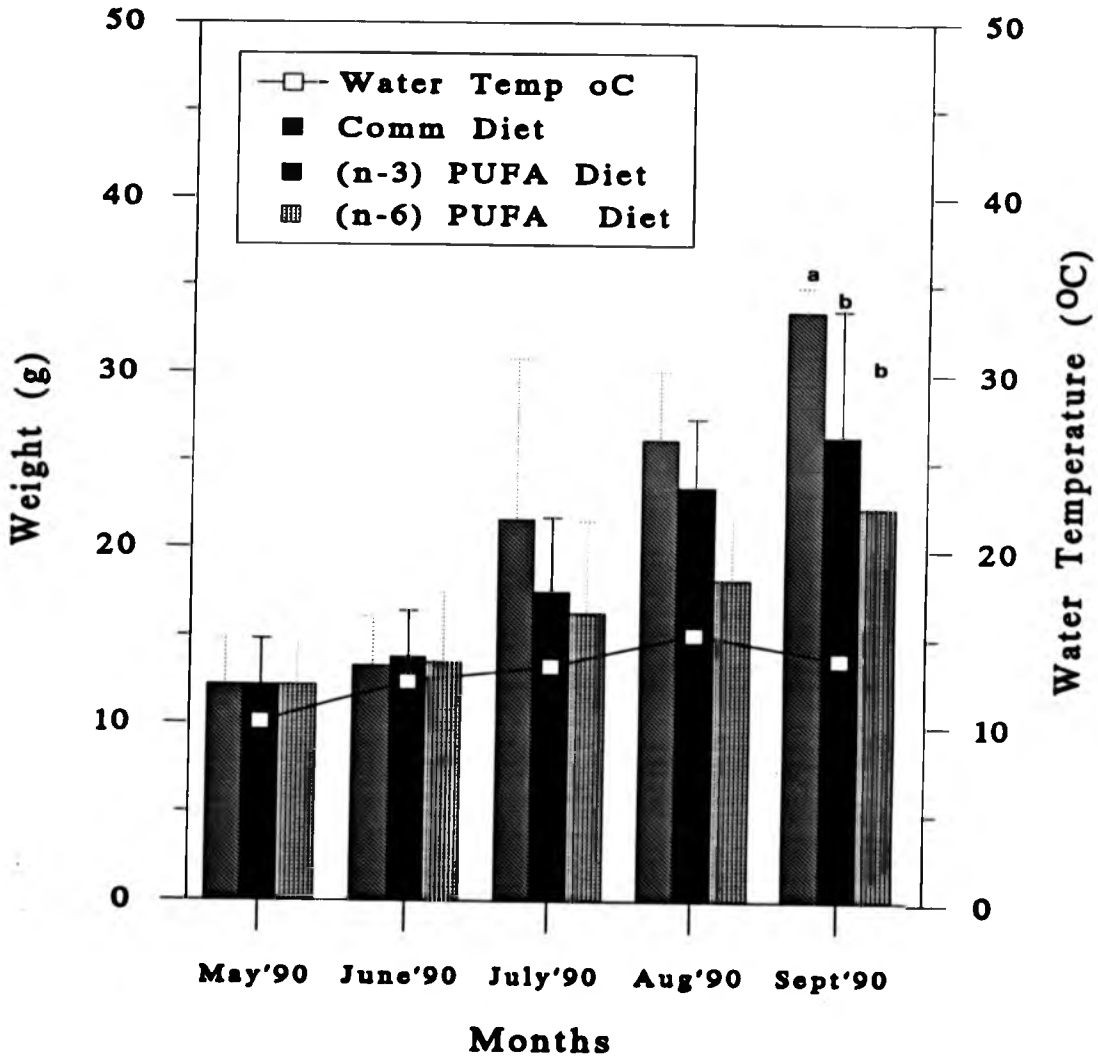


Figure 5.1 Weight gained by fish over the duration of dietary trial 1, and ambient water temperature during this period.

Values are expressed as mean weight (g) ±S.D. Numbers sampled for each group was 18. * Water temperature (°C). Values with different superscript letters are significantly different ($p < 0.05$) by ANOVA

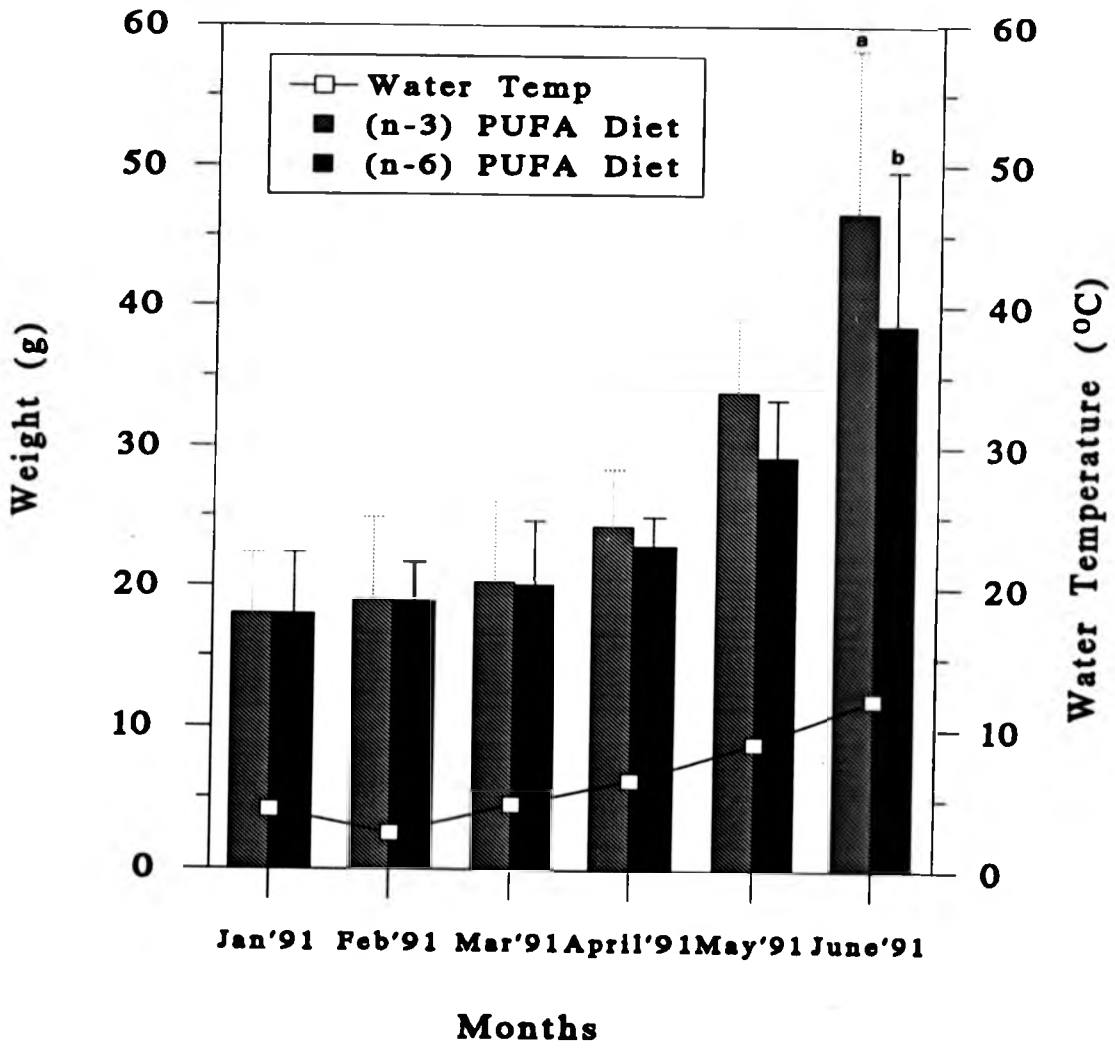


Figure 5.2 Weight gained by fish over the duration of dietary trial 2, and ambient water temperature during this period.

Values are expressed as mean weight (g) ±S.D. Numbers sampled for each group was 38. * Water temperature (°C). Values with different superscript letters are significantly different ($p < 0.05$) by ANOVA

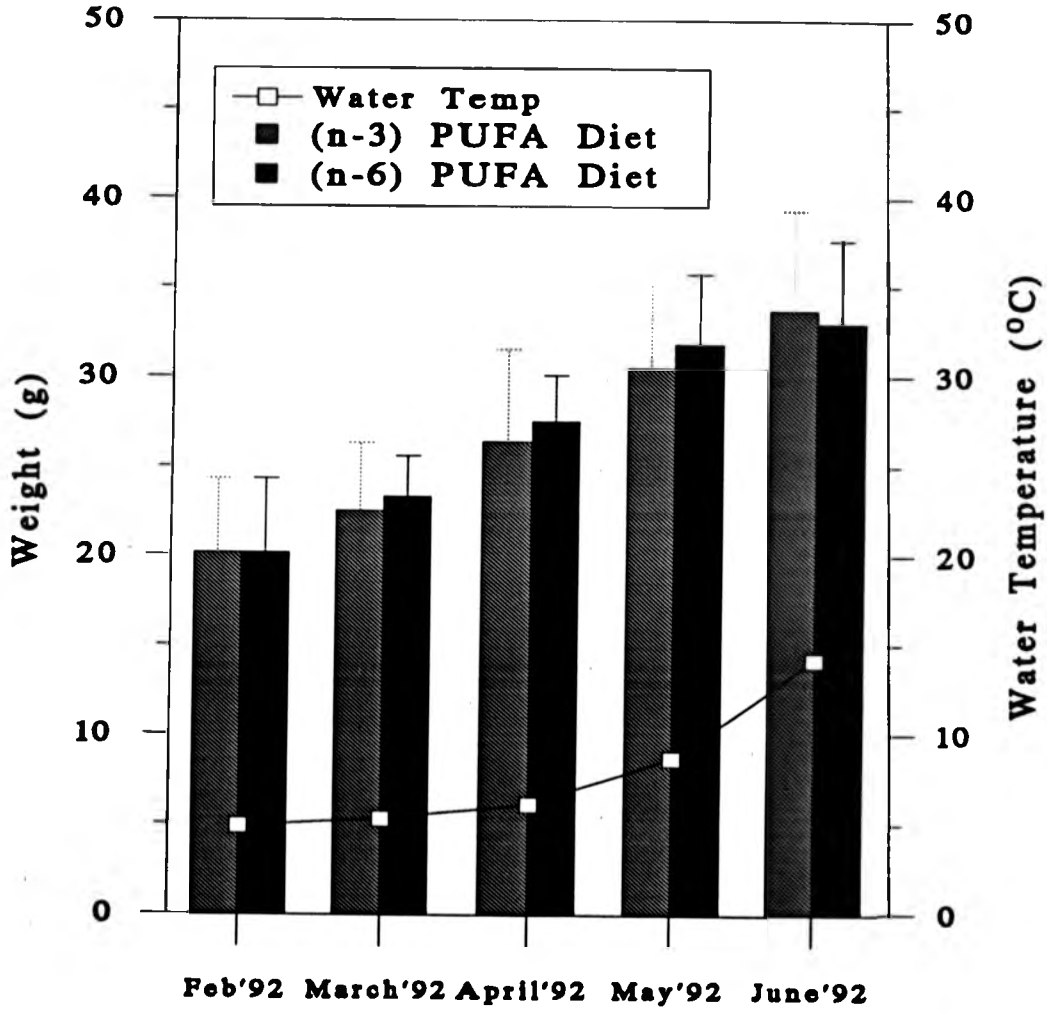


Figure 5.3 Weight gained by fish over the duration of dietary trial 3, and ambient water temperature during this period.

Values are expressed as mean weight (g) ±S.D. Numbers sampled for each group was 27.

Table 5.1

Weight and condition of fish on different dietary (n-3)/(n-6) PUFA ratios on completion of trials 1-3.

Dietary groups	Weight (g)	Length (cm)	C.F.	H.I.	Sp.I.	mortalities
<u>Trial 1</u>						
Commercial diet	33.5 ±1.4[a]	14.7 ±1.1[c]	1.05 ±0.0	0.9 ±0.1[e]	-	2%
(n-3) diet	26.6 ±7.7[b]	13.5 ±1.2[d]	1.07 ±0.1	1.2 ±0.2[f]	-	4%
(n-6) diet	22.4 ±7.9[b]	12.6 ±1.8[d]	1.07 ±0.1	1.1 ±0.2[f]	-	6%
<u>Trial 2</u>						
(n-3) diet	48.8 ±12.2[a]	17.6 ±1.6	0.9 ±0.1[a]	1.0 ±0.05[a]	0.7 ±0.05[a]	3%
(n-6) diet	38.7 ±11.0[b]	16.3 ±2.4	0.8 ±0.1[b]	1.4 ±0.4[b]	0.9 ±0.1[b]	7%
<u>Trial 3</u>						
(n-3) diet	33.7 ±5.6	15.1 ±0.8	1.0 ±0.1	-	-	3%
(n-6) diet	33.0 ±4.6	15.1 ±0.8	1.0 ±0.1	-	-	6%

Results expressed as mean ±S.D. Values with different superscript letters are significantly different ($p < 0.05$) by ANOVA for Trial 1 and ($p < 0.05$) by Student 't' test for Trials 2 and 3. n = 18, 38 & 27 in Trials 1, 2 & 3 respectively. H.I. values: n =12 in trial 1 and 14 in trial 2. Sp.I. values: n =18 in trial 2.

weights ($P < 0.05$), resulting in a higher C.F value. In the same trial (n-6) PUFA dietary fish had significantly higher H.I and Sp.I values ($P < 0.05$).

5.2.2 Blood cell numbers and haematocrits

Blood cells counts for each group of fish from the dietary trials are presented in Table 5.2. The water temperature at the time of measuring blood cells counts was 13.7°C, 12°C, and 14.2°C for trials 1 to 3, respectively. Values for differential blood cell counts for fish of trial 2 at day 150, are presented in Table 5.3. Haematocrits, shown in Table 5.4, were measured from the same blood samples as used for blood counts. No significant difference due to dietary PUFA was observed in blood cell numbers, differential blood cell counts or haematocrits between the different fish populations.

Table 5.2

Blood cell numbers of Atlantic salmon at end of dietary trial period

	(n-3) PUFA diet	(n-6) PUFA diet	Commercial diet
		Blood cell numbers ml^{-1}	
Trial 1			
Leucocytes	$1.8 \pm 0.6 \times 10^7$	$2.1 \pm 0.5 \times 10^7$	$2.2 \pm 0.2 \times 10^7$
Erythrocytes	$1.2 \pm 3.1 \times 10^9$	$1.1 \pm 2.0 \times 10^9$	$1.3 \pm 0.6 \times 10^9$
Trial 2			
Leucocytes	$1.5 \pm 0.8 \times 10^7$	$1.4 \pm 0.6 \times 10^7$	-
Erythrocytes	$1.3 \pm 0.5 \times 10^9$	$1.4 \pm 0.6 \times 10^9$	-
Trial 3			
Leucocytes	$2.3 \pm 0.5 \times 10^7$	$2.2 \pm 0.4 \times 10^7$	-
Erythrocytes	$1.4 \pm 0.7 \times 10^9$	$1.4 \pm 0.4 \times 10^9$	-

Results expressed as mean ±S.D. Number of fish sampled for leucocyte values = 15, 14 and 12 in trials 1, 2 and 3 respectively. n=8 for erythrocyte values.

Table 5.3

Differential cell counts of Atlantic salmon in trial 2 at day 150.

Cell Type	Differential cell count (%)	
	(n-3) PUFA diet	(n-6) PUFA diet
thrombocytes	32.7 ±9.0	35.8 ±12.9
lymphocytes	61.5 ±8.4	58.3 ±9.7
monocytes	1.1 ±0.4	1.4 ±0.7
granulocytes	4.0 ±1.2	4.6 ±3.5

Results expressed as mean % ±S.D. (n=3) for each sample.

Table 5.4

Haematocrits of Atlantic salmon at end of dietary trial period

Trial	Haematocrit (%)		
	(n-3) PUFA diet	(n-6) PUFA diet	Commercial diet
1	47.2 ±6.1	49.0 ±7.7	48.2 ±8.7
2	51.2 ±6.2	50.9 ±5.9	-
3	48.3 ±7.4	47.1 ±8.1	-

Results expressed as mean % ±S.D. n = 18, 22 and 20 in trials 1, 2 and 3 respectively.

5.3 LIPID COMPOSITION

5.3.1 The fatty acid composition of commercial and laboratory prepared fish diets

Details of the preparation of experimental diets can be found in Section 3.6.2. The fatty acid profiles of both the commercial and experimental diets used throughout the reported work, are found in Table 5.5 and Table 5.6. The similarity observed between the fatty acid profile of herring meal, the commercial (Ewos) and the experimental fish oil diets was not surprising since herring meal was the basic constituent of the diets. Oil from the herring meal contributed 7 % of the total lipid yield in the experimental diets. Both the trout (Ewos) and the BP mainstream pellets contained more 18:2(n-6) fatty acid than the commercial salmon diets, suggesting they had been supplemented with vegetable oil. This was reflected by the lower (n-3)/(n-6) PUFA ratios observed in the lipid of the Ewos 5 and BP diets. The oils used to supplement the experimental diets were composed almost entirely of TAGs.

Variations in fatty acids between batches of experimental diets were small. The (n-3) PUFA diet was always comparable to the salmon fingerling diet (No.3) shown in Table 5.5, having a (n-3)/(n-6) PUFA ratio of around 5.3, while the lipid of the (n-6) PUFA diet contained high levels of 18:2(n-6) fatty acid acquired from the added sunflower oil, resulting in a low (n-3)/(n-6) PUFA ratio of around 0.3.

Table 5.5

The percentage fatty acid composition of lipid from herring meal and commercially prepared fish diets

Fatty acids (%)	Herring Meal	Ewos No. 3	Ewos No. 4	Ewos* No. 5	BP mainstream
14:0	5.6 ±0.1	5.6 ±0.0	5.7 ±0.1	5.7 ±0.1	4.6 ±0.2
16:0	16.9 ±0.1	16.5 ±0.1	15.6 ±0.2	14.9 ±0.1	16.2 ±0.3
18:0	3.0 ±0.0	3.6 ±0.0	3.3 ±0.4	3.1 ±0.1	3.7 ±0.1
Σ Saturates	**26.9 ±0.3**	**25.7 ±0.2**	**25.9 ±1.0**	**24.8 ±0.6**	**25.9 ±1.0**
16:1	5.2 ±0.0	5.8 ±0.0	5.7 ±0.0	5.9 ±0.3	6.3 ±0.2
18:1	13.9 ±0.1	14.7 ±0.2	14.9 ±0.3	15.3 ±0.7	16.8 ±0.1
20:1	8.4 ±0.1	6.8 ±0.1	8.5 ±0.1	8.7 ±0.3	7.1 ±0.1
22:1	13.8 ±0.2	9.7 ±0.3	13.2 ±0.2	12.7 ±0.2	9.5 ±0.2
24:1	1.5 ±0.1	1.0 ±0.2	1.1 ±0.0	1.1 ±0.1	0.9 ±0.2
Σ monoenes	**42.9 ±0.4**	**38.2 ±0.6**	**43.5 ±0.5**	**43.7 ±1.1**	**40.6 ±0.4**
18:2	1.7 ±0.0	2.3 ±0.1	2.7 ±0.0	4.5 ±0.6	6.2 ±0.1
20:4	0.8 ±0.1	0.9 ±0.0	0.9 ±0.2	0.7 ±0.1	1.1 ±0.1
22:2	0.7 ±0.1	0.5 ±0.1	0.4 ±0.0	0.5 ±0.2	0.4 ±0.0
22:5	0.5 ±0.0	0.4 ±0.1	0.3 ±0.0	0.4 ±0.1	0.3 ±0.1
Σ(n-6) PUFA	**4.6 ±0.1**	**5.5 ±0.2**	**5.4 ±0.2**	**7.0 ±0.6**	**9.1 ±0.0**
18:3	1.2 ±0.0	1.1 ±0.0	1.3 ±0.1	1.4 ±0.1	1.5 ±0.1
18:4	2.4 ±0.0	2.9 ±0.0	2.9 ±0.1	2.8 ±0.1	2.0 ±0.0
20:4	0.8 ±0.0	0.9 ±0.0	0.8 ±0.0	0.7 ±0.1	0.6 ±0.0
20:5	7.2 ±0.2	9.9 ±0.1	7.5 ±0.4	7.5 ±0.2	8.2 ±0.3
22:5	1.4 ±0.1	1.6 ±0.1	1.3 ±0.1	1.4 ±0.1	1.4 ±0.1
22:6	11.9 ±0.4	12.0 ±0.4	10.6 ±0.9	9.8 ±1.0	9.7 ±0.7
Σ(n-3) PUFA	**25.5 ±0.8**	**29.9 ±0.6**	**25.4 ±1.5**	**24.5 ±1.1**	**24.4 ±1.1**
(n-3)/(n-6)	**5.5 ±0.3**	**5.4 ±0.4**	**4.8 ±0.4**	**3.5 ±0.4**	**2.7 ±0.1**

In addition to the fatty acids shown above, Σ saturates include 15:0, 20:0, 22:0; Σ (n-6) PUFA include 16:2, 18:3, 20:2, 20:3 and Σ (n-3) PUFA include 16:3, 16:4; where one or more of the 5 values is < 0.5. Values are expressed as means (%) ±S.D. of triplicate samples. *Trout diet

Table 5.6

The percentage fatty acid composition of lipid from laboratory-prepared fish diets

Fatty	(n-3) PUFA Diet			(n-6) PUFA Diet		
acids (%)	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
14:0	7.2 ±0.1	6.9 ±0.6	5.9 ±0.4	2.3 ±0.1	2.1 ±0.3	2.3 ±0.0
16:0	20.0 ±0.2	17.1 ±0.2	16.2 ±0.7	10.8 ±0.2	10.1 ±0.2	10.8 ±0.3
18:0	3.7 ±0.1	3.6 ±0.1	2.7 ±0.1	4.5 ±0.1	4.7 ±0.1	2.8 ±1.7
Σ Saturates	**32.0 ±0.1**	**28.5 ±0.6**	**25.8 ±0.6**	**18.2 ±0.4**	**17.9 ±0.2**	**17.5 ±0.7**
16:1	9.1 ±0.1	8.6 ±0.2	9.4 ±0.4	2.5 ±0.2	2.8 ±0.0	5.0 ±0.3
18:1	15.0 ±0.1	12.9 ±0.4	11.6 ±1.9	19.5 ±0.2	18.7 ±0.4	18.2 ±0.5
20:1	5.5 ±0.1	4.7 ±0.2	7.6 ±1.3	4.0 ±0.1	3.2 ±0.1	5.5 ±0.2
22:1	3.0 ±2.9	5.6 ±0.3	5.4 ±0.1	5.6 ±0.3	4.2 ±0.1	4.4 ±0.1
24:1	0.8 ±0.5	1.1 ±0.2	0.8 ±0.1	0.4 ±0.0	0.5 ±0.0	0.5 ±0.1
Σ monoenes	**33.4 ±3.2**	**32.9 ±0.9**	**34.8 ±2.6**	**32.0 ±0.1**	**29.4 ±0.5**	**33.7 ±0.2**
18:2	2.0 ±0.1	1.8 ±0.2	2.2 ±0.9	39.8 ±0.7	39.9 ±0.4	36.6 ±1.2
20:4	1.0 ±0.1	1.1 ±0.0	0.7 ±0.1	0.4 ±0.0	0.6 ±0.0	0.1 ±0.1
22:2	0.5 ±0.0	0.6 ±0.0	0.5 ±0.0	0.1 ±0.0	0.1 ±0.0	0.0 ±0.1
Σ (n-6)	**5.4 ±0.3**	**6.2 ±0.5**	**6.1 ±1.7**	**40.8 ±0.7**	**41.5 ±0.4**	**37.2 ±1.2**
16:4	1.7 ±0.2	1.7 ±0.1	1.5 ±0.1	0.3 ±0.1	0.5 ±0.0	0.3 ±0.1
18:3	0.9 ±0.1	0.8 ±0.0	0.8 ±0.1	0.4 ±0.0	0.5 ±0.0	0.6 ±0.2
18:4	2.6 ±0.2	2.8 ±0.0	2.9 ±0.1	0.6 ±0.1	1.0 ±0.0	1.1 ±0.0
20:4	0.7 ±0.1	0.8 ±0.0	0.8 ±0.1	0.2 ±0.0	0.2 ±0.1	0.1 ±0.1
20:5	11.8 ±1.3	12.9 ±0.1	12.5 ±0.5	3.1 ±0.3	3.6 ±0.0	3.6 ±0.0
22:5	1.4 ±0.0	1.6 ±0.0	1.4 ±0.1	0.4 ±0.0	0.5 ±0.0	0.3 ±0.0
22:6	9.6 ±1.4	10.6 ±0.2	12.0 ±0.6	3.8 ±0.3	4.4 ±0.0	5.4 ±0.0
Σ (n-3) PUFA	**29.3 ±3.1**	**32.4 ±0.3**	**33.3 ±0.6**	**9.0 ±0.7**	**11.2 ±0.0**	**11.7 ±0.3**
(n-3)/(n-6)	**5.4 ±0.8**	**5.2 ±0.5**	**5.5 ±1.6**	**0.2 ±0.0**	**0.3 ±0.0**	**0.3 ±0.0**

In addition to the fatty acids shown, Σ saturates include 15:0, 20:0, 22:0; Σ (n-6) PUFA include 16:2, 18:3, 20:2, 20:3, 22:5 and Σ (n-3) PUFA include 16:3; where one or more of the six samples is < 0.5. Values are expressed as means (%) ±S.D. of triplicate samples.

5.3.2 Lipid composition of erythrocytes, leucocytes and serum:

Change with time

The period of time required for dietary fatty acids to alter the total fatty acid composition of salmon erythrocytes, leucocytes and serum was investigated by analysing the fatty acid composition of the cells and serum at intervals throughout trial 2. Changes in the 18:2(n-6) fatty acid level, in the total (n-6) and total (n-3) PUFA levels, and in the (n-3)/(n-6) PUFA ratios of erythrocytes, leucocytes and serum from the dietary fish are illustrated in Figures 5.4, 5.5, 5.6 and 5.7, respectively. Insufficient lipid was obtained for the 16 week serum samples so analysis was carried out on a pooled lipid sample from three fish at this time period, while other samples were the mean of three fish. Water temperatures over this period have been shown previously in Figure 5.2.

The first evidence of dietary manipulation was seen 4 weeks from the onset of the trial, as an increased 18:2(n-6) fatty acid level in the total lipid of serum and leucocytes from fish fed the high (n-6) diet (Figure 5.4). The leucocytes of fish in this dietary group gradually incorporated 18:2 (n-6) until a (n-3)/(n-6) PUFA ratio of 0.8 was maintained after 16 weeks (Figure 5.7). Leucocytes were found to include greater proportions of dietary 18:2(n-6) in their lipid than erythrocytes, which by comparison, did not show extensive dietary manipulation until 16 weeks, at which point the 18:2(n-6) fatty acid level was observed to plateau (Figure 5.4). The (n-3)/(n-6) PUFA ratio fell from 12.7 ±2.2 to 4.4 ±0.2 in the erythrocytes of fish fed the (n-6) diet (Figure 5.5), from 2.6 ±0.3 to 0.8 ±0.0 in their leucocytes (Figure 5.6) and from 7.4 ±0.9 to 1.4 ±1.2 in their serum (Figure 5.7).

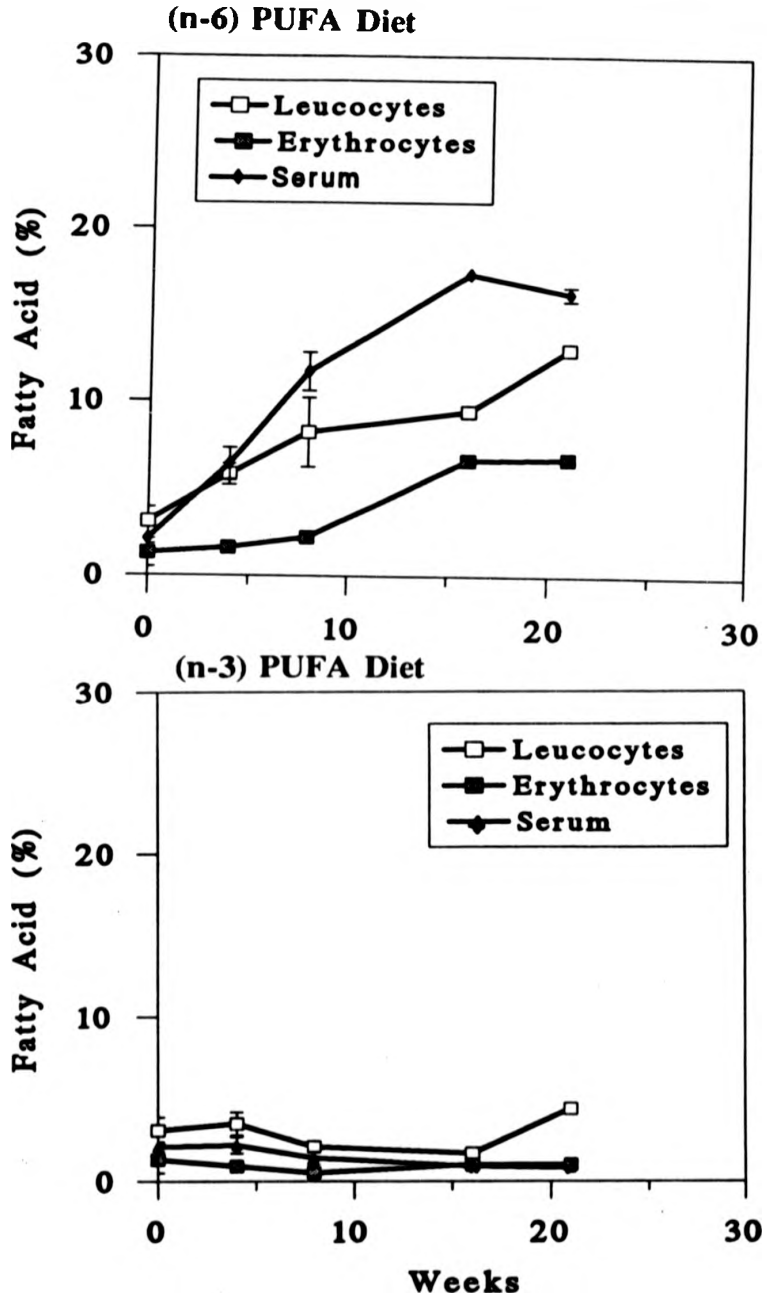


Figure 5.4

Changes in 18:2(n-6) fatty acid levels in the total lipid of erythrocytes, leucocytes and serum of Atlantic salmon over the course of trial 2. Values are expressed as means (%) ±S.D. of triplicate samples.

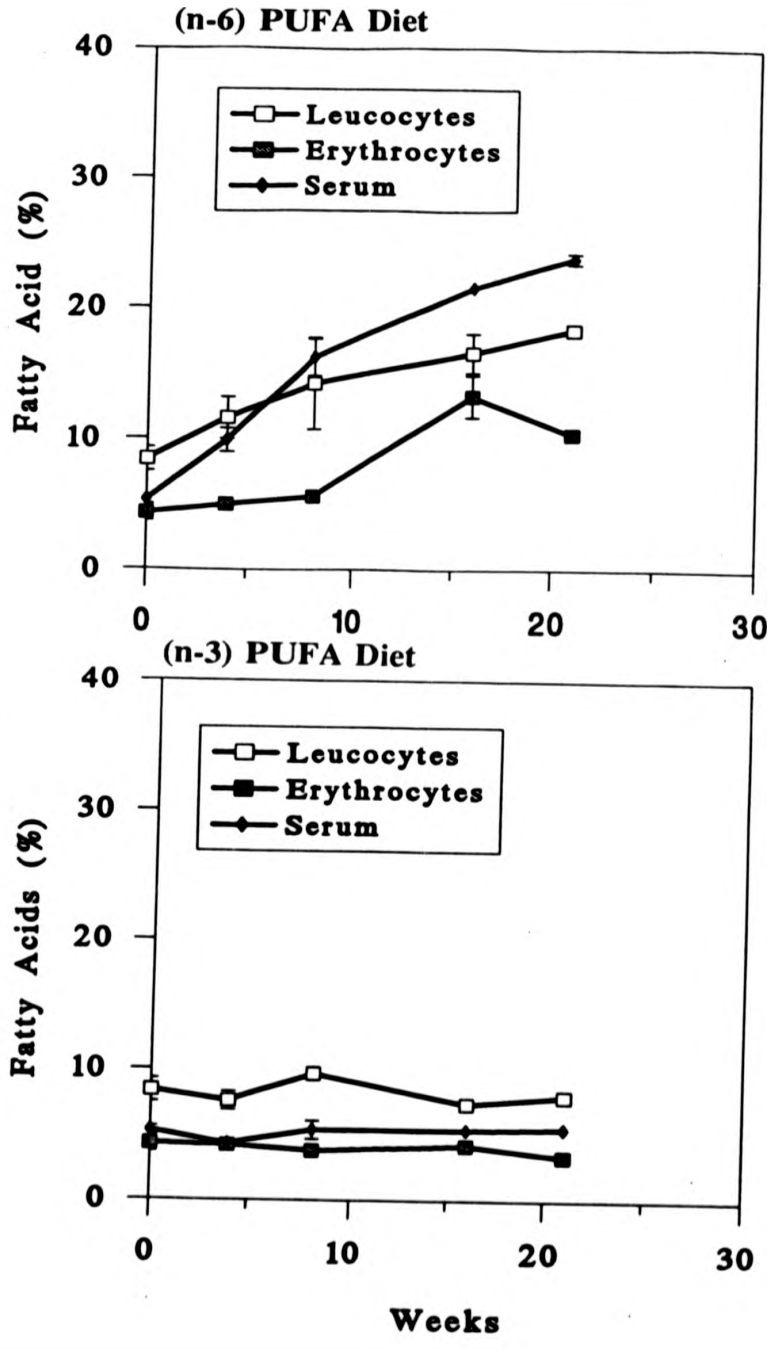


Figure 5.5

Changes in total (n-6) PUFA levels in the total lipid of erythrocytes, leucocytes and serum from Atlantic salmon over the course of trial 2. Values are expressed as means (%) ±S.D. of triplicate samples.

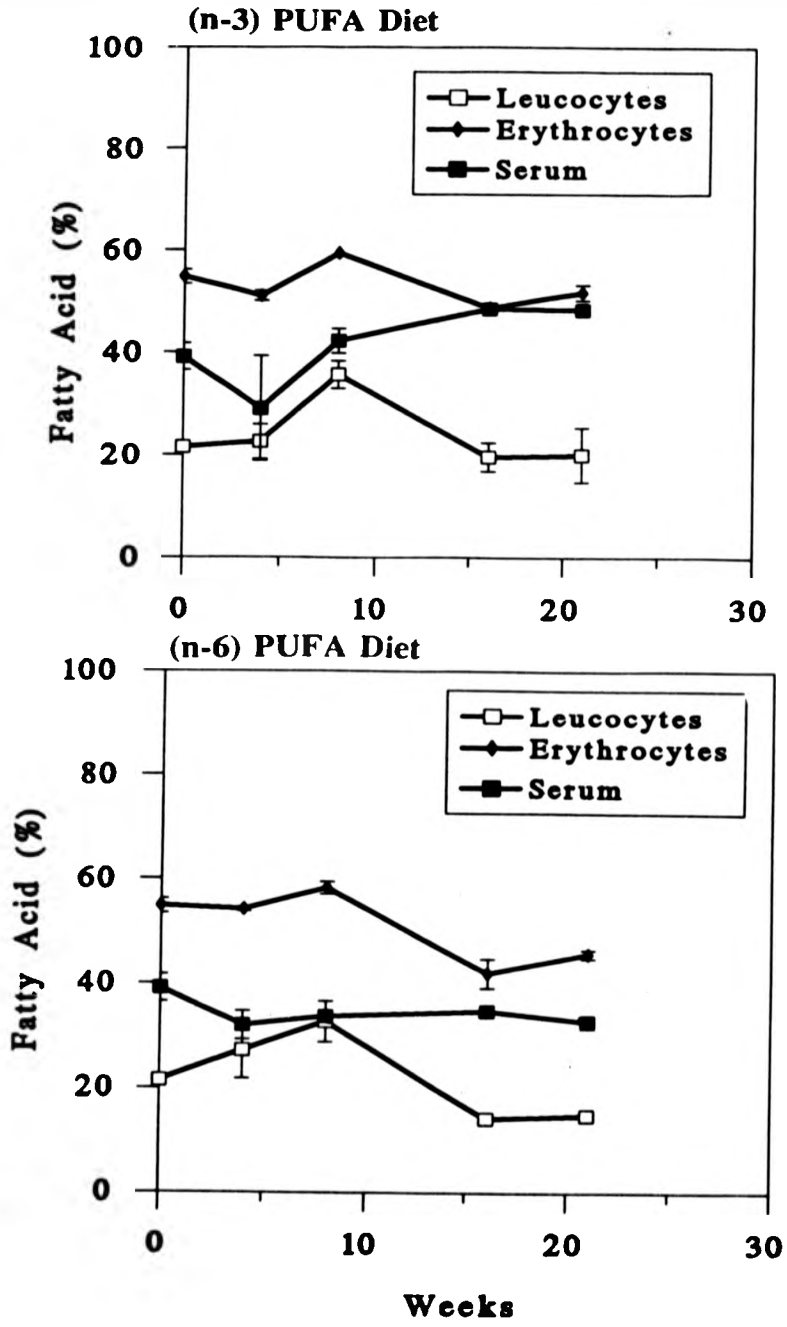


Figure 5.6

Changes in total (n-3) fatty acid levels in the total lipid of erythrocytes, leucocytes and serum of Atlantic salmon over the course of trial 2. Values are expressed as means (%) ±S.D. of triplicate samples.

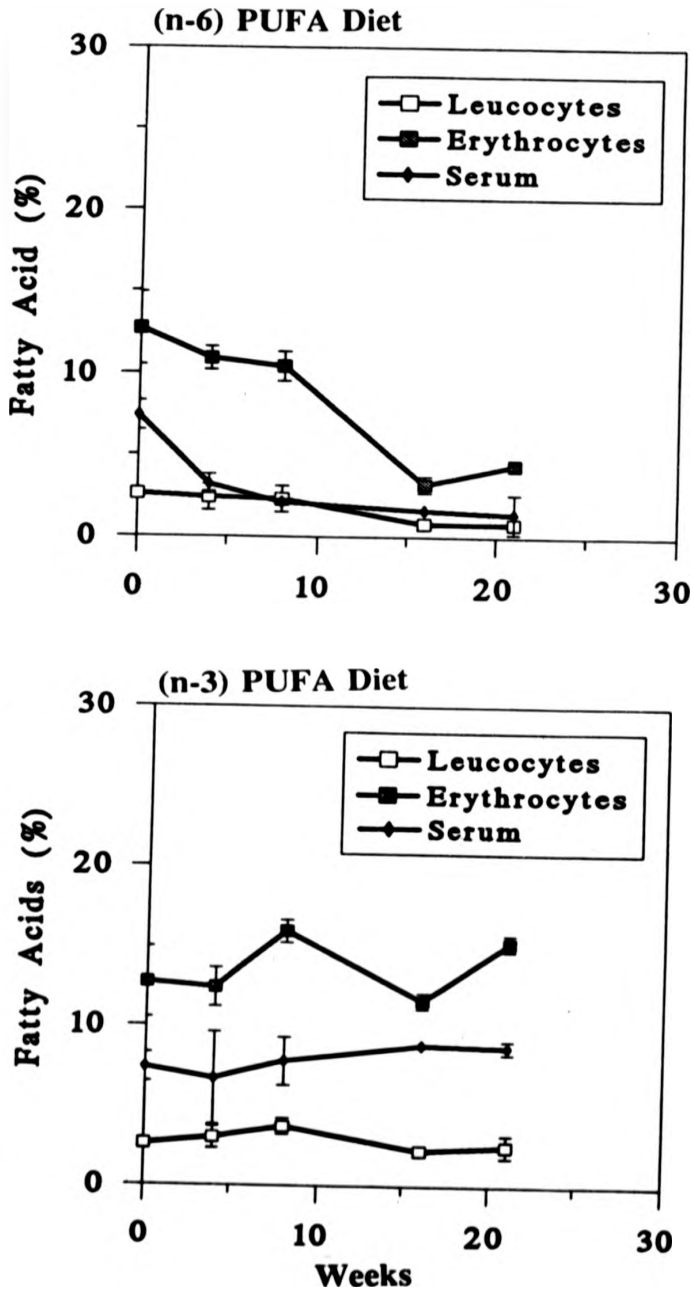


Figure 5.7

Changes in (n-3)/(n-6) PUFA ratios in the total lipid of erythrocytes, leucocytes and serum from Atlantic salmon over the course of trial 2. Values are expressed as means (%) ±S.D. of triplicate samples.

5.3.3 Lipid composition of immunocompetent tissues and cell

The present section, using fish which had been maintained on the experimental diets differing in (n-3)/(n-6) PUFA ratios, examined firstly, the lipid class and fatty acid profiles of peripheral blood erythrocytes, leucocytes, serum and headkidney macrophages and B and T cells, and secondly, lipid class and fatty acid profiles of thymus, spleen, kidney, gills and leucocytes isolated from these tissues. Liver was also examined. Component fatty acids of phospholipid classes were also analysed for each of the above tissues. Sampling took place at the end of each trial, prior to vaccination and/or challenging with bacteria and was carried out on days 105, 150, and 112 for trials 1, 2 and 3, respectively. Water temperatures at the time of sampling were 13.7°C, 12.0°C and 14.2°C. Identical lipid analyses were not performed for every trial, and the trial corresponding to a particular analysis is indicated with the analysis data.

5.3.3.1 Blood

The lipid and protein contents of peripheral blood erythrocytes and leucocytes, and of serum from fish maintained in trial 3 are shown in Table 5.7. There was no evidence for dietary PUFA influencing lipid or protein content.

The lipid classes of the erythrocytes from trial 2 are shown in Tables 5.8, while the fatty acid composition of the same cells is given in Table 5.9. Erythrocyte lipid class composition was found to be independent of diet, while the component fatty acids exhibited a strong dietary influence. Thus, levels of 18:2(n-6) fatty acid were significantly higher in the total lipid extracted from erythrocytes of the (n-6) PUFA dietary group than from those maintained on the high (n-3) PUFA diet(6.7 % vs. 1.0 %).

Table 5.7

The lipid and protein content of peripheral blood erythrocytes and leucocytes, and of serum from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 3.

	Lipid		Protein	
	(n-3) PUFA Diet	(n-6) PUFA Diet	(n-3) PUFA Diet	(n-6) PUFA Diet
Erythrocytes	0.58 ±0.3	0.65 ±0.4	11.0 ±2.7	14.9 ±2.2
Leucocytes	12.0 ±8.0	12.0 ±1.0	18.0 ±3.0	25.0 ±5.0
Serum	15.8 ±3.0	15.8 ±3.0	52.4 ±3.9	58.0 ±8.2

Values are expressed as μg per 10^6 cells and mg per ml serum for 3 fish

Table 5.8

The percentage lipid class composition of erythrocytes from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 2

Lipid class	Dietary Group	
(% Total lipid)	(n-3) PUFA	(n-6) PUFA
Σ polar lipid	50.1 ±1.9	51.4 ±3.6
Σ neutral lipid	49.9 ±1.9	48.6 ±3.6
Polar lipid:		
LPC	-	0.3 ±0.1
Sphingomyelin	-	3.0 ±0.8
PC	33.3 ±2.1	37.0 ±3.7
PS	0.8 ±0.5	0.4 ±0.1
PI	0.1 ±0.0	0.1 ±0.1
Cardiolipin	-	0.6 ±0.1
PE	14.9 ±2.1	13.8 ±0.6
Sulphatides	0.1 ±0.0	-
Cerebrosides	0.9 ±0.1	-
Neutral lipid:		
Cholesterol	27.7 ±2.1	26.5 ±0.9
Free fatty acids	13.1 ±1.1	12.9 ±0.7
Triacylglycerols	4.7 ±0.5	5.9 ±0.4
Sterol esters	4.2 ±1.0	2.8 ±2.1
Unknowns	0.2 ±0.2	0.4 ±0.3

Abbreviations:- LPC-Lysophosphatidylcholine; PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine. Values are expressed as means (%) ±S.D. of triplicate samples.

Table 5.9

The percentage fatty acid composition of total lipid of erythrocytes from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 2.

Fatty acids (%)	Dietary Group	
	(n-3) PUFA	(n-6) PUFA
14:0	0.8 ±0.0	0.5 ±0.1
16:0	26.5 ±0.8	24.9 ±0.7
18:0	5.4 ±0.1	6.5 ±0.1
Σ saturates	**33.6 ±1.0**	**33.0 ±1.0**
16:1	1.3 ±0.1	0.9 ±0.1
18:1	5.7 ±0.4	6.5 ±0.4
20:1	1.1 ±0.1	0.8 ±0.0
22:1	0.6 ±0.1	0.5 ±0.0
24:1	2.0 ±0.1	2.0 ±0.3
Σ monoenes	**11.1 ±0.5**	**10.9 ±0.8**
18:2	1.0 ±0.1***	6.7 ±0.0***
20:2	0.1 ±0.0	0.7 ±0.1
20:3	0.1 ±0.0	0.7 ±0.1
20:4	1.3 ±0.0	1.5 ±0.2
Σ (n-6) PUFA	**3.4 ±0.0*** **	**10.4 ±0.2*** **
20:4	0.8 ±0.4	0.8 ±0.6
20:5	7.8 ±0.3**	5.1 ±0.2**
22:5	2.3 ±0.0**	1.7 ±0.0**
22:6	40.3 ±0.9**	37.6 ±0.8**
Σ (n-3) PUFA	**51.8 ±1.5*** **	**45.6 ±1.6*** **
(n-3)/(n-6)	**15.2 ±0.5*** **	**4.4 ±0.2*** **

In addition to the fatty acids shown, Σ saturates include 15:0, 20:0, 22:0, 24:0; Σ monoenes include 14:1; Σ (n-6) PUFA include 16:2, 18:3, 22:2, 22:5 and Σ (n-3) PUFA include 16:3, 16:4, 18:3, 18:4; where one or more of the 2 values is < 0.5. Values are expressed as means (%) ±S.D. of triplicate samples. ** = 0.01 > p > 0.001, *** = p < 0.001 by Student 't' test.

The level of 20:3(n-6) was also higher in leucocytes of the (n-6) PUFA dietary group. As a result of an overall increase in total (n-6) PUFA in erythrocytes from the (n-6) PUFA dietary group of fish, the (n-3) PUFA level and consequently the (n-3)/(n-6) ratio were observed to fall significantly in this group, in comparison with the (n-3) group.

The fatty acid compositions of the phospholipid classes of erythrocytes from dietary fish of trial 1 are shown in Table 5.10 (a), (b) and (c). Values were from the analysis of 3 pooled samples, which meant that no statistical analysis could be performed between the dietary groups. Similar patterns were found between the dietary groups for saturated and unsaturated fatty acids in individual phospholipid classes. Thus, the PC fraction always contained high levels of (n-3) PUFA unsaturation, but not as high as levels found in the PE fraction. However, since the PE class always had higher (n-6) PUFA levels than in PC class, a lower (n-3)/(n-6) PUFA ratio resulted in the PE fraction. The level of saturated fatty acid was consistently lowest in the PE fraction (17.2 - 26.5 % compared with 32.4 -37.3 % in PC), while the level of (n-3) PUFA unsaturation in this phospholipid was the highest amongst the phospholipid classes examined (54.2 - 67.4 % compared with 43.4 -49.5 % in PC) regardless of diet. The PC and PE fractions of (n-6) PUFA dietary group erythrocytes had increased levels of 18:2(n-6) PUFA (7.6 and 5.8 % respectively, compared with 0.6 and 0.7 % in PC and PE of (n-3) PUFA dietary group erythrocytes) (Table 5.10c and b). PS did not appear to be influenced by dietary PUFA to the same extent as PC and PE. This fraction contained 2.3 % 18:2(n-6) compared with 7.6 % or 5.8 % in the PC and PE fractions of erythrocytes from the (n-6) PUFA dietary group (Table 5.10c). The PC and PE lipid classes from this dietary group contained as high levels of 22:6(n-3) as the same phospholipids in erythrocytes

Table 5.10 (a)

The percentage fatty acid composition of the phospholipid classes of erythrocytes from Atlantic salmon maintained on a commercial diet in trial 1.

Fatty acids (%)	Commercial Diet			
	PC	PS	PI	PE
14:0	0.8	0.2	0.2	0.2
16:0	31.0	14.0	14.1	8.0
18:0	3.2	17.6	19.0	8.6
24:0	1.6	0.3	0.4	-
Σ saturates	**37.3**	**33.2**	**34.3**	**17.2**
16:1	1.6	1.5	1.9	0.8
18:1	5.7	5.7	6.9	4.1
20:1	1.4	0.2	1.6	3.4
22:1	0.8	0.8	0.6	2.0
24:1	0.2	0.5	0.5	-
Σ monoenes	**10.2**	**8.8**	**12.3**	**11.1**
18:2	0.4	0.8	1.0	0.6
18:3	0.1	0.4	0.3	0.2
20:2	0.1	0.4	-	0.2
20:3	0.1	0.2	0.2	0.2
20:4	1.4	7.3	13.6	1.8
22:2	0.2	0.5	1.0	0.4
Σ (n-6)	**2.4**	**9.9**	**16.5**	**3.6**
20:4	0.2	0.4	0.4	1.2
20:5	6.6	5.4	4.1	6.0
22:5	1.1	1.5	1.3	2.6
22:6	41.0	37.3	26.9	56.6
Σ (n-3) PUFA	**49.5**	**46.4**	**34.4**	**67.4**
(n-3)/(n-6)	**20.7**	**4.7**	**2.1**	**18.7**

Abbreviations:- PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; Σ saturates also include 15:0, 17:0, 20:0, 22:0; Σ monoenes include 14:1; Σ (n-6) PUFA include 16:2, 22:5 and Σ (n-3) PUFA include 16:3, 16:4, 18:3, 18:4; where one or more of the 4 values is < 0.5. Values are expressed as 1 analysis of 3 pooled samples.

Table 5.10 (b)

The percentage fatty acid composition of the phospholipid classes of erythrocytes from Atlantic salmon maintained on a high (n-3) PUFA diet in trial 1.

Fatty acids (%)	(n-3) PUFA Diet			
	PC	PS	PI	PE
14:0	3.0	0.2	0.4	0.2
16:0	27.9	14.3	8.0	11.9
18:0	3.6	23.3	37.0	12.7
24:0	-	0.8	0.9	1.2
Σ saturates	**35.1**	**38.9**	**47.3**	**26.5**
16:1	4.3	2.1	1.7	1.6
18:1	9.2	6.8	2.8	6.6
20:1	1.3	3.5	1.1	3.0
22:1	1.0	3.5	0.7	1.5
24:1	1.7	0.7	1.1	0.8
Σ monoenes	**13.3**	**16.8**	**7.4**	**13.5**
18:2	0.6	0.5	-	0.7
18:3	0.1	1.6	0.5	1.3
20:3	0.2	-	0.2	0.2
20:4	0.8	3.1	25.8	1.6
22:2	0.4	2.2	2.8	2.0
Σ (n-6) PUFA	**2.8**	**7.8**	**29.4**	**6.2**
16:4	1.0	1.0	0.8	0.6
18:4	1.4	0.9	0.5	0.6
20:4	0.4	0.4	0.3	0.7
20:5	13.0	3.3	6.7	6.6
22:5	1.3	1.4	0.6	2.3
22:6	25.5	27.6	5.8	41.5
Σ (n-3) PUFA	**43.4**	**35.9**	**16.0**	**54.2**
(n-3)/(n-6)	**15.8**	**4.6**	**0.5**	**8.7**

Abbreviations:- PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; Σ saturates include 15:0, 20:0, 22:0; Σ monoenes include 14:1; Σ (n-6) PUFA include 16:2, 20:2, 22:5; Σ (n-3) PUFA include 16:3, 18:3; where one or more of the 4 values is < 0.5. Values are expressed as 1 analysis of 3 pooled samples.

Table 5.10 (c)

The percentage fatty acid composition of the phospholipid classes of erythrocytes from Atlantic salmon maintained on a high (n-6) PUFA diet in trial 1.

Fatty acids	(n-6) PUFA Diet			
(%)	PC	PS	PI	PE
14:0	0.5	0.3		0.2
16:0	25.6	12.6	nd	8.1
18:0	5.5	21.6		9.8
24:0	0.3	1.5		0.1
Σ saturates	**32.4**	**37.2**		**18.8**
16:1	1.1	2.0		1.8
18:1	7.0	4.8		4.8
20:1	1.2	2.1		3.0
22:1	0.5	1.2		1.6
24:1	1.6	1.8		1.5
Σ monoenes	**11.4**	**11.9**		**12.6**
18:2	7.6	2.3		5.8
18:3	0.3	1.4		0.2
20:2	0.5	0.6		1.6
20:3	1.0	0.7		1.5
20:4	1.3	1.2		1.4
22:2	0.4	2.9		1.3
Σ (n-6) PUFA	**11.6**	**9.6**		**13.1**
18:3	1.2	0.3		0.2
18:4	0.1	0.7		0.8
20:4	0.1	0.4		0.7
20:5	3.7	1.7		3.9
22:5	0.4	1.0		1.6
22:6	38.4	35.2		45.7
Σ (n-3) PUFA	**44.2**	**40.9**		**54.0**
(n-3)/(n-6)	**3.8**	**4.3**		**4.1**

Abbreviations:- PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; nd- not done; Σ saturates also include 15:0, 20:0, 22:0; Σ monoenes include 14:1; Σ (n-6) PUFA include 16:2, 22:5 and Σ (n-3) PUFA include 16:3, 16:4; where one or more of the 4 values is < 0.5. Values are expressed as 1 analysis of 3 pooled samples.

from fish fed (n-3) PUFA. PC had a higher 16:0 fatty acid content than the other phospholipid classes. Both the PS and PI fractions contained higher levels of 18:0 than 16:0, and the PI fraction also had a high 20:4(n-6) content in fish of all three dietary groups.

The lipid class composition for peripheral blood leucocytes from salmon of trial 2 was found to be independent of the dietary lipid fatty acid composition, with no significant differences being observed between the (n-3) and the (n-6) PUFA dietary groups (Table 5.11). The fatty acid profiles of total lipid from these cells are presented in Table 5.12. Leucocytes of the (n-6) PUFA dietary group contained higher proportions of 18:2(n-6) than the (n-3) PUFA dietary group (13.0 % compared with 4.4 %), and higher levels of 18:3(n-6) were also observed in the former group. The lipid of leucocytes was apparently influenced more by dietary 18:2(n-6) than that of erythrocytes. It was shown previously in Chapter 4 that the Atlantic salmon leucocytes of the commercial fish had a higher level of 18:2(n-6) than the erythrocytes prior to diet manipulation (1.7 % compared to 0.5 % (see Table 4.3)). The fatty acid composition of the (n-3) PUFA group leucocytes from this section did not differ from the commercial group shown in Table 4.3.

Tables 5.13 (a) and (b) show the fatty acid composition of the leucocyte lipid classes from trial 2 and are the analysis of 3 pooled samples. There was insufficient lipid for analysis of individual PS and PI fractions and the two lipid classes were therefore subsequently pooled before analysing. Similar patterns of saturated and unsaturated fatty acids were found to those observed for the erythrocytes, except lower levels of

Table 5.11

The percentage lipid class composition of leucocytes from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 2.

Lipid class	Dietary Group	
(% Total lipid)	(n-3) PUFA	(n-6) PUFA
Σ polar lipid	26.1 ±2.3	22.9 ±2.4
Σ neutral lipid	73.9 ±2.3	77.1 ±2.4
Polar lipid:		
LPC	0.4 ±0.1	-
Sphingomyelin	2.8 ±0.3	-
PC	17.8 ±1.8	14.7 ±2.4
PS	0.9 ±0.1	0.9 ±0.1
PI	1.1 ±0.3	0.8 ±0.2
Cardiolipin	0.5 ±0.1	-
PE	1.3 ±0.2	1.3 ±0.8
Sulphatides	0.4 ±0.2	0.4 ±0.1
Cerebrosides	0.6 ±0.9	1.1 ±0.2
Neutral lipid:		
Cholesterol	23.5 ±1.3	23.1 ±1.3
Free fatty acids	3.2 ±0.5	2.5 ±0.7
Triacylglycerols	12.5 ±2.0	15.7 ±2.3
Sterol esters	34.0 ±3.3	34.9 ±1.5
Unknowns	0.7 ±0.3	0.8 ±0.6

Abbreviations:- LPC-Lysophosphatidylcholine; PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine. Values are expressed as means (%) ±S.D. of triplicate samples.

Table 5.12

The percentage fatty acid composition of leucocyte total lipid from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 2

Fatty acids (%)	Dietary Group	
	(n-3) PUFA diet	(n-6) PUFA diet
14:0	2.8 ±0.4	1.4 ±0.5
15:0	1.3 ±0.3	0.7 ±0.2
16:0	26.7 ±0.3**	23.9 ±0.9**
18:0	7.2 ±0.9	7.1 ±0.4
24:0	0.7 ±0.2	0.2 ±0.2
Σ saturates	**39.6 ±2.4****	**34.0 ±1.5****
16:1	6.2 ±1.5	3.3 ±0.3
18:1	17.4 ±2.1	19.2 ±0.8
20:1	2.0 ±0.1	2.8 ±0.4
22:1	1.8 ±0.2	2.9 ±0.9
24:1	2.4 ±0.5	2.5 ±0.2
Σ monoenes	**32.3 ±3.5**	**32.9 ±0.7**
18:2	4.4 ±0.3**	13.0 ±0.2**
18:3	0.2 ±0.1	0.4 ±0.1
20:2	0.5 ±0.1**	1.7 ±0.1**
20:3	0.3 ±0.0*	0.9 ±0.2*
20:4	1.6 ±0.2	1.9 ±0.2
22:5	0.4 ±0.1	0.2 ±0.1
Σ (n-6) PUFA	**8.0 ±0.2*****	**18.3 ±0.1*****
18:3	0.3 ±0.1	0.2 ±0.0
18:4	0.3 ±0.1	0.2 ±0.0
20:4	0.3 ±0.1	0.1 ±0.1
20:5	3.5 ±1.0	2.5 ±0.1
22:5	1.0 ±0.2	0.7 ±0.1
22:6	13.8 ±4.7	10.8 ±1.0
Σ (n-3) PUFA	**20.1 ±5.3**	**14.8 ±0.9**
(n-3)/(n-6)	**2.5 ±0.7*****	**0.8 ±0.0*****

In addition to the fatty acids shown, Σ saturates include 17:0, 20:0, 22:0; Σ monoenes include 14:1; Σ (n-6) PUFA include 16:2, 22:2 and Σ (n-3) PUFA include 16:3, 18:3; where one or more of the 2 values is < 0.5. Values are expressed as mean (%) ±S.D for triplicate samples. ** = 0.01 > p > 0.001, *** = p < 0.001 by Student 't' test.

Table 5.13 (a)

The fatty acid composition of the lipid classes of leucocytes from Atlantic salmon maintained on high (n-3) PUFA diets in trial 2

Fatty acids	(n-3) PUFA Diet		
(%)	PC	PS/PI	PE
14:0	2.3	1.0	0.7
15:0	0.8	1.3	1.5
16:0da	-	-	4.9
16:0	35.1	17.5	13.5
18:0da	-	-	3.3
18:0	4.2	21.5	8.7
Σ saturates	**42.7**	**41.2**	**32.7**
14:1	1.1	2.7	3.2
16:1	4.2	5.0	2.5
18:1da	-	-	3.9
18:1	18.3	14.6	11.0
20:1	1.3	2.3	1.1
22:1	0.6	1.7	-
24:1	1.9	2.9	1.2
Σ monoenes	**27.4**	**29.2**	**22.9**
16:2	0.8	1.3	1.0
18:2	2.9	2.4	2.6
18:3	0.4	1.3	-
20:2	0.4	1.0	1.1
20:4	1.8	1.7	1.7
22:2	0.8	-	1.4
Σ (n-6) PUFA	**7.8**	**7.8**	**7.8**
16:3	0.7	3.3	1.9
20:5	7.4	1.2	3.8
22:5	1.1	1.3	1.5
22:6	12.1	16.0	29.4
Σ (n-3) PUFA	**22.2**	**21.7**	**36.6**
(n-3)/(n-6)	**2.8**	**2.8**	**4.7**

Abbreviations:- PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; da-dimethyl acetals; Σ saturates also include 20:0; Σ (n-6) PUFA include 20:3, 22:5 and Σ (n-3) PUFA include 18:3, 18:4, 20:4; where one or more of the 3 values is < 0.5. Values are one analysis of 3 pooled samples for trial 2

Table 5.13 (b)

The fatty acid composition of the lipid classes of leucocytes from Atlantic salmon maintained on high (n-6) PUFA diets in trial 2

Fatty acids	(n-6) PUFA Diet		
(%)	PC	PS/PI	PE
14:0	2.0	1.2	1.3
15:0	-	1.1	1.3
16:0da	-	4.8	0.0
16:0	29.0	13.1	11.5
18:0	8.8	11.8	1.3
20:0	0.9	0.9	-
Σ saturates	**42.1**	**33.5**	**21.3**
14:1	1.0	4.7	0.0
16:1	3.2	2.3	4.3
18:1	20.2	9.4	18.3
20:1	0.9	0.8	0.9
22:1	0.6	0.7	0.9
24:1	2.5	2.0	2.1
Σ monoenes	**28.3**	**20.0**	**26.4**
16:2	0.6	0.9	1.7
18:2	8.2	9.3	6.4
18:3	1.2	0.4	3.8
20:2	1.1	1.2	6.4
20:3	0.9	0.5	0.9
20:4	1.3	3.1	3.0
22:2	2.6	3.3	-
Σ (n-6) PUFA	**16.0**	**19.1**	**22.1**
16:3	0.8	1.7	1.7
18:3	-	1.2	-
18:4	-	-	2.1
20:4	1.3	0.5	-
20:5	3.1	2.0	1.7
22:5	0.6	0.9	1.3
22:6	7.8	21.0	23.4
Σ (n-3) PUFA	**13.6**	**27.4**	**30.2**
(n-3)/(n-6)	**0.8**	**1.4**	**1.4**

Abbreviations:- PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; da-dimethyl acetals; Σ (n-6) PUFA also include 22:5; where one or more of the 3 values is < 0.5. Values are expressed as 1 analysis of 3 pooled samples for trial 2

(n-3) PUFA were obtained in the leucocyte phospholipids. The PC fraction had a higher 16:0 fatty acid content than the other phospholipid classes. The PS and PI combination contained higher 18:0 than 16:0 only in the (n-3) PUFA dietary group leucocytes, but the levels of both these fatty acids in this fraction were reduced in the (n-6) PUFA dietary group (Table 5.13 a and b). It was not possible to establish if the PI fraction had a high 20:4(n-6) content, since its presence was masked by PS component fatty acids. The incorporation of dietary 18:2(n-6) into the PC, PS/PI and PE lipid classes from (n-6) PUFA dietary group leucocytes resulted in lower (n-3)/(n-6) PUFA ratios compared with the (n-3) PUFA dietary group (Table 5.13 (b)).

T and B cells were isolated from headkidney of fish in each dietary group as described in Section 3.2.7. The B cell purity was determined by labelling with clone 2G9 (a monoclonal against α salmon IgM), then α-mouse IgG-FITC as described in Section 3.7.4. Plate 5.1 illustrates positively labelled B cells. Due to insufficient material, the lipid classes of headkidney T and B cells could not be determined, but fatty acid analysis was carried out using pooled samples from three fish. Unfortunately, this meant that significance differences between dietary groups could not be determined. Values of percentage fatty acid for the T and B cells are given in Table 5.14 and indicate that there is no large difference in fatty acid content between the two cell types. Both T and B leucocytes from the (n-6) PUFA dietary fish showed notably higher levels of 18:2(n-6) in their lipids than T and B leucocytes from fish in the (n-3) PUFA dietary group and the corresponding (n-3)/(n-6) ratios were consequently lower.

The lipid class composition of serum was not affected by the PUFA composition of the

diet (Table 5.15), whereas very large differences were notable in the fatty acid composition of the serum lipid in the two dietary groups (Table 5.16). Overall serum from trial 2 fish reflected the fatty acid composition of the diet more closely than the erythrocytes and leucocytes (Tables 5.15 and 5.16).

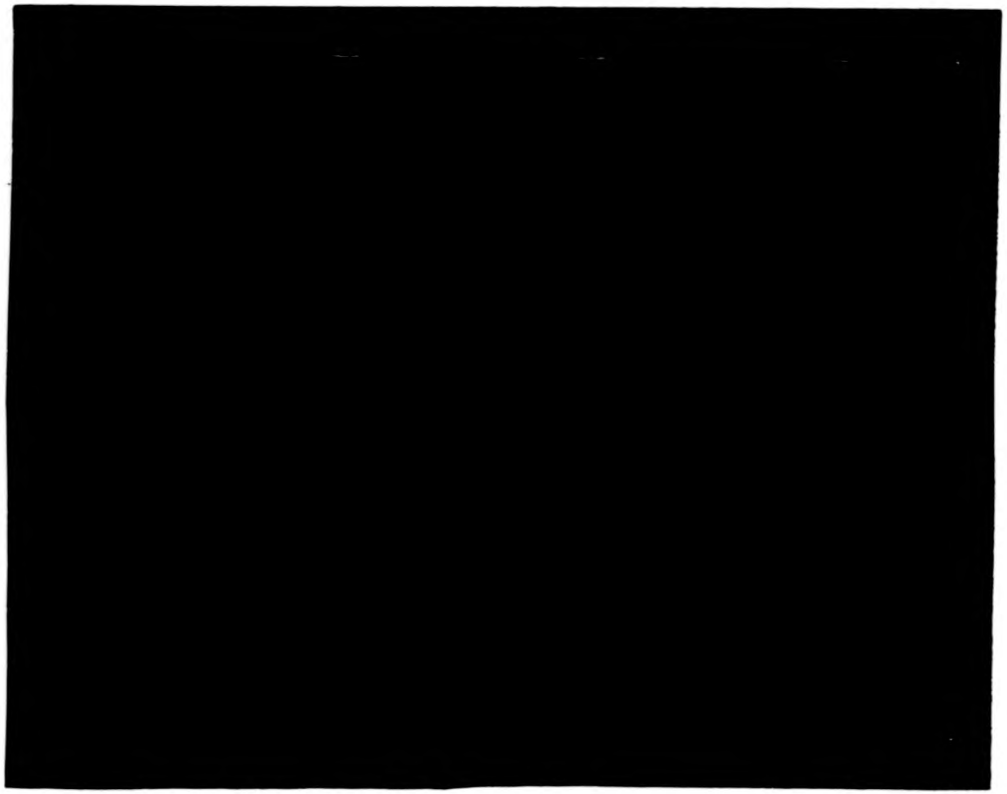


Plate 5.1 Headkidney B cells from Atlantic salmon labelled with clone 2G9 (a monoclonal against α salmon IgM), then α-mouse IgG-FITC.

Table 5.14

The fatty acid composition of total lipid from headkidney T and B leucocytes from Atlantic salmon maintained on high (n-6) PUFA diets in trial 3

Fatty acids	(n-3) PUFA Diet		(n-6) PUFA Diet	
(%)	T cell	B cell	T cell	B cell
14:0	1.7	2.7	2.2	2.1
15:0	0.8	1.6	2.3	2.1
16:0	26.8	30.2	26.9	24.4
18:0	8.4	10.1	8.2	10.0
20:0	0.4	0.4	0.4	0.8
22:0	0.4	0.2	0.8	0.8
24:0	0.4	0.3	0.5	1.2
Σ saturates	**39.0**	**45.4**	**41.2**	**41.2**
16:1	5.9	8.0	7.7	4.8
18:1	15.6	18.8	15.2	16.0
20:1	2.7	0.8	2.7	1.7
22:1	0.8	1.2	1.3	0.7
24:1	1.1	1.2	1.0	4.5
Σ monoenes	**26.5**	**29.9**	**27.9**	**27.7**
16:2	0.3	0.5	-	-
18:2	1.9	4.1	9.7	8.3
18:3	0.8	0.2	1.0	2.2
20:2	0.1	0.6	1.3	-
20:3	0.2	1.5	-	-
20:4	3.5	1.5	0.7	3.5
22:2	0.2	0.3	-	1.1
Σ (n-6) PUFA	**7.3**	**8.8**	**12.7**	**15.1**
18:3	0.4	0.3	0.8	0.8
20:4	0.5	0.4	-	1.1
20:5	7.3	3.4	3.8	3.2
22:5	1.0	0.6	0.9	-
22:6	17.0	10.8	12.7	10.6
Σ (n-3) PUFA	**27.2**	**15.9**	**18.1**	**15.7**
(n-3)/(n-6)	**3.9**	**1.8**	**1.4**	**1.1**

In addition to the fatty acids shown, Σ monoenes include 14:1; Σ (n-6) PUFA include 22:5 and Σ (n-3) PUFA include 16:3, 18:4; where one or more of the 4 values is < 0.5. Values are expressed as 1 analysis of 3 pooled samples for trial 2.

Table 5.15

The lipid class composition of serum from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 2.

Lipid class (% Total lipid)	Dietary Group	
	(n-3) PUFA Diet	(n-6) PUFA Diet
Σ polar lipid	32.1 ±6.0	31.8 ±2.4
Σ neutral lipid	67.9 ±6.0	68.2 ±2.4
Polar lipid:		
LPC	0.7 ±0.7	1.6 ±0.1
Sphingomyelin	5.0 ±1.8	6.5 ±0.2
PC	23.4 ±7.3	21.5 ±1.8
PS	0.8 ±0.4	1.3 ±0.2
PI	1.0 ±1.0	-
PE	0.7 ±0.2	0.8 ±0.3
Sulphatides	0.4 ±0.4	-
Cerebrosides	0.1 ±0.1	-
Neutral lipid:		
Cholesterol	15.0 ±0.7	15.6 ±0.0
Free fatty acids	2.6 ±0.9	2.3 ±0.1
Triacylglycerols	13.5 ±0.8	14.8 ±2.9
Sterol esters	36.5 ±6.7	34.1 ±0.3
Unknowns	1.5 ±0.3	-

Abbreviations:- LPC-Lysophosphatidylcholine; PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine. Values are expressed as means (%) ±S.D. of triplicate samples.

Table 5.16

The fatty acid composition of serum total lipid from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 2.

Fatty acids (%)	Dietary Group	
	(n-3) PUFA	(n-6) PUFA
14:0	1.9 ±0.1	1.0 ±0.2
15:0	0.5 ±0.1	0.4 ±0.1
16:0	21.5 ±0.4**	18.2 ±0.2**
17:0	-	-
18:0	2.9 ±0.5	3.8 ±0.1
Σ saturates	**27.6 ±0.8****	**24.2 ±0.5****
14:1	0.3 ±0.2	0.6 ±0.0
16:1	3.7 ±0.1	2.4 ±0.6
18:1	9.5 ±0.0***	12.6 ±0.6***
20:1	1.8 ±0.2	1.6 ±0.1
22:1	1.8 ±0.1	1.2 ±0.1
24:1	1.3 ±0.1	1.0 ±0.1
Σ monoenes	**18.4 ±0.4**	**19.3 ±1.2**
16:2	0.9 ±0.0	0.5 ±0.2
18:2	0.8 ±0.0***	16.2 ±0.4***
18:3	0.2 ±0.0	0.4 ±0.1
20:2	0.2 ±0.0***	2.1 ±0.3***
20:3	0.2 ±0.0***	2.1 ±0.1***
20:4	2.5 ±0.1	2.2 ±0.2
22:5	0.5 ±0.0	0.3 ±0.0
Σ (n-6)	**5.5 ±0.1*****	**23.8 ±0.4*****
16:3	0.4 ±0.0	0.4 ±0.2
18:3	0.3 ±0.0	0.2 ±0.0
18:4	0.6 ±0.1	0.3 ±0.0
20:4	1.0 ±0.0**	2.2 ±0.2**
20:5	14.2 ±1.1***	5.0 ±0.3***
22:5	1.2 ±1.5	1.5 ±0.1
22:6	30.8 ±1.0***	23.2 ±1.3***
Σ (n-3) PUFA	**48.4 ±1.2*****	**32.7 ±1.2*****
(n-3)/(n-6)	**8.7 ±0.4*****	**1.4 ±1.2*****

In addition to the fatty acids shown, Σ saturates include 20:0, 22:0, 24:0; Σ (n-6) PUFA include 22:2 and Σn (n-3) PUFA include 16:2; where one or more of the 2 values is < 0.5. Values are expressed as mean (%) ±S.D for triplicate samples. ** = 0.01 > p > 0.001, *** = p < 0.001 by Student 't' test.

5.3.3.2 Thymus

The lipid content of thymus tissue was not significantly influenced by the type of PUFA in the diet. The lipid yield from the thymocytes was too small to measure and so consequently only the fatty acid content of the cells was determined. This was 6.9 ±6.0 $\mu g/10^6$ cells for the thymocytes from (n-3) PUFA group fish and 13.1 ±1.5 $\mu g/10^6$ cells for the (n-6) PUFA group from trial 2. The fatty acid content of the thymocytes were not significantly influenced by the type of PUFA in the diet.

The lipid classes of thymus from trial 1, and thymus and thymocytes from trial 2 are shown in Tables 5.17 (a) and (b) respectively, while the fatty acid composition of the tissue and cells are presented in Tables 5.18 (a) and (b). Fatty acid profiles of the thymus tissue lipid classes are shown in Table 5.19 (a) and (b).

Small, but significant (i.e. $p < 0.05$) differences were found in the lipid classes of the thymus between the different dietary groups of trial 1 and trial 2 (Tables 5.17 (a) and (b)). In both trials, the lipid of (n-6) dietary PUFA thymus contained significantly higher levels of neutral lipid than other groups. In trial 2, the intact thymus contained similar levels of phospholipid in total lipid to those found in the isolated thymocytes in only the (n-3) dietary PUFA group. Isolated cells contained higher proportions of cholesterol and lower levels of TAG than the tissue from which they were taken. Although the difference in the cholesterol levels between the cells and tissues was significant, the difference in the proportion of TAG was less obvious. The thymocytes of both dietary groups exhibited a lipid class profile characteristic of those found previously with peripheral blood leucocytes (Table 5.11), albeit the thymocyte lipid

Table 5.17 (a)

The lipid class composition of thymus from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets or a commercial diet in trial 1.

Lipid class	Diet		
(% Total lipid)	(n-3) PUFA	(n-6) PUFA	Commercial
Lipid Content	4.4 ±1.3	3.5 ±1.7	2.9 ±0.8
Σ polar lipid	28.0 ±0.4[a]	25.6 ±0.9[b]	31.8 ±3.2[a]
Σ neutral lipid	72.0 ±0.4[a]	74.4 ±0.9[b]	68.2 ±3.2[a]
Polar lipid:			
Sphingomyelin	0.6 ±0.0	0.5 ±0.1	0.6 ±0.4
PC	12.8 ±0.8[a]	11.8 ±0.1[b]	16.4 ±1.8[c]
PS	1.4 ±0.1[a]	1.1 ±0.2[a]	1.7 ±0.1[b]
PI	2.3 ±0.2	2.0 ±0.1	2.4 ±0.3
Cardiolipin	0.1 ±0.2[a]	0.9 ±0.1[b]	0.9 ±0.4[b]
PE	9.0 ±0.0	8.6 ±0.4	9.2 ±0.8
Sulphatides	0.4 ±0.1	0.2 ±0.2	0.2 ±0.2
Cerebrosides	0.6 ±0.2	0.5 ±0.1	0.2 ±0.2
Neutral lipid:			
Cholesterol	11.1 ±0.1	9.1 ±1.8	11.4 ±1.0
Free fatty acids	5.6 ±0.3[a]	5.0 ±1.0[a]	3.0 ±0.2[b]
Triacylglycerols	44.0 ±0.4[a]	49.8 ±3.2[b]	46.5 ±4.1[ab]
Sterol esters	11.0 ±0.6[a]	9.9 ±1.8[ab]	7.1 ±1.5[b]
Unknowns	0.8 ±0.3	0.6 ±0.4	1.1 ±0.4

Abbreviations:- LPC-Lysophosphatidylcholine; PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; Values are expressed as means (%) ±S.D. of triplicate samples. Values with different superscript letters are significantly different ($p < 0.05$) by ANOVA Lipid Content (mg g^{-1} wet weight of tissue)

Table 5.17 (b)

The lipid class composition of thymus and thymus leucocytes from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 2.

Lipid class		Thymus		Thymocytes	
(% Total lipid)	*Diet:*	(n-3) PUFA	(n-6) PUFA	(n-3) PUFA	(n-6) PUFA
Lipid Content		1.8 ±0.3	3.3 ±1.6	-	-
Σ polar lipid		38.2 ±1.2^{a}	31.2 ±0.9^{b}	34.3 ±2.1^{b}	18.8 ±2.9^{c}
Σ neutral lipid		61.8 ±1.2^{a}	68.8 ±0.9^{b}	65.7 ±2.1^{b}	81.2 ±2.9^{c}
Polar lipid:					
LPC		1.3 ±0.3^{a}	1.1 ±0.0^{a}	0.3 ±0.4^{b}	0.1 ±0.2^{b}
Sphingomyelin		1.1 ±0.2	1.2 ±0.3	1.9 ±1.2	1.4 ±0.2
PC		17.1 ±1.0^{a}	12.3 ±0.5^{b}	12.4 ±3.4^{bc}	8.7 ±1.2^{bc}
PS		2.1 ±0.0^{a}	1.3 ±0.1^{b}	2.2 ±0.2^{a}	0.8 ±0.1^{c}
PI		3.0 ±0.3^{a}	1.4 ±0.3^{b}	1.6 ±1.4^{ab}	0.3 ±0.1^{bc}
Cardiolipin		1.9 ±0.1	1.4 ±0.3	3.5 ±2.0	1.5 ±0.8
PE		10.5 ±0.1^{a}	8.6 ±0.3^{b}	7.3 ±2.5^{b}	3.1 ±0.9^{c}
Sulphatides		0.2 ±0.2	0.9 ±1.1	1.5 ±0.9	1.0 ±0.2
Cerebrosides		1.0 ±0.1	2.9 ±1.6	3.5 ±2.4	1.9 ±1.1
Neutral lipid:					
Cholesterol		9.4 ±0.9^{a}	11.7 ±1.3^{a}	28.4 ±10.5^{b}	22.2 ±3.6^{b}
Free fatty acids		5.4 ±0.7^{a}	9.5 ±2.2^{b}	3.1 ±1.4^{a}	7.0 ±2.4^{ab}
Triacylglycerols		47.0 ±0.3]	36.8 ±3.4	23.8 ±8.0	35.4 ±10.3
Sterol esters		]	7.7 ±0.5	10.0 ±3.4	16.6 ±7.3
Unknowns		-	3.1 ±1.0	0.4 ±0.6	-

Abbreviations:- LPC-Lysophosphatidylcholine; PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; Values are expressed as means (%) ±S.D. of triplicate samples. Values with different superscript letters are significantly different ($p < 0.05$) by ANOVA. Sterol esters and TAG were unresolved for the thymus from the (n-3) PUFA group. Lipid Content (mg g^{-1} wet weight of tissue)

Table 5.18 (a)

The fatty acid composition of thymus from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets or on a commercial diet in trial 1.

Fatty		thymus		
acids (%)	*Diet:*	(n-3) PUFA	(n-6) PUFA	commercial
14:0		3.7 ±0.3^{a}	2.0 ±0.1^{b}	0.4 ±0.3^{c}
16:0		18.8 ±0.7^{a}	15.4 ±1.5^{b}	17.6 ±0.8^{ab}
18:0		5.0 ±0.3	5.3 ±0.2	4.5 ±0.4
Σ saturates		**20.5 ±1.6**	**23.6 ±2.0**	**23.1 ±1.7**
16:1		6.5 ±0.2^{a}	3.6 ±0.2^{b}	4.9 ±0.5^{c}
18:1		17.4 ±0.8	18.6 ±2.0	17.3 ±3.1
20:1		5.4 ±0.2^{ab}	4.6 ±0.4^{a}	6.8 ±1.1^{b}
22:1		5.3 ±0.2	4.4 ±0.6	6.4 ±1.2
24:1		1.2 ±0.1	2.4 ±2.4	1.2 ±0.1
Σ monoenes		**36.0 ±1.6**	**33.8 ±5.2**	**36.8 ±6.0**
16:2		0.8 ±0.2	0.5 ±0.2	0.6 ±0.1
18:2		2.3 ±0.0^{a}	18.4 ±1.7^{b}	2.6 ±0.2^{a}
18:3		0.3 ±0.0^{a}	1.4 ±0.4^{b}	0.4 ±0.2^{a}
20:2		0.2 ±0.0^{a}	1.0 ±0.1^{b}	0.4 ±0.0^{a}
20:3		0.2 ±0.1^{a}	0.7 ±0.1^{b}	0.2 ±0.0^{a}
20:4		1.0 ±0.1	1.1 ±0.1	1.1 ±0.1
Σ (n-6)		**5.4 ±0.5^{a}**	**23.6 ±2.6^{b}**	**5.9 ±1.1^{a}**
18:3		0.6 ±0.2^{ab}	0.4 ±0.1^{b}	0.8 ±0.1^{a}
18:4		1.6 ±0.2^{a}	0.7 ±0.1^{b}	1.6 ±0.2^{a}
20:4		0.8 ±0.1^{a}	0.4 ±0.2^{b}	0.9 ±0.0^{a}
20:5		5.7 ±0.3^{a}	2.4 ±0.2^{b}	4.8 ±0.2^{c}
22:5		1.8 ±0.2^{a}	0.9 ±0.1^{b}	1.8 ±0.1^{a}
22:6		18.7 ±0.4^{a}	15.1 ±0.7^{b}	20.9 ±2.2^{a}
Σ (n-3) PUFA		**29.8 ±1.4^{a}**	**20.4 ±1.5^{b}**	**31.4 ±3.0^{a}**
(n-3)/(n-6)		**5.5 ±0.3^{a}**	**0.9 ±0.2^{b}**	**5.3 ±0.6^{a}**

In addition to the fatty acids show, Σ saturates include 15:0, 17:0, 20:0, 22:0; Σ monoenes include 14:1; Σ (n-6) PUFA include 22:2, 22:5 and Σ (n-3) PUFA include 16:3, 16:4; where one or more of the 3 values is < 0.5. Values are expressed as mean (%) ±S.D for triplicate samples. Values with different superscript letters are significantly different (p <0.05) by ANOVA.

Table 5.18 (b)

The fatty acid composition of thymus and thymus leucocytes from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 2.

Fatty		Thymus		Thymocytes	
acids (%)	*Diet:*	(n-3) PUFA	(n-6) PUFA	(n-3) PUFA	(n-6) PUFA
16:0da		0.3 ±0.1	0.2 ±0.0	-	-
14:0		3.5 ±0.2^{a}	1.7 ±0.2^{b}	2.0 ±0.3^{b}	1.4 ±0.4^{b}
16:0		19.1 ±1.0^{a}	14.5 ±0.5^{b}	22.9 ±0.5^{c}	24.9 ±2.6^{c}
18:0		4.0 ±0.2^{a}	5.0 ±0.2^{b}	8.5 ±1.4^{c}	26.9 ±12.2^{d}
Σ saturates		**27.2 ±1.1^{a}**	**21.8 ±0.6^{b}**	**36.8 ±2.7^{c}**	**56.0 ±11.7^{d}**
18:1da		0.2 ±0.1	-	4.8 ±1.7	2.4 ±1.0
16:1		4.5 ±0.3	2.2 ±0.3	6.2 ±0.4	3.3 ±1.4
18:1		17.3 ±0.3	18.2 ±0.4	19.2 ±1.9	13.6 ±4.5
20:1		6.0 ±0.4	3.4 ±0.6	4.1 ±2.9	1.8 ±0.9
22:1		6.2 ±0.3	3.4 ±0.7	3.3 ±2.9	1.3 ±0.6
24:1		0.9 ±0.1	0.9 ±0.6	4.5 ±2.8	1.6 ±0.6
Σ monoenes		**35.0 ±0.9^{a}**	**28.1 ±1.7^{b}**	**42.1 ±5.2^{c}**	**23.9 ±7.8^{b}**
18:2		2.6 ±0.2^{a}	23.3 ±1.0^{b}	2.8 ±0.2^{a}	8.0 ±4.1^{c}
20:2		0.3 ±0.0	0.9 ±0.0	0.1 ±0.1	0.5 ±0.2
20:3		-	0.5 ±0.0	0.1 ±0.1	0.2 ±0.2
20:4		0.9 ±0.0	1.0 ±0.1	2.1 ±0.9	1.1 ±0.3
Σ (n-6)		**4.5 ±0.3^{a}**	**26.5 ±1.0^{b}**	**6.8 ±1.5^{c}**	**11.1 ±4.5^{c}**
18:3		0.8 ±0.1	0.4 ±0.0^{b}	0.5 ±0.1	0.2 ±0.1
18:4		1.5 ±0.0^{a}	0.6 ±0.1^{b}	0.5 ±0.2^{b}	0.1 ±0.1^{c}
20:4		0.9 ±0.1^{a}	0.4 ±0.0^{b}	1.4 ±1.3^{ab}	0.6 ±0.3^{ab}
20:5		5.6 ±0.5^{a}	3.0 ±0.2^{b}	3.0 ±0.3^{b}	1.2 ±0.4^{c}
22:5		1.9 ±0.1^{a}	1.3 ±0.1^{b}	0.9 ±0.1^{c}	0.5 ±0.2^{d}
22:6		22.2 ±0.0^{a}	17.7 ±1.2^{b}	7.1 ±1.2^{c}	5.6 ±3.4^{c}
Σ (n-3) PUFA		**33.3 ±0.4^{a}**	**23.6 ±1.1^{b}**	**14.3 ±1.6^{c}**	**9.0 ±3.9^{d}**
(n-3)/(n-6)		**7.4 ±0.6^{a}**	**0.9 ±0.1^{b}**	**2.1 ±0.7^{c}**	**0.8 ±0.7^{bc}**

da - dimethyl acetals. In addition to the fatty acids shown, Σ saturates include 15:0; Σ monoenes include 14:1; Σ (n-6) PUFA include 16:2, 18:3; 22:2, 22:5 and Σ (n-3) PUFA include 16:3, 16:4; where one or more of the 4 values is < 0.5. Values are expressed as mean (%) ±S.D for triplicate samples. Values with different superscript letters are significantly different (p <0.05) by ANOVA.

Table 5.19 (a)

The fatty acid composition of lipid classes of thymus from Atlantic salmon maintained on high (n-3) PUFA diets in trial 2.

Fatty acids (%)	(n-3) PUFA Diet			
	PC	PS	PI	PE
16:0da	0.2	-	0.3	3.6
14:0	1.4	0.9	0.4	0.5
16:0	31.9	15.2	9.0	12.1
18:0	2.0	17.0	25.2	7.5
Σ saturates	**35.9**	**34.0**	**35.4**	**24.6**
18:1da	-	-	-	3.6
16:1	2.3	1.3	0.6	0.9
18:1	12.3	15.0	8.6	9.5
20:1	0.6	2.6	1.0	2.2
22:1	0.2	0.4	0.3	1.3
24:1	0.3	6.5	4.4	4.2
Σ monoenes	**15.6**	**25.9**	**14.9**	**21.6**
18:2	2.2	1.9	1.1	1.8
18:3	1.0	0.6	0.8	0.3
20:2	0.2	0.6	0.4	0.3
20:3	0.2	0.2	0.2	0.2
20:4	1.2	0.9	12.1	2.1
Σ (n-6)	**5.4**	**5.9**	**15.8**	**5.6**
16:3	0.3	0.6	0.5	0.3
18:3	0.2	0.4	0.3	0.3
20:4	0.4	0.3	0.2	0.5
20:5	6.9	2.8	6.9	5.4
22:5	1.1	2.8	1.8	2.0
22:6	33.9	27.2	24.2	39.6
Σ (n-3) PUFA	**43.1**	**34.3**	**33.9**	**48.2**
(n-3)/(n-6)	**8.0**	**5.8**	**2.3**	**8.6**

Abbreviations:- PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; da-dimethyl acetals. In addition to the fatty acids shown, Σ saturates include 15:0, 20:0, 22:0; Σ (n-6) PUFA include 16:2, 22:2, 22:5 and Σ (n-3) PUFA include 18:4, 20:4; where one or more of the 4 values is < 0.5. Values are expressed as 1 analysis of 3 pooled samples for trial 2.

Table 5.19 (b)

The fatty acid composition of lipid classes of thymus from Atlantic salmon maintained on high (n-6) PUFA diets in trial 2

Fatty acids (%)	(n-6) PUFA Diet			
	PC	PS	PI	PE
16:0da	0.8	0.3	0.2	2.3
14:0	0.3	0.3	0.9	0.2
16:0	25.7	12.7	10.3	8.6
18:0	2.4	15.5	18.3	7.1
Σ saturates	**29.4**	**29.3**	**29.9**	**18.4**
18:1da	-	0.5	0.5	2.5
16:1	1.4	1.2	2.0	0.5
18:1	12.7	15.3	11.9	9.1
20:1	1.0	3.7	5.9	1.6
22:1	0.2	0.9	3.6	0.1
24:1	0.3	8.6	4.7	1.2
Σ monoenes	**15.6**	**30.2**	**28.6**	**15.1**
18:2	10.0	5.6	4.6	7.7
18:3	0.1	0.9	0.9	0.2
20:2	1.0	1.0	0.7	1.3
20:3	0.4	0.4	0.3	0.6
20:4	1.4	1.0	5.8	2.3
Σ (n-6)	**13.6**	**10.4**	**13.6**	**13.2**
20:4	0.3	0.2	0.2	0.7
20:5	5.2	2.7	4.7	4.2
22:5	1.2	3.1	1.7	2.3
22:6	34.1	22.7	20.6	45.3
Σ (n-3) PUFA	**41.4**	**30.0**	**27.9**	**53.4**
(n-3)/(n-6)	**3.0**	**2.9**	**2.0**	**4.0**

Abbreviations:- PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; da-dimethyl acetals. In addition to the fatty acids shown, Σ saturates include 15:0, 20:0, 22:0; Σ (n-6) PUFA include 16:2, 22:2, 22:5; Σ (n-3) PUFA include 16:3, 18:3, 18:4; where one or more of the 4 values is < 0.5. Values are expressed as 1 analysis of 3 pooled samples for trial 2.

classes from the two dietary PUFA groups lay at either end of the established range.

Data obtained in trial 1 showed that the fish from the (n-6) PUFA dietary group incorporated high levels of 18:2(n-6) into the lipid of their thymus resulting in a low (n-3)/(n-6) PUFA ratio of 0.9 (Table 5.18(a)). The ratios for the (n-3) PUFA and commercial diet groups were 5.5 and 5.3 respectively. Intact thymus tissue lipid contained more dietary 18:2(n-6) fatty acid than that of the isolated thymocyte cells. In fish fed the (n-3) PUFA diet in trial 2, thymocytes had a significantly higher (n-3) PUFA level than the (n-6) PUFA group, but while the (n-3)/(n-6) PUFA ratio was apparently higher in the former, it was not statistically significantly different from that of the (n-6) PUFA group. Thymocytes from fish in trial 2 contained higher proportions of 16:0 and 18:0 than was found in the corresponding thymus (Table 5.18 (b)), thus resulting in a higher overall level of saturated fatty acid.

Overall, the lipid of thymocytes was more saturated and less polyunsaturated than that of the thymus tissue from which they were isolated. Phospholipid classes of thymus had fatty acid compositions similar to those reported for erythrocytes and leucocytes (see Tables 5.9 and 5.12), thus, regardless of diet, the PC fraction had the highest level of 16:0 and high levels of 18:1, while PS and PI were rich in 16:0, 18:0 and 18:1. PI was characterised by its high content of 18:0 and 20:4(n-6). The lowest level of saturation was always found in the PE fraction which was compensated for by its having the highest level of (n-3) PUFA unsaturation, and therefore the highest (n-3)/(n-6) PUFA ratio. The influence of dietary PUFA composition was most evident in the PC and PE fractions in which the levels of 18:2(n-6) and its elongation product 20:2(n-6) were

notably higher in fish fed the (n-6) PUFA than in those fed (n-3) PUFA (Table 5.19 (b)). Nevertheless, the PI fraction from the (n-3) dietary PUFA group thymus (Table 5.19 (a)) contained notably higher levels of 20:4(n-6) than that of the (n-6) dietary PUFA group (12.1 % vs. 5.8 %)(Table 5.19 (b)), and the PE fraction of the thymus of the latter group contained a higher 22:6(n-3) level (53.4 %) than that of the (n-3) dietary PUFA group (48.2 %).

5.3.3.3 Spleen

The amount of lipid extracted from spleen tissue from dietary trial 1 and 2 salmon is presented in Tables 5.20 (a) and (b). Only the fatty acid content of spleen cells from trial 2 was measured, which was 2.7 ±1.0 $\mu g/10^6$ cells for the (n-3) PUFA group fish, and 3.6 ±1.8 $\mu g/10^6$ cells for the (n-6) PUFA group. The lipid content of spleen and fatty acid content of spleen cells were not significantly influenced by PUFA composition of the diet.

The lipid classes of spleen from trial 1, and spleen and spleen cells from trial 2 are shown in Table 5.20 (a) and (b) respectively. Spleen lipid from fish in all dietary groups of trial 1 had similar levels of polar lipid to those found in the isolated spleen leucocytes of trial 2, while intact spleen tissue from trial 2 contained higher levels of polar lipid, although the latter was only significantly different from the (n-3) PUFA dietary spleen cells. Trial 2 spleen leucocytes contained lower levels of polar lipid than their parent tissue due to lower PE levels, and with the (n-3) PUFA dietary group also due to lower PC levels. Conversely, these cells contained higher proportions of cholesterol and sterol esters than the tissue from which they were taken.

The total lipid of spleen from fish in trials 1 and 2 contained lower proportions of 16:0 and 18:0 than found in corresponding isolated spleen cells, which resulted in an overall lower saturated fatty acid level (Table 5.21). It was observed for both dietary groups that the tissue lipid contained more dietary 18:2(n-6) fatty acid than the isolated cells. There was no significant difference in proportions of 20:4(n-6) PUFA between the lipid of intact spleens of the different dietary groups and between their spleen cells. Noticeable differences existed between the (n-3)/(n-6) PUFA ratios of the spleens from different dietary groups, but no differences were evident with spleen cells.

Individual lipid classes of spleen had fatty acid patterns similar to those reported for other tissues in this section. In both dietary groups in trial 2, the PC fraction had the highest level of 16:0 (44.4 % for the (n-3) PUFA group and 31.4% for the (n-6) PUFA group) and also the highest levels of 18:1 (31.2 % for the (n-3) PUFA group and 19.5 % for the (n-6) PUFA group) (Tables 5.22 (a) and (b)). The PS and PI phospholipid classes had high levels of 16:0, 18:0 and 18:1. The 18:0 level was higher in the PI fraction than in the PE fraction. The PI lipid class also contained a typically high level of 20:4(n-6). The lowest level of saturation was again found in the PE fraction which was compensated for by the highest level of (n-3) PUFA unsaturation, however, since PS had a high (n-3) PUFA level and a low (n-6) PUFA level, it had the highest (n-3)/(n-6) PUFA ratio for both of the dietary groups. The influence of dietary fatty acid was most evident in the PC and PE fractions, in which 18:2(n-6), and 20:3(n-6) derived from it, were present in high levels in the spleen lipid of the fish from the (n-6) PUFA group.

Table 5.20 (a)

The lipid class composition of spleen from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets or a commercial diet in trial 1.

Lipid class	Diet		
(% Total lipid)	(n-3) PUFA	(n-6) PUFA	Commercial
Lipid Content	1.5 ±0.6	1.9 ±0.9	1.3 ±0.6
Σ polar lipid	26.1 ±4.2	19.3 ±5.7	19.0 ±6.7
Σ neutral lipid	73.9 ±4.2	80.7 ±5.7	81.0 ±6.7
Polar lipid:			
LPC	0.3 ±0.2	-	0.1 ±0.1
Sphingomyelin	2.0 ±0.6	1.0 ±0.1	1.0 ±0.2
PC	9.6 ±1.7	7.2 ±1.0	8.7 ±3.4
PS	3.1 ±1.1	4.0 ±3.7	1.4 ±0.4
PI	1.2 ±0.7	0.9 ±0.4	0.9 ±0.6
Cardiolipin	0.4 ±0.2	0.2 ±0.2	0.4 ±0.4
PE	8.0 ±1.8	5.6 ±1.1	6.0 ±2.1
Sulphatides	0.3 ±0.0	0.1 ±0.1	0.1 ±0.1
Cerebrosides	1.0 ±0.1^{a}	0.1 ±0.1^{b}	0.2 ±0.2^{b}
Neutral lipid:			
Cholesterol	16.3 ±2.4^{a}	14.1 ±4.0^{ab}	10.8 ±1.5^{b}
Free fatty acids	13.3 ±1.4^{a}	9.0 ±5.5^{ab}	6.4 ±1.2^{b}
Triacylglycerols	29.1 ±3.5^{a}	46.3 ±17.0^{ab}	55.2 ±6.0^{b}
Sterol esters	13.2 ±3.0^{a}	11.0 ±2.6a^{b}	7.7 ±1.7^{b}
Unknowns	2.2 ±0.7	0.7 ±0.6	1.0 ±0.8

Abbreviations:- LPC-Lysophosphatidylcholine; PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; Values are expressed as means (%) ±S.D. of triplicate samples. Values with different superscript letters are significantly different ($p < 0.05$) by ANOVA. Lipid Content (mg g^{-1} wet weight of tissue)

Table 5.20 (b)

The lipid class composition of spleen and spleen leucocytes from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 2

Lipid class		Spleen		Spleen Leucocytes	
(% Total lipid)	*Diet:*	(n-3) PUFA	(n-6) PUFA	(n-3) PUFA	(n-6) PUFA
Lipid Content		2.7 ±0.8	2.0 ±0.6	-	-
Σ polar lipid		37.2 ±4.8^{a}	34.3 ±5.9^{a}	14.3 ±3.8^{b}	26.1 ±8.3^{ab}
Σ neutral lipid		62.8 ±4.8^{a}	65.7 ±5.9^{a}	85.7 ±3.8^{b}	73.9 ±8.3^{ab}
Polar lipid:					
LPC		0.4 ±0.1	1.2 ±1.1	-	-
Sphingomyelin		2.7 ±0.3^{a}	2.1 ±0.8^{ab}	1.2 ±0.3^{b}	2.8 ±1.5^{ab}
PC		10.2 ±0.8^{a}	9.4 ±1.3^{a}	5.9 ±1.1^{b}	8.2 ±3.0^{ab}
PS		4.4 ±0.6^{a}	3.5 ±0.7^{a}	0.6 ±0.4^{b}	1.5 ±0.8^{b}
PI		4.3 ±1.6^{a}	2.0 ±0.4^{b}	0.4 ±0.3^{c}	1.4 ±1.1^{abc}
Cardiolipin		1.4 ±0.3^{a}	2.2 ±0.7^{a}	0.7 ±0.6^{a}	4.2 ±1.4^{b}
PE		12.0 ±1.7^{a}	11.2 ±1.8^{a}	1.7 ±0.9^{b}	3.0 ±1.4^{b}
Sulphatides		0.3 ±0.1^{a}	0.6 ±0.5^{ab}	1.0 ±0.3^{b}	1.3 ±0.4^{b}
Cerebrosides		1.5 ±0.4^{a}	2.1 ±1.5^{ab}	2.9 ±0.5^{b}	3.6 ±1.0^{b}
Neutral lipid:					
Cholesterol		18.6 ±1.3^{a}	17.7 ±1.0^{a}	20.7 ±0.5^{b}	27.0 ±2.3^{c}
Free fatty acids		12.4 ±1.0^{a}	14.5 ±1.4^{a}	9.1 ±0.7^{b}	8.5 ±0.8^{b}
Triacylglycerols		31.4 ±6.8}	25.2 ±8.0	26.2 ±0.8	18.7 ±4.1
Sterol esters		}	6.0 ±2.2	29.6 ±5.7	19.7 ±6.0
Unknown		-	2.3 ±1.4	-	-

Abbreviations:- LPC-Lysophosphatidylcholine; PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; Values are expressed as means (%) ±S.D. of triplicate samples. Values with different superscript letters are significantly different ($p < 0.05$) by ANOVA. TAG and sterol esters were unresolved by HPTLC for the spleen from the (n-3) dietary PUFA group. Lipid Content (mg g^{-1} wet weight of tissue).

Table 5.21 (a)

The fatty acid composition of spleen from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets or a commercial diet in trial 1.

Fatty acids (%)	Dietary Group		
	Commercial	(n-3) PUFA	(n-6) PUFA
14:0	5.1 ±0.4[a]	3.7 ±0.7[a]	2.1 ±0.5[c]
16:0	18.3 ±1.1	20.0 ±2.0	19.4 ±1.2
18:0	4.1 ±0.1[a]	4.8 ±0.7[a]	7.1 ±0.7[b]
Σ saturates	**28.4 ±1.6**	**29.7 ±1.6**	**29.8 ±1.7**
16:1	6.7 ±0.7	5.1 ±0.8	3.9 ±0.2
18:1	18.8 ±1.6	18.8 ±1.9	17.0 ±0.8
20:1	6.8 ±1.1	5.2 ±1.5	3.3 ±0.4
22:1	7.1 ±1.0	5.2 ±1.9	3.5 ±0.3
24:1	0.9 ±0.0	0.9 ±0.1	1.1 ±0.4
Σ monoenes	**41.5 ±2.6[a]**	**35.5 ±2.6[a]**	**29.3 ±1.6[b]**
16:2	1.1 ±0.4	0.7 ±0.2	0.4 ±0.2
18:2	3.0 ±0.2[a]	2.9 ±0.7[a]	12.1 ±3.0[b]
18:3	0.9 ±0.4[a]	0.6 ±0.2[a]	5.2 ±0.2[b]
20:2	0.4 ±0.3[a]	0.2 ±0.1[a]	1.0 ±0.0[b]
20:3	0.2 ±0.0[a]	0.2 ±0.0[a]	1.0 ±0.1[b]
20:4	1.0 ±0.1[a]	1.8 ±0.7[ab]	2.7 ±0.8[b]
22:2	0.8 ±0.2	1.8 ±1.0	0.8 ±1.0
Σ (n-6)	**7.6 ±0.4[a]**	**8.5 ±0.3[a]**	**18.8 ±3.0[b]**
16:3	1.2 ±0.9	0.5 ±0.1	0.4 ±0.1
18:4	2.0 ±0.1[a]	1.1 ±0.5[b]	0.5 ±0.2[b]
20:4	1.0 ±0.1[a]	1.8 ±0.7[a]	0.4 ±0.1[b]
20:5	4.7 ±0.6[a]	5.6 ±1.1[a]	2.8 ±0.4[b]
22:5	1.6 ±0.1[a]	0.3 ±0.0[b]	0.7 ±0.0[c]
22:6	12.0 ±1.0	16.0 ±2.4	15.8 ±4.4
Σ (n-3) PUFA	**23.6 ±1.2**	**26.3 ±1.2**	**21.4 ±4.8**
(n-3)/(n-6)	**3.1 ±0.3[a]**	**3.2 ±0.8[a]**	**1.4 ±0.3[b]**

In addition to the fatty acids shown, Σ saturates include 15:0, 17:0, 20:0, 22:0, 24:0; Σ monoenes include 14:1; Σ (n-6) PUFA include 22:5 and Σ (n-3) PUFA include 16:4, 18:3; where one or more of the 3 values is < 0.5. Values are expressed as means (%) ±S.D. of triplicate samples. Values with different superscript letters are significantly different ($p < 0.05$) by ANOVA.

Table 5.21 (b)

The fatty acid composition of spleen and spleen leucocytes from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 2.

Fatty		Spleen		Spleen Leucocytes	
acids (%)	*Diet:*	(n-3) PUFA	(n-6) PUFA	(n-3) PUFA	(n-6) PUFA
14:0		3.1 ±1.1^{a}	1.3 ±0.1^{b}	1.4 ±0.3^{b}	1.4 ±0.5^{b}
16:0		19.1 ±0.8^{a}	16.1 ±2.4^{a}	25.1 ±0.7^{b}	27.6 ±2.2^{b}
18:0		5.4 ±0.2^{a}	6.7 ±0.8^{a}	10.9 ±1.3^{b}	11.9 ±0.3^{b}
Σ saturates		**29.0 ±1.1^{a}**	**24.9 ±3.3^{a}**	**40.8 ±1.8^{b}**	**43.6 ±2.6^{b}**
18:1da		0.7 ±0.3	0.8 ±0.2	-	-
16:1		3.1 ±0.8^{a}	1.8 ±0.4^{a}	5.8 ±0.0^{b}	5.0 ±0.6^{b}
18:1		18.0 ±1.4^{a}	17.4 ±0.5^{a}	25.3 ±4.0^{b}	23.7 ±1.8^{b}
20:1		4.4 ±1.2	2.6 ±0.8	2.1 ±0.7	3.4 ±1.2
22:1		3.7 ±1.3^{a}	2.3 ±0.8^{ab}	1.1 ±0.4^{b}	2.3 ±0.8^{ab}
24:1		1.0 ±0.3^{a}	0.5 ±0.3^{a}	2.8 ±0.5^{b}	2.5 ±0.5^{b}
Σ monoenes		**30.9 ±4.3^{a}**	**25.4 ±1.7^{a}**	**42.7 ±5.8^{b}**	**40.2 ±2.9^{b}**
18:2		5.0 ±3.7^{a}	21.0 ±5.5^{b}	4.0 ±1.6^{a}	5.1 ±1.2^{a}
20:2		0.2 ±0.2^{a}	1.1 ±0.1^{b}	- ^{a}	0.4 ±0.1^{a}
20:3		0.2 ±0.5^{a}	0.8 ±0.1^{b}	- ^{a}	0.1 ±0.1^{a}
20:4		2.5 ±0.1	2.4 ±0.5	2.5 ±1.1	1.7 ±0.7
Σ (n-6)		**8.7 ±4.1^{a}**	**26.0 ±5.0^{b}**	**8.0 ±1.2^{a}**	**8.6 ±2.0^{a}**
16:3		0.3 ±0.1^{a}	0.3 ±0.0^{a}	0.9 ±0.3^{b}	0.7 ±0.1^{b}
18:3		0.6 ±0.1^{a}	0.3 ±0.0^{b}	1.5 ±0.8^{a}	1.0 ±1.1^{a}
18:4		0.9 ±0.4^{a}	0.3 ±0.0^{b}	0.2 ±0.2^{c}	0.1 ±0.1^{c}
20:4		0.7 ±0.1^{a}	0.3 ±0.1^{b}	0.4 ±0.1^{b}	0.9 ±1.1^{ab}
20:5		5.4 ±0.4^{a}	3.1 ±0.2^{c}	1.4 ±0.4^{b}	1.1 ±0.4^{b}
22:5		1.6 ±0.1^{a}	1.2 ±0.1^{a}	0.4 ±0.2^{b}	0.2 ±0.1^{b}
22:6		22.0 ±3.1^{a}	18.2 ±3.3^{a}	3.7 ±1.5^{b}	3.6 ±1.6^{b}
Σ (n-3) PUFA		**31.5 ±2.6^{a}**	**23.7 ±3.4^{b}**	**8.5 ±3.4^{c}**	**7.6 ±3.3^{c}**
(n-3)/(n-6)		**3.6 ±2.0^{a}**	**0.9 ±0.3^{b}**	**1.1 ±0.6^{ab}**	**0.9 ±0.3^{b}**

da - dimethyl acetals. In addition to the fatty acids shown, Σ saturates include 15:0, 17:0, 20:0, 22:0, 24:0; Σ monoenes include 14:1 and Σ (n-6) PUFA include 16:2, 18:3, 22:2, 22:5; where one or more of the 4 values is < 0.5. Values are expressed as means (%) ±S.D. of triplicate samples. Values with different superscript letters are significantly different (p <0.05) by ANOVA.

Table 5.22 (a)

The fatty acid composition of lipid classes of spleen from Atlantic salmon maintained on high (n-3) PUFA diets in trial 2.

Fatty acids	(n-3) PUFA diet			
(%)	PC	PS	PI	PE
16:0da	0.2	1.1	0.2	5.2
14:0	2.8	0.3	0.4	0.2
15:0	0.9	0.3	0.3	0.1
16:0	44.4	13.5	10.0	11.3
18:0	2.6	20.8	24.8	6.9
Σ saturates	**51.0**	**36.1**	**35.7**	**24.5**
18:1da	-	-	2.0	5.2
16:1	2.3	0.7	0.8	0.5
18:1	31.2	9.1	8.6	9.3
20:1	0.9	2.5	1.0	1.8
22:1	-	0.1	0.5	0.2
24:1	0.3	1.2	0.3	0.5
Σ monoenes	**34.7**	**13.6**	**13.3**	**17.4**
16:2	0.2	0.2	1.9	0.8
18:2	0.5	0.7	0.7	0.8
18:3	0.2	0.4	0.4	-
20:4	1.3	0.7	22.0	2.8
Σ (n-6)	**2.2**	**2.6**	**25.8**	**5.4**
16:3	0.2	0.9	1.2	0.4
20:5	2.9	1.3	4.0	4.3
22:5	0.5	2.0	1.0	1.5
22:6	8.4	43.0	18.9	45.9
Σ (n-3)	**12.2**	**47.8**	**25.2**	**52.7**
(n-3)/(n-6)	**5.5**	**18.4**	**1.0**	**9.8**

Abbreviations:- PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; da-dimethyl acetals. In addition to the fatty acids shown, Σ saturates include 20:0; Σ (n-6) PUFA include 20:2, 20:3, 22:2, 22:5; Σ (n-3) PUFA include 18:3, 18:4, 20:4; where one or more of the 4 values is < 0.5. Values are expressed as 1 analysis of 3 pooled samples for trial 2.

Table 5.22 (b)

The fatty acid composition of lipid classes of spleen from Atlantic salmon maintained on high (n-6) PUFA diets from trial 2.

Fatty acids	(n-6) PUFA Diet			
(%)	PC	PS	PI	PE
16:0da	0.2	0.3	0.2	3.1
18:0da	-	-	-	0.7
14:0	0.7	0.3	0.2	0.1
16:0	31.4	14.4	7.7	9.6
18:0	3.8	21.6	26.3	9.2
Σ saturates	**36.5**	**36.9**	**34.5**	**22.8**
18:1da	0.1	0.1	1.2	5.4
16:1	1.0	0.5	0.3	0.2
18:1	19.5	7.8	6.6	6.9
20:1	0.7	1.2	1.2	1.1
24:1	0.3	1.0	0.8	0.1
Σ monoenes	**21.8**	**10.7**	**10.1**	**14.0**
16:2	0.3	0.3	0.3	0.8
18:2	9.2	4.5	3.5	7.4
18:3	0.1	0.1	0.2	0.1
20:2	0.9	0.7	0.5	0.9
20:3	1.0	1.6	1.5	0.7
20:4	2.6	1.4	21.4	4.0
Σ (n-6)	**14.4**	**8.0**	**27.4**	**14.3**
18:3	0.1	0.6	-	0.1
20:4	0.3	0.4	0.5	0.2
20:5	5.4	2.2	3.7	5.0
22:5	0.8	1.5	3.7	1.3
22:6	20.3	39.1	21.6	42.1
Σ (n-3) PUFA	**27.4**	**39.1**	**21.6**	**49.0**
(n-3)/(n-6)	**1.9**	**4.9**	**0.8**	**3.4**

Abbreviations:- PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; da-dimethyl acetals. In addition to the fatty acids shown, Σ saturates include 15:0; Σ monoenes include 22:1; Σ (n-6) PUFA include 22:2, 22:5; Σ (n-3) PUFA include 16:3, 18:4; where one or more of the 4 values is < 0.5. Values are expressed as 1 analysis of 3 pooled samples for trial 2.

5.3.3.4 Kidney

The amount of lipid extracted from kidney tissue from dietary trial 1 and 2 salmon is presented in Tables 5.23 (a) and (b). The fatty acid content of the kidney cells from trial 2 was determined and found to be 3.5 ±1.5 $\mu g/10^6$ cells for the (n-3) PUFA group of fish, and 2.0 ±0.5 $\mu g/10^6$ cells for the (n-6) PUFA group fish. The lipid content of kidney and fatty acid content of kidney cells were not significantly influenced by PUFA composition of the diet.

Headkidney macrophages had been isolated according to Section 3.2. Their fatty acid content was not determined since no internal standard had been added during the methyl ester preparation.

Lipid classes of headkidney from trial 1 are shown in Tables 5.23 (a), and headkidney from trial 2 and the leucocytes isolated from it are shown in Tables 5.23 (b). Kidney contained a higher percentage of polar lipid in its total lipid, both in trial 1 and trial 2, than was found in the leucocytes isolated from it. Macrophages, by comparison, contained a higher percentage of polar lipid than the kidney tissue (Table 5.26). The differences observed in polar lipid between the head kidney tissue and their leucocytes were due both to a lower PE fraction and a higher cholesterol level in the leucocyte lipid. Conversely, the macrophages had a higher percentage of PC and cerebrosides than the kidney tissue, although the higher cerebroside fractions were only found for the (n-6) PUFA and commercial group macrophages. The leucocytes from both dietary groups (Table 5.23 (b)) exhibited lipid class profiles characteristic to those found previously with peripheral blood leucocytes (Table 5.11).

Fatty acid compositions of tissue and cells are given in Table 5.24 (a) and (b) respectively. Headkidney leucocytes from trial 2 contained in their lipid higher proportions of 16:0 and 18:0 than was found in either the corresponding tissue, or the kidneys in trial 1, thus resulting in a higher saturated fatty acid level. Both the tissue and the cells contained similar levels of 18:2(n-6) derived from the diet, and its elongation products 20:2(n-6) and 20:3(n-6). The (n-3) PUFA group leucocytes had a significantly higher (n-3) PUFA level in total lipid than the (n-6) PUFA group, but while the (n-3)/(n-6) PUFA ratio was higher, it was not significantly different from the (n-6) PUFA group (2.3 vs 1.0). The fatty acid composition of the lipid of macrophages was comparable to that of the leucocytes (Table 5.27).

Profiles of lipid class fatty acids of headkidney and headkidney leucocytes from dietary trial 2 fish can be found in Table 5.25 (a-d). The PC fraction of headkidney lipid always contained the highest level of 16:0 and 18:1 fatty acids, while PS and PI had high levels of 16:0, 18:0 and 18:1. The 18:0 level was higher in the PI fraction than in PS fraction, while the PS lipid class had a higher 16:0 level. The PI lipid class, as found with the previous tissues, contained a typically high level of 20:4(n-6). The lowest level of saturation was found in the PE fraction which was compensated for by the highest level of (n-3) PUFA unsaturation, but as it had a higher proportion of (n-6) PUFA, PE had a lower (n-3)/(n-6) PUFA ratio than was found for PC and PS in both dietary groups. Fatty acid analysis of lipids of thymus and spleen tissues from dietary fish indicated that dietary 18:2(n-6) is present in highest proportions in the PC and PE fractions. Lipids of the headkidney were no exception with higher levels of 18:2(n-6) and its elongation product 20:2(n-6) being observed in these two phospholipids.

Since there was insufficient lipid obtained from the headkidney leucocytes to analyse the PS and PI fractions separately, the two lipid classes were subsequently pooled before analysing their fatty acid composition. Similar patterns of saturated fatty acids were found to those observed in the intact kidney tissue, but the phospholipids of isolated leucocytes contained higher levels of (n-6) PUFA and lower levels of (n-3) PUFA (see Tables 5.25 (a-d)). The combined PS and PI fractions comprised of higher 16:0 and 18:0 fatty acid levels in the lipid of (n-3) PUFA dietary group leucocytes than those of the (n-6) PUFA group. As with the spleen cells, it was not possible to establish if the PI fraction had a high 20:4(n-6) content, since its presence was masked by PS component fatty acids. The incorporation of dietary 18:2(n-6) into the PC, PS/PI and PE of the (n-6) PUFA dietary group leucocytes resulted in lower (n-3)/(n-6) PUFA ratios in these phospholipids compared with the (n-3) PUFA dietary group.

Table 5.23 (a)

The lipid class composition of kidney from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets or a commercial diet in trial 1.

Lipid class		Kidney		
(% Total lipid)	*Diet:*	(n-3) PUFA	(n-6) PUFA	Commercial
Lipid Content		4.0 ±1.3	3.0 ±1.4	6.6 ±1.6
Σ polar lipid		45.8 ±1.4	42.8 ±1.3	39.4 ±3.9
Σ neutral lipid		54.2 ±1.4	57.2 ±1.3	60.6 ±3.9
Polar lipid:				
Sphingomyelin		3.2 ±1.1	3.2 ±0.4	3.1 ±0.4
PC		17.0 ±1.2	16.7 ±0.2	15.6 ±1.1
PS		4.2 ±0.1	3.7 ±0.1	3.7 ±0.6
PI		3.6 ±0.3	2.7 ±0.6	3.2 ±0.3
Cardiolipin		2.0 ±0.4	2.0 ±0.9	1.1 ±0.8
PE		12.9 ±0.5	12.3 ±0.5	11.6 ±0.7
Sulphatides		0.2 ±0.0	0.1 ±0.1	0.1 ±0.0
Cerebrosides		1.9 ±0.3	2.0 ±0.4	0.9 ±0.6
Neutral lipid:				
Cholesterol		16.2 ±0.1	17.7 ±2.0	14.6 ±1.4
Free fatty acids		4.8 ±0.9	5.7 ±0.5	4.3 ±0.9
Triacylglycerols		31.6 ±0.5]	33.8 ±2.4]	41.1 ±6.3]
Sterol esters		]	]	]
Unknowns		2.0 ±0.0	1.2 ±0.0	0.6 ±0.2

Abbreviations:- PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine. Values are expressed as means (%) ±S.D. of triplicate samples. Lipid Content (mg g^{-1} wet weight of tissue).

Table 5.23 (b)

The lipid class composition of kidney and kidney leucocytes from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 2.

Lipid class	Kidney		Kidney Leucocytes	
(% Total lipid)	(n-3) PUFA	(n-6) PUFA	(n-3) PUFA	(n-6) PUFA
Lipid Content	2.9 ±0.2	2.6 ±0.3	-	-
Σ polar lipid	42.2 ±5.2^{a}	46.8 ±4.3^{a}	31.2 ±1.3^{b}	28.3 ±2.5^{b}
Σ neutral lipid	57.8 ±5.2^{a}	53.2 ±4.3^{a}	68.8 ±1.3^{b}	71.7 ±2.5^{b}
Polar lipid:				
LPC	0.7 ±0.1^{a}	0.9 ±0.1^{a}	-	0.5 ±0.1^{b}
Sphingomyelin	3.7 ±0.4^{a}	3.8 ±0.2^{a}	2.8 ±0.5^{b}	2.5 ±0.1^{b}
PC	13.3±0.2^{bc}	16.8 ±2.1^{a}	13.3±0.2^{bc}	12.3±1.4^{bc}
PS	3.5 ±0.7^{ab}	4.2 ±0.4^{ac}	2.1 ±0.7^{b}	2.0 ±0.4^{b}
PI	2.7 ±0.4^{a}	2.7 ±0.4^{a}	1.2 ±0.2^{b}	1.0 ±0.1^{b}
Cardiolipin	3.1 ±0.6^{a}	2.8 ±0.3^{a}	2.8 ±0.5^{a}	2.0 ±0.1^{b}
PE	14.0 ±1.8^{a}	14.7 ±1.2^{a}	7.7 ±1.3^{b}	6.4 ±1.0^{b}
Sulphatides	- ^{a}	0.4 ±0.1^{a}	1.0 ±0.4^{b}	1.0 ±0.1^{b}
Cerebrosides	1.2 ±0.5^{a}	1.0 ±0.1^{a}	0.3 ±0.2^{b}	0.5 ±0.3^{b}
Neutral lipid:				
Cholesterol	14.6 ±0.7^{a}	16.6 ±0.9^{a}	25.3 ±1.9^{b}	22.8 ±0.6^{b}
Free fatty acids	10.8 ±0.4^{a}	12.2 ±1.9^{a}	13.6 ±2.7^{a}	18.9 ±1.8^{b}
Triacylglycerols	27.4 ±5.8	18.7 ±6.8	21.7 ±4.2	16.8 ±4.7
Sterol esters.	4.8 ±0.8^{a}	5.2 ±1.0^{a}	8.2 ±0.5^{b}	13.2 ±5.8^{b}
Unknowns	0.2 ±0.2	0.5 ±0.1	-	1.4 ±0.4

Abbreviations:- LPC-Lysophosphatidylcholine; PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; Values are expressed as means (%) ±S.D. of triplicate samples. Values with different superscript letters are significantly different ($p < 0.05$) by ANOVA. Lipid Content (mg g^{-1} wet weight of tissue).

Table 5.24 (a)

The fatty acid composition of kidney from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets or a commercial diet in trial 1.

Fatty		Kidney		
acids (%)	*Diet:*	(n-3) PUFA	(n-6) PUFA	commercial
14:0		2.8 ±1.8^{ab}	1.9 ±0.1^{b}	3.9 ±0.6^{a}
16:0		19.2 ±1.2^{a}	14.8 ±1.3^{b}	17.4 ±1.3^{ab}
18:0		4.1 ±0.4	4.8 ±0.4	3.9 ±0.4
Σ saturates		**26.7 ±3.5**	**22.2 ±1.8**	**26.0 ±2.6**
16:1		5.6 ±1.1^{a}	2.8 ±0.2^{b}	5.2 ±0.8^{a}
18:1		15.7 ±4.2	17.9 ±1.1	17.6 ±1.4
20:1		4.8 ±1.3^{ab}	4.6 ±0.7^{b}	7.4 ±1.6^{a}
22:1		4.2 ±1.2^{ab}	3.8 ±0.6^{b}	6.3 ±1.7^{a}
24:1		1.2 ±0.2	0.7 ±0.5	1.0 ±0.4
Σ monoenes		**31.8 ±8.0**	**30.1 ±3.1**	**37.8 ±6.1**
18:2		2.0 ±0.4^{a}	21.9 ±3.3^{b}	2.4 ±0.3^{a}
18:3		0.3 ±0.0^{a}	0.9 ±0.2^{b}	0.2 ±0.0^{a}
20:2		0.2 ±0.1^{a}	1.2 ±0.1^{b}	0.3 ±0.0^{a}
20:3		0.1 ±0.1^{a}	1.0 ±0.1^{b}	0.2 ±0.0^{a}
20:4		2.6 ±1.0	2.5 ±0.7	2.1 ±0.9
Σ (n-6)		**6.9 ±1.9^{a}**	**28.5 ±4.6^{b}**	**7.2 ±2.1^{a}**
18:3		0.6 ±0.0	0.4 ±0.0	0.7 ±0.4
18:4		1.4 ±0.4^{a}	0.4 ±0.2^{b}	1.6 ±0.3^{a}
20:4		0.8 ±0.1^{a}	0.4 ±0.0^{b}	2.1 ±0.9^{c}
20:5		9.1 ±1.0^{a}	3.2 ±0.6^{b}	6.1 ±1.0^{c}
22:5		1.7 ±0.3^{a}	0.8 ±0.0^{b}	1.6 ±0.1^{a}
22:6		19.6 ±2.8^{a}	13.4 ±2.3^{b}	17.6 ±2.5^{ab}
Σ (n-3) PUFA		**33.1 ±5.9^{a}**	**18.9 ±3.4^{b}**	**30.4 ±5.5^{a}**
(n-3)/(n-6)		**4.8 ±2.6^{a}**	**0.7 ±0.51^{b}**	**4.2 ±1.2^{a}**

In addition to the fatty acids shown, Σ saturates include 15:0, 17:0, 20:0, 22:0; Σ monoenes include 14:1; Σ (n-6) PUFA include 16:2, 22:2, 22:5; Σ (n-3) PUFA include 16:3, 16:4; where one or more of the 3 values is < 0.5. Values are expressed as means (%) ±S.D. of triplicate samples. Values with different superscript letters are significantly different (p <0.05) ANOVA.

Table 5.24 (b)

The fatty acid composition of kidney and kidney leucocytes from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 2.

Fatty		Kidney		Kidney Leucocytes	
acids (%)	*Diet:*	(n-3) PUFA	(n-6) PUFA	(n-3) PUFA	(n-6) PUFA
14:0		2.6 ±0.5^{a}	1.0 ±0.3^{b}	2.2 ±0.4^{a}	1.6 ±0.4^{ab}
16:0		19.9 ±0.4^{a}	17.0 ±1.1^{b}	26.2 ±2.6^{c}	22.8 ±1.0^{d}
18:0		4.5 ±0.1^{a}	5.8 ±0.1^{b}	6.8 ±0.2^{c}	7.6 ±0.4^{d}
Σ saturates		**28.0 ±0.8^{a}**	**24.5 ±1.1^{b}**	**37.9 ±3.7^{c}**	**33.7 ±1.5^{c}**
18:1da		0.4 ±0.1	0.7 ±0.0	1.1 ±0.6	1.1 ±0.6
16:1		3.2 ±0.6	1.2 ±0.3	4.1 ±0.5	3.5 ±0.2
18:1		16.3 ±1.1	13.8 ±1.9	16.4 ±0.5	16.6 ±0.5
20:1		5.2 ±0.7	2.3 ±0.7	1.5 ±0.9	2.1 ±0.1
22:1		4.3 ±0.4	2.0 ±0.6	1.0 ±0.5	1.0 ±0.4
24:1		1.6 ±0.2	1.2 ±0.2	1.6 ±0.0	2.3 ±1.1
Σ monoenes		**31.0 ±2.8^{a}**	**21.2 ±3.5^{b}**	**25.7 ±0.9^{bc}**	**26.6 ±0.1^{c}**
18:2		2.2 ±0.2^{a}	16.0 ±2.7^{b}	5.1 ±3.7^{a}	13.1 ±3.1^{b}
18:3		0.1 ±0.1	0.2 ±0.1	0.3 ±0.0	0.2 ±0.0
20:2		0.2 ±0.0^{a}	1.0 ±0.1^{b}	0.5 ±0.3^{a}	1.0 ±0.3^{b}
20:3		0.1 ±0.0^{a}	0.7 ±0.1^{b}	0.4 ±0.3^{ab}	1.0 ±0.1^{c}
20:4		3.1 ±0.4	4.4 ±0.6^{a}	3.4 ±0.2^{b}	3.5 ±0.5
Σ (n-6)		**6.4 ±0.2^{a}**	**23.5 ±2.0^{b}**	**11.0 ±5.1^{a}**	**19.6 ±2.9^{b}**
18:3		0.6 ±0.1^{a}	0.3 ±0.1^{b}	0.3 ±0.0^{b}	0.3 ±0.0^{b}
18:4		0.8 ±0.2	0.3 ±0.1	0.5 ±0.5	0.4 ±0.1
20:4		0.6 ±0.1^{a}	0.3 ±0.0^{b}	0.6 ±0.1^{a}	0.4 ±0.1^{ab}
20:5		7.8 ±0.5^{a}	5.9 ±1.5^{ab}	7.0 ±0.6^{a}	5.0 ±0.9^{b}
22:5		1.5 ±0.1	1.2 ±0.1	1.5 ±0.1	1.2 ±0.2
22:6		22.9 ±3.3^{a}	22.7 ±4.0^{a}	14.8 ±1.1^{b}	12.4 ±1.7^{b}
Σ (n-3) PUFA		**34.6 ±3.3^{a}**	**30.9 ±4.0^{ab}**	**25.4 ±1.9^{b}**	**20.2 ±2.9^{c}**
(n-3)/(n-6)		**5.4 ±0.7^{a}**	**1.3 ±0.3^{b}**	**2.3 ±1.2^{b}**	**1.0 ±0.3^{b}**

da-dimethyl acetals. Σ saturates also include 16:0da, 15:0, 20:0, 22:0, 24:0; Σ (n-6) PUFA include 16:2, 22:2, 22:5; Σ (n-3) PUFA include 16:3, 16:4; where one or more of the 4 values is < 0.5. Values are 4 expressed as means (%) ±S.D. of triplicate samples. Values with different superscript letters are significantly different (p <0.05) ANOVA.

Table 5.25 (a)

The fatty acid composition of lipid classes of kidney from Atlantic salmon maintained on high (n-3) PUFA diets in trial 2

Fatty acids (%)	(n-3) PUFA Diet			
	PC	PS	PI	PE
14:0	2.3	0.5	0.2	0.3
16:0	36.2	24.5	11.2	17.0
18:0	1.6	11.7	21.8	4.3
Σ saturates	**40.8**	**37.2**	**33.4**	**24.3**
18:1da	-	-	-	2.5
16:1	1.9	0.5	0.7	1.7
18:1	15.4	9.1	6.5	11.2
20:1	1.1	2.8	1.7	3.1
22:1	0.2	0.1	0.1	0.2
24:1	0.5	0.2	0.4	0.1
Σ monoenes	**19.2**	**12.8**	**9.4**	**19.0**
16:2	0.3	0.1	0.1	1.5
18:2	0.6	0.7	0.7	1.1
18:3	-	0.2	0.1	0.1
20:2	0.2	0.2	0.1	0.2
20:3	-	0.1	0.2	0.1
20:4	2.3	3.0	17.5	5.6
22:5	0.3	1.0	0.3	0.4
Σ (n-6)	**3.7**	**5.3**	**19.0**	**9.0**
16:3	0.2	0.7	0.6	0.3
18:3	-	0.3	0.4	0.3
20:4	0.3	0.2	0.2	0.3
20:5	9.6	7.4	7.2	13.9
22:5	1.0	1.6	1.0	1.1
22:6	25.0	34.4	28.9	31.8
Σ (n-3) PUFA	**36.3**	**44.7**	**38.2**	**47.7**
(n-3)/(n-6)	**9.8**	**8.4**	**2.0**	**5.3**

Abbreviations:- PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; da-dimethyl acetals. In addition to the fatty acids shown, Σ saturates include da16:0, 15:0, 20:0, 22:0, 24:0; Σ (n-6) PUFA include 22:2 and Σ (n-3) PUFA include 18:4; where one or more of the 4 values is < 0.5. Values are expressed as 1 analysis of 3 pooled samples for trial 2.

Table 5.25 (b)

The fatty acid composition of lipid classes of kidney from Atlantic salmon maintained on high (n-6) PUFA diets in trial 2

Fatty acids (%)	(n-6) PUFA Diet			
	PC	PS	PI	PE
16:0da	0.2	0.1	0.1	1.8
14:0	0.6	0.2	0.1	0.1
15:0	0.3	0.1	0.1	0.1
16:0	30.9	18.7	8.0	11.6
18:0	2.7	19.8	25.5	6.8
Σ saturates	**34.7**	**39.0**	**33.8**	**22.3**
18:1da	0.1	-	0.4	3.4
16:1	0.5	0.2	0.2	0.2
18:1	13.0	5.6	5.0	7.1
20:1	0.5	1.5	0.9	1.2
22:1	0.1	-	0.1	0.1
24:1	0.6	2.1	0.9	0.3
Σ monoenes	**14.7**	**9.4**	**7.5**	**12.3**
16:2	0.3	0.2	0.1	1.1
18:2	10.4	3.7	5.0	11.9
20:2	0.8	0.5	0.8	0.9
20:3	0.9	0.8	1.2	0.7
20:4	3.2	4.1	16.7	7.2
22:5	0.2	0.6	0.3	0.4
Σ (n-6)	**16.1**	**10.1**	**24.1**	**22.3**
16:3	0.2	0.5	0.3	0.2
18:3	0.1	0.2	0.2	0.1
20:4	0.2	-	0.1	0.2
20:5	9.1	5.6	5.2	10.6
22:5	1.0	1.6	1.0	1.0
22:6	23.9	33.7	27.9	32.8
Σ (n-3) PUFA	**34.5**	**41.5**	**34.7**	**45.0**
(n-3)/(n-6)	**2.1**	**4.1**	**1.4**	**2.0**

Abbreviations:- PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; da-dimethyl acetals. Σ (n-6) PUFA also includes 18:3, 22:2 and Σ (n-3) PUFA include 18:4; where one or more of the 4 values is < 0.5. Values are expressed as 1 analysis of 3 pooled samples for trial 3.

Table 5.25 (c)

The fatty acid composition of the lipid classes of kidney leucocytes from Atlantic salmon maintained on high (n-3) PUFA diets in trial 2

Fatty acids	(n-3) PUFA Diet		
(%)	PC	PS/PI	PE
14:0	2.3	1.4	1.5
15:0	1.9	1.0	1.3
16:0da	-	-	2.5
16:0	33.3	17.2	16.0
18:0	4.0	11.0	7.4
20:0	0.3	0.7	0.3
22:0	0.3	0.9	0.6
Σ saturates	**42.4**	**32.7**	**30.7**
14:1	3.3	3.7	2.8
16:1	4.2	4.2	6.2
18:1	15.7	12.2	10.9
20:1	1.2	1.1	1.0
22:1	0.4	1.4	0.7
24:1	1.4	2.8	2.8
Σ monoenes	**26.2**	**25.4**	**24.5**
16:2	1.2	0.7	1.1
18:2	4.4	6.0	3.0
20:4	1.9	5.3	2.7
22:2	1.0	4.1	2.3
22:5	0.3	0.9	1.0
Σ (n-6) PUFA	**10.6**	**18.5**	**11.6**
16:3	0.9	1.0	1.3
18:3	0.8	1.0	1.5
20:4	0.5	0.8	0.6
20:5	6.7	3.9	4.7
22:5	0.8	1.3	1.3
22:6	10.5	15.5	23.4
Σ (n-3) PUFA	**20.8**	**23.5**	**33.2**
(n-3)/(n-6)	**2.0**	**1.3**	**2.9**

Abbreviations:- PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; da-dimethyl acetals. In addition to the fatty acids shown, Σ saturates include 24:0; Σ (n-6) PUFA include 18:3; 20:3; 22:2 and Σ (n-3) PUFA include 18:4; where one or more of the 3 values is < 0.5. Values are expressed as 1 analysis of 3 pooled samples for trial 2.

Table 5.25 (d)

The fatty acid composition of the lipid classes of kidney leucocytes from Atlantic salmon maintained on high (n-6) PUFA diets in trial 2.

Fatty acids	(n-6) PUFA Diet		
(%)	PC	PS/PI	PE
14:0	1.2	1.2	1.3
15:0	1.1	1.5	1.3
16:0da	3.2	-	-
16:0	16.5	15.9	11.5
18:0	12.8	7.4	1.3
20:0	0.6	0.6	-
24:0	0.2	3.2	0.9
Σ saturates	**40.1**	**39.0**	**28.1**
14:1	3.1	1.7	2.6
16:1	3.2	7.5	4.9
18:1	15.9	11.6	16.3
20:1	0.8	1.2	1.1
22:1	0.5	1.4	1.2
24:1	2.8	3.2	4.5
Σ monoenes	**26.2**	**26.7**	**30.7**
16:2	0.7	1.1	1.2
18:2	7.6	6.2	5.5
20:2	1.0	0.9	0.7
20:3	0.9	0.9	3.1
20:4	2.0	4.5	2.7
22:2	1.5	3.1	-
22:5	0.4	0.9	0.7
Σ (n-6) PUFA	**14.2**	**17.5**	**14.8**
16:3	1.2	1.2	1.5
20:5	5.5	2.1	4.3
22:5	0.9	0.9	1.3
22:6	11.4	12.1	18.8
Σ (n-3) PUFA	**19.5**	**16.7**	**26.4**
(n-3)/(n-6)	**1.4**	**1.0**	**1.8**

Abbreviations:- PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; da-dimethyl acetals. In addition to the fatty acids shown, Σ saturates include 22:0; Σ (n-6) PUFA include 18:3 and Σ (n-3) PUFA include 18:3, 20:4; where one or more of the 3 values is < 0.5. Values are expressed as 1 analysis of 3 pooled samples for trial 2.

Table 5.26

The lipid class composition of headkidney macrophages from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 3, and on a commercial diet.

Lipid class	Diet		
(% Total lipid)	(n-3) PUFA	(n-6) PUFA	Commercial
Σ polar lipid	58.1 ±7.0	52.4 ±1.2	58.8 ±5.3
Σ neutral lipid	41.9 ±7.0	47.6 ±1.2	41.2 ±5.3
Polar lipid:			
LPC	0.1 ±0.1	-	0.5 ±0.3
Sphingomyelin	2.4 ±0.5	2.5 ±0.3	3.3 ±0.7
PC	29.2 ±2.4	21.1 ±1.2	20.6 ±1.3
PS	4.1 ±0.8	2.6 ±0.5	3.1 ±1.4
PI	3.4 ±1.1	2.5 ±0.5	2.7 ±0.9
Cardiolipin	4.7 ±0.4	-	0.8 ±1.2
PE	10.5 ±7.5	5.5 ±0.5	7.8 ±2.6
Sulphatides	2.1 ±0.3	6.5 ±0.2	7.3 ±0.7
Cerebrosides	1.6 ±0.7	11.7 ±0.3	12.5 ±3.7
Neutral lipid:			
Cholesterol	14.2 ±2.9	19.1 ±2.8	18.1 ±1.7
Free fatty acids	5.8 ±0.7	11.1 ±0.5	9.7 ±1.8
Triacylglycerols	13.6 ±5.1	3.4 ±0.4	5.7 ±2.0
Sterol esters	7.2 ±1.1	14.1 ±3.1	7.7 ±4.8
Unknowns	1.1 ±0.5	-	-

Abbreviations:- LPC-Lysophosphatidylcholine; PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; Values are expressed as means (%) ±S.D. of triplicate samples.

Table 5.27

The fatty acid composition of headkidney macrophages from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 3, and on a commercial diet.

Fatty acids (%)	Diet		
	(n-3) PUFA	(n-6) PUFA	Commercial *
14:0	2.7	1.3	2.1 ±0.9
15:0	0.7	1.4	2.5 ±1.4
16:0	26.3	24.1	17.7 ±1.8
18:0	9.5	9.5	13.0 ±3.2
20:0	0.6	-	0.5 ±0.2
22:0	0.7	-	1.0 ±0.6
24:0	1.4	1.1	-
Σ saturates	**42.7**	**38.1**	**37.3 ±7.4**
16:1	11.5	8.4	5.9 ±1.0
18:1da	-	-	0.7 ±0.3
18:1	17.7	13.6	20.6 ±2.7
20:1	1.5	1.3	1.8 ±0.1
22:1	0.9	-	0.6 ±0.2
24:1	1.9	0.9	3.7 ±1.1
Σ monoenes	**33.4**	**24.1**	**33.5 ±5.1**
16:2	0.4	0.8	0.2 ±0.3
18:2	4.4	7.7	4.3 ±0.8
18:3	0.5	1.7	0.4 ±0.3
20:2	1.0	0.4	1.5 ±0.3
20:3	-	0.5	1.3 ±0.4
20:4	0.7	1.5	2.3 ±0.4
22:2	-	1.3	-
Σ (n-6)	**7.0**	**14.1**	**10.1 ±1.7**
16:3	0.4	0.9	0.7 ±0.6
18:3	0.4	0.9	0.3 ±0.2
20:5	2.8	4.3	3.8 ±1.1
22:5	0.8	0.7	0.5 ±0.4
22:6	12.5	17.0	13.2 ±1.8
Σ (n-3) PUFA	**16.9**	**23.7**	**19.1 ±2.2**
(n-3)/(n-6)	**2.4**	**1.7**	**1.9 ±0.5**

da-dimethyl acetals. Σ (n-6) PUFA also includes 22:5; Σ (n-3) PUFA also includes 20:4; where one or more of the 3 values is < 0.5. Values are expressed as 1 analysis of 3 pooled samples for trial 3. * Values are expressed as means (%) ±S.D. of triplicate samples

5.3.3.5 Gills

The lipid classes of gills from salmon in trial 1 are shown in Tables 5.28, while the fatty acid composition of the tissue are given in Table 5.29. The lipid content of gill tissue was found to be independent of diet (Table 5.28).

Lipid of gill tissue from salmon in all three dietary groups was very rich in neutral lipid and low in polar lipid. While TAGs and sterol esters, which together comprised 59.6 % to 68.2 % of total lipid, were unresolved by densitometry, the value of the TAG/sterol ester peak as assessed by visual inspection of the chromatogram was mostly attributed to the TAG level. The sterol ester / TAG content of the gill lipid from fish fed (n-6) PUFA was significantly higher than that of the two dietary groups.

The gills of the (n-6) PUFA dietary group reflected the high input of 18:2(n-6) in their fatty acid profiles, and contained high levels of 18:2(n-6) fatty acid and its derivatives, 18:3(n-6), 20:2(n-6) and 20:3(n-6). Thus, 18:2(n-6) accounted for 23.2 % of the gill fatty acids in the (n-6) PUFA group but less than 4 % of those in fish fed the (n-3) PUFA and commercial diets. As a result of this (n-6) PUFA uptake, the percentage of the other fatty acid series fell to compensate for the increase and resulted in a low (n-3)/(n-6) ratio of 0.5 in the (n-6) PUFA group.

Table 5.28

The lipid class composition of gills from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets and on a commercial diet in trial 1.

Lipid class	Diet		
(% Total lipid)	(n-3) PUFA	(n-6) PUFA	Commercial
Lipid Content	5.4 ±0.5	5.5 ±0.6	6.9 ±0.4
Σ polar lipid	18.0 ±3.3^{ab}	14.4 ±0.8^{b}	20.4 ±1.6^{a}
Σ neutral lipid	82.0 ±3.3^{ab}	85.6 ±0.8^{b}	79.6 ±1.6^{a}
Polar lipid:			
LPC	0.4 ±0.1^{ab}	0.2 ±0.0^{b}	0.4 ±0.0^{a}
Sphingomyelin	1.3 ±0.2	1.1 ±0.2	1.6 ±0.2
PC	7.7 ±1.4^{ab}	6.2 ±0.6^{b}	8.4 ±0.5^{a}
PS	0.9 ±0.2	1.0 ±0.1	1.4 ±0.3
PI	0.5 ±0.3^{ab}	0.4 ±0.0^{b}	0.7 ±0.2^{a}
Cardiolipin	0.5 ±0.1	0.4 ±0.0	0.5 ±0.0
PE	5.0 ±0.9^{ab}	4.1 ±0.3^{b}	5.6 ±0.1^{a}
Sulphatides	-	-	0.1 ±0.0
Cerebrosides	1.4 ±0.8	0.9 ±0.4	1.4 ±0.4
Neutral lipid:			
Cholesterol	14.7 ±5.1^{a}	11.4 ±0.8^{b}	13.4 ±0.5^{ac}
Free fatty acids	7.0 ±3.1	5.6 ±1.0	4.7 ±0.5
Triacylglycerols	59.6 ±1.6]^{a}	68.2 ±1.5]^{b}	61.1 ±1.5]^{a}
Sterol esters	]	]	]
Unknowns	1.0 ±0.5	1.3 ±0.3	0.8 ±0.6

Abbreviations:- LPC-Lysophosphatidylcholine; PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; Values are expressed as means (%) ±S.D. of triplicate samples. Values with different superscript letters are significantly different ($p < 0.05$) by ANOVA Triacylglycerols and sterol esters were unresolved by TLC. Lipid Content (mg g^{-1} wet weight of tissue).

Table 5.29

The fatty acid composition of total lipid of gills from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets and on a commercial diet in trial 1.

Fatty acids (%)	Diet		
	(n-3) PUFA	(n-6) PUFA	Commercial
14:0	5.6 ±0.9[a]	2.8 ±0.1[b]	4.9 ±0.1[a]
16:0	15.1 ±0.3[a]	11.2 ±0.3[b]	14.8 ±0.4[a]
18:0	3.5 ±0.1[a]	4.0 ±0.0[b]	3.5 ±0.2[a]
Σ saturates	**25.6 ±1.8[a]**	**19.0 ±0.8[b]**	**23.9 ±1.0[a]**
16:1	7.4 ±0.6[a]	4.5 ±0.1[b]	7.1 ±0.7[a]
18:1	17.7 ±2.1[a]	20.7 ±0.1[b]	20.2 ±1.1[b]
20:1	7.6 ±0.6[a]	6.3 ±0.2[b]	8.8 ±1.0[a]
22:1	7.6 ±1.6	6.0 ±0.2[b]	8.4 ±0.7[a]
Σ monoenes	**41.6 ±5.0[ab]**	**38.5 ±0.8[b]**	**45.6 ±3.6[a]**
18:2	3.3 ±0.2[a]	23.2 ±1.6[b]	3.2 ±0.1[a]
18:3	0.5 ±0.2[a]	1.0 ±0.1[b]	0.3 ±0.0[a]
20:2	0.4 ±0.1[a]	1.3 ±0.1[b]	0.3 ±0.0[a]
20:3	0.1 ±0.0[a]	0.6 ±0.1[b]	0.2 ±0.0[a]
20:4	0.6 ±0.0[a]	0.4 ±0.0[b]	0.6 ±0.0[a]
Σ (n-6)	**6.0 ±0.9[a]**	**27.2 ±1.9[b]**	**5.5 ±0.2[a]**
18:3	1.2 ±0.2	1.0 ±0.0	1.0 ±0.0
18:4	2.6 ±0.4[a]	1.4 ±0.1[b]	2.4 ±0.1[a]
20:4	1.0 ±0.1[a]	0.6 ±0.0[b]	1.3 ±0.0[c]
20:5	5.0 ±0.9[a]	2.0 ±0.1[b]	5.5 ±0.1[a]
22:5	1.9 ±0.2[a]	1.0 ±0.1[b]	2.1 ±0.1[a]
22:6	12.7 ±0.9[a]	7.8 ±0.6[b]	13.7 ±0.4[a]
Σ (n-3) PUFA	**25.7 ±0.9[a]**	**14.6 ±0.9[b]**	**27.1 ±0.7[a]**
(n-3)/(n-6)	**4.2 ±0.2[a]**	**0.5 ±0.3[b]**	**5.0 ±0.5[c]**

In addition to the fatty acids shown, Σ saturates include 15:0, 17:0, 20:0, 22:0; Σ monoenes include 14:1, 24:1; Σ (n-6) PUFA include 16:2, 22:5 and Σ (n-3) PUFA include 16:3, 16:4; where one or more of the 3 values is < 0.5. Values are expressed as means (%) ±S.D. of triplicate samples. Values with different superscript letters are significantly different ($p < 0.05$) ANOVA

5.3.3.6 Liver

The lipid content of liver was not influenced by dietary lipid (Table 5.30). Liver lipid, in comparison with that of the immunological tissues examined, contained lower proportions of TAG and sterol esters, and conversely higher proportions of polar lipid, particularly PC and PE. The lipid classes of liver from trial 2 are shown in Table 5.30. There were significant differences in lipid class composition between the (n-3) and (n-6) dietary PUFA groups in that the proportions of PC, PI, cardiolipin, PE and cerebrosides were all higher in the liver of the (n-3) group fish.

Liver lipid had higher levels of (n-3) PUFA and lower levels of monoenoic fatty acids, than other tissues which have been examined previously. The fatty acid composition of the tissue are given in Table 5.31. Higher 18:2(n-6) fatty acid levels were observed in the lipid of (n-6) PUFA fish livers, as well as elevated 20:2(n-6) and 20:3(n-6) levels derived from the 18:2(n-6),than in liver lipid of fish fed (n-3) PUFA. Proportions of the other fatty acid classes fell to compensate for the increase in the (n-6) PUFA level, and consequently, the (n-3)/(n-6) ratio in the (n-6) PUFA group was approximately six times less than the (n-3) group.

The fatty acids profiles found in the lipid classes of liver of the two dietary groups resembled those found in the other tissues examined (Table 5.32). The PC fraction characteristically had high 16:0 levels, and, although it had lower levels of (n-3) PUFA than PE, it still had a higher (n-3)/(n-6) PUFA ratio, since PE contained higher proportions of (n-6) PUFA in both dietary groups. The PS fraction had high levels of 16:0 and 18:0, but PI had a higher 18:0 level than the PS fraction. The PI lipid class

was again characterised by a high 20:4(n-6) level content: 24.7 % in fish of the (n-3) PUFA group and 21.6 % in the (n-6) PUFA fish. Liver PI from the (n-6) dietary PUFA group contained notably higher levels of 18:2(n-6), 20:2(n-6) and 20:3(n-6) than liver PI of the (n-3) salmon.

5.3.3.7 A summary of 18:2(n-6) and (n-3)/(n-6) PUFA ratios in total lipid from dietary tissues and leucocytes.

A comparison of 18:2(n-6) fatty acids levels and (n-3)/(n-6) PUFA ratios in total lipid of tissues and leucocytes, examined from the two dietary groups in trials 1, 2 and 3, are presented in Figure 5.8 and Figure 5.9 respectively. Erythrocytes and serum from the (n-3) PUFA dietary group contained the lowest levels of 18:2(n-6) fatty acid (1.0 and 0.8 % of total fatty acids, respectively), while there were no great differences in 18:2(n-6) fatty acid between tissues and their leucocytes for thymus: (2.6 % vs 2.8 %), spleen: (5.0 % vs. 4.0 %), and kidney: (2.2 % vs. 5.1 %) from the (n-3) dietary group. In contrast, tissues and erythrocytes from the (n-3) dietary group always possessed higher (n-3)/(n-6) PUFA ratios than their leucocytes, while the (n-3)/(n-6) PUFA ratios were found to be lower and similar between the tissues and leucocytes of the (n-6) PUFA dietary group. This was because tissues of the (n-6) dietary group apparently incorporated much more 18:2(n-6) dietary fatty acid than their leucocytes. Erythrocytes from this group did not incorporated as much dietary 18:2(n-6) fatty acid as the peripheral blood leucocytes and consequently contained a higher (n-3)/(n-6) ratio.

Table 5.30

The percentage lipid class composition of liver from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 2.

Lipid class	Diet	
(% Total lipid)	(n-3) PUFA	(n-6) PUFA
Lipid Content	2.8 ±0.1	2.1 ±0.6
Σ polar lipid	59.1 ±2.8**	48.1 ±5.0**
Σ neutral lipid	40.9 ±2.8**	51.9 ±5.0**
Polar lipid:		
LPC	2.5 ±0.2	1.3 ±1.9
Sphingomyelin	2.5 ±0.3	2.8 ±1.0
PC	24.1 ±1.3**	19.9 ±1.9**
PS	3.6 ±0.1*	3.3 ±0.2*
PI	6.2 ±0.4**	4.8 ±0.5**
Cardiolipin	4.6 ±0.2**	3.0 ±0.3**
PE	14.5 ±1.2**	11.9 ±0.6**
Cerebrosides	1.2 ±0.2*	0.7 ±0.1*
Neutral lipid:		
Cholesterol	15.1 ±0.4*	16.8 ±0.1*
Free fatty acids	13.5 ±1.9***	19.3 ±0.9***
Triacylglycerols	5.8 ±2.6	7.0 ±3.4
Sterol esters	4.9 ±1.2	6.9 ±1.5
Unknowns	1.5 ±0.4	1.8 ±0.7

Abbreviations:- LPC-Lysophosphatidylcholine; PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine. Values are expressed as means (%) ±S.D. of triplicate samples. * = $0.05 > p > 0.01$, ** = $0.01 > p > 0.001$, *** = $p < 0.001$ by Student 't' test. Lipid Content (mg g^{-1} wet weight of tissue).

Table 5.31

The percentage fatty acid composition of liver from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 2.

Fatty acids (%)	Diet	
	(n-3) PUFA	(n-6) PUFA
14:0	2.5 ±0.1**	0.8 ±0.1**
16:0	22.3 ±0.3**	15.7 ±0.6**
18:0	3.9 ±0.2**	5.0 ±0.2**
Σ saturates	**29.0 ±0.4*****	**21.9 ±0.6*****
16:1	2.6 ±0.3**	1.0 ±0.2**
18:1	12.1 ±1.0	12.2 ±0.8
20:1	2.2 ±0.2*	1.4 ±0.2*
22:1	1.3 ±0.3	1.2 ±0.6
24:1	1.1 ±0.2*	0.5 ±0.0*
Σ monoenes	**19.4 ±1.7****	**16.3 ±1.1****
18:2	1.7 ±0.2***	19.5 ±1.8***
18:3	0.3 ±0.1	0.5 ±0.1
20:2	0.2 ±0.0**	1.6 ±0.2**
20:3	0.2 ±0.0***	2.1 ±0.4***
20:4	2.8 ±0.1	3.1 ±0.2
Σ (n-6) PUFA	**5.7 ±0.3*****	**27.3 ±2.1*****
18:3	0.5 ±0.0*	0.2 ±0.1*
18:4	0.3 ±0.1	0.2 ±0.0
20:4	0.9 ±0.1*	0.4 ±0.3*
20:5	6.8 ±0.4**	3.7 ±0.5**
22:5	2.0 ±0.2**	1.4 ±0.1**
22:6	35.0 ±2.4**	28.4 ±2.6**
Σ (n-3) PUFA	**45.9 ±1.8*****	**34.4 ±3.0*****
(n-3)/(n-6)	**8.0 ±0.2*****	**1.3 ±0.7*****

Σ saturates include 15:0, 20:0, 22:0; Σ (n-6) PUFA include 16:2, 22:2; Σ (n-3) PUFA include 16:3, 16:4; where one or more of the 2 values is < 0.5. Values are expressed as means (%) ±S.D. of triplicate samples. * = 0.05 > p > 0.01, ** = 0.01 > p > 0.001, *** = p < 0.001 by Student 't' test.

Table 5.32 (a)

The percentage fatty acid composition of the lipid classes of liver from Atlantic salmon maintained on high (n-3) PUFA diets in trial 2.

Fatty acids (%)	(n-3) PUFA Diet			
	PC	PS	PI	PE
14:0	2.7	1.0	0.2	0.3
16:0	32.9	40.8	7.6	20.0
18:0	1.3	17.3	27.6	4.8
Σ saturates	**37.8**	**59.5**	**35.6**	**25.4**
16:1	1.4	1.3	0.4	0.6
18:1	8.5	18.9	7.2	13.0
20:1	0.8	4.2	1.2	3.4
22:1	0.3	0.2	0.2	0.4
24:1	0.4	0.3	0.8	0.2
Σ monoenes	**11.3**	**24.7**	**9.8**	**17.8**
16:2	0.8	1.3	-	0.8
18:2	0.7	2.5	0.5	1.7
20:2	0.1	0.4	-	0.3
20:3	-	0.3	0.4	0.2
20:4	1.6	0.5	24.7	2.8
Σ (n-6) PUFA	**3.3**	**5.3**	**25.7**	**6.4**
16:3	0.3	1.3	0.5	0.4
20:4	0.4	0.2	0.3	0.4
20:5	8.6	0.8	3.4	7.5
22:5	3.0	0.5	2.2	1.6
22:6	35.2	7.1	22.2	40.2
Σ (n-3) PUFA	**47.6**	**10.4**	**29.0**	**50.4**
(n-3)/(n-6)	**14.4**	**2.0**	**1.1**	**7.9**

Abbreviations:- PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; Σ saturates include 15:0, 20:0, 22:0; Σ (n-6) PUFA include 22:2; Σ (n-3) PUFA include 18:3, 18:4; where one or more of the 4 values is < 0.5. Values are expressed as 1 analysis of 3 pooled samples for trial 2.

Table 5.32 (b)

The percentage fatty acid composition of the lipid classes of liver from Atlantic salmon maintained on high (n-6) PUFA diets in trial 2

Fatty acids (%)	(n-6) PUFA Diet			
	PC	PS	PI	PE
14:0	0.1	0.3	0.1	0.1
16:0	11.4	18.6	4.9	11.9
18:0	6.3	17.1	27.5	6.5
Σ saturates	**17.9**	**36.5**	**32.5**	**18.8**
16:1	0.2	0.5	0.1	0.2
18:1	7.8	8.2	5.6	1.3
20:1	1.3	1.2	0.6	1.7
22:1	0.2	0.2	0.1	0.3
Σ monoenes	**9.9**	**10.4**	**6.7**	**4.0**
16:2	0.8	1.0	0.2	0.9
18:2	13.0	13.3	4.7	13.9
20:2	1.9	1.5	0.9	2.0
20:3	2.5	0.9	3.9	2.6
20:4	4.1	1.9	21.6	4.4
22:2	0.2	0.8	0.2	0.3
Σ (n-6) PUFA	**22.6**	**21.1**	**31.8**	**25.2**
20:4	-	0.1	0.9	0.1
20:5	3.8	1.7	2.8	4.2
22:5	2.0	1.5	1.8	2.1
22:6	43.5	27.9	23.3	45.1
Σ (n-3) PUFA	**49.6**	**32.1**	**28.9**	**52.0**
(n-3)/(n-6)	**2.2**	**1.5**	**0.9**	**2.1**

Abbreviations:- PC-Phosphatidylcholine; PS-Phosphatidylserine; PI-Phosphatidylinositol; PE-Phosphatidylethanolamine; da-dimethyl acetals. Σ saturates include 16:0da., 15:0, 20:0; Σ monoenes include 14:1, 24:1; Σ (n-6) PUFA include 18:3, 22:5; Σn (n-3) PUFA include 16:2, 18:3, 18:4; where one or more of the 4 values is < 0.5. Values are expressed as 1 analysis of 3 pooled samples for trial 2.

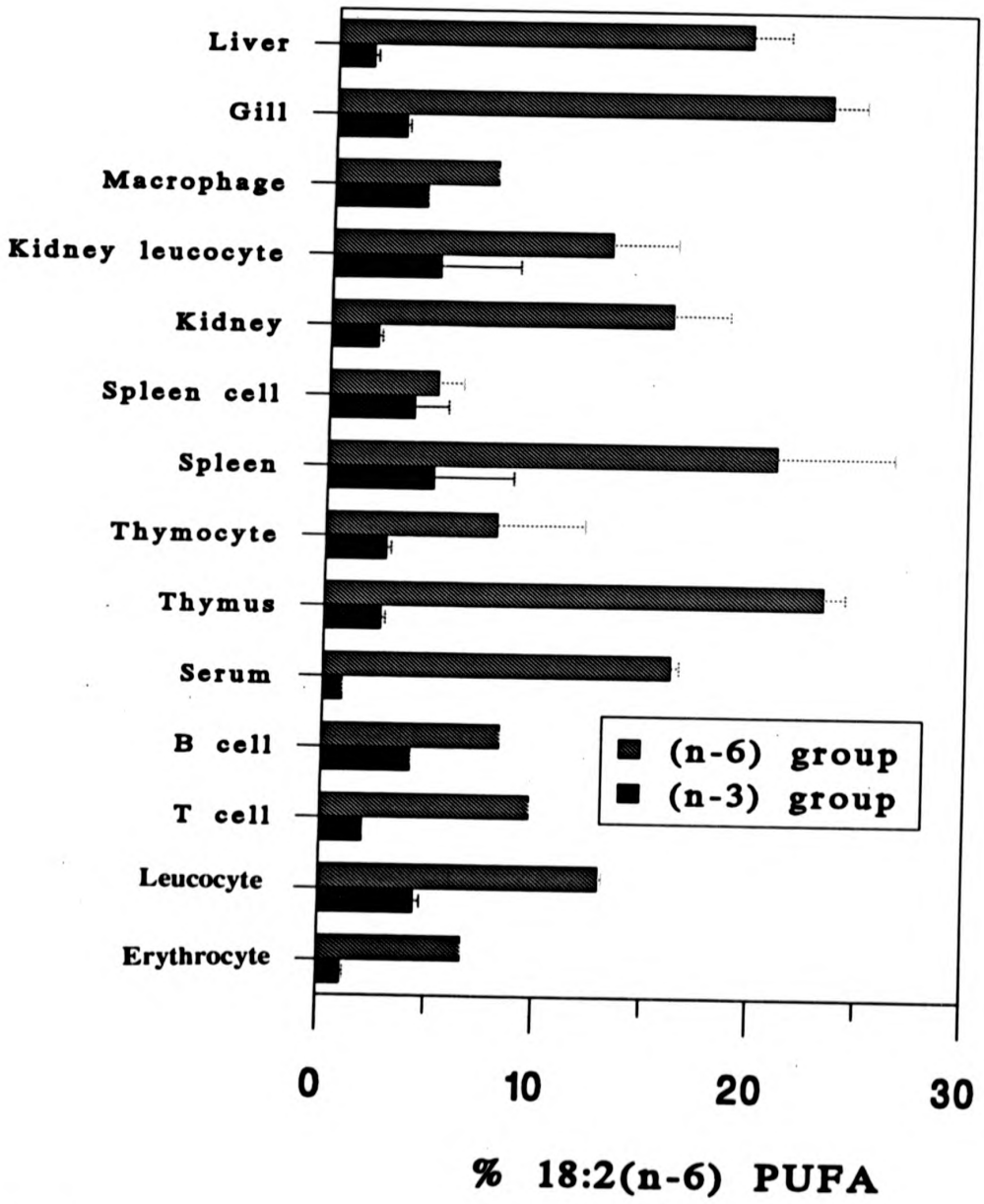


Figure 5.8

A comparison of 18:2(n-6) PUFA levels between tissues and their leucocytes from Atlantic salmon maintained on high (n-3) or (n-6) PUFA diets.

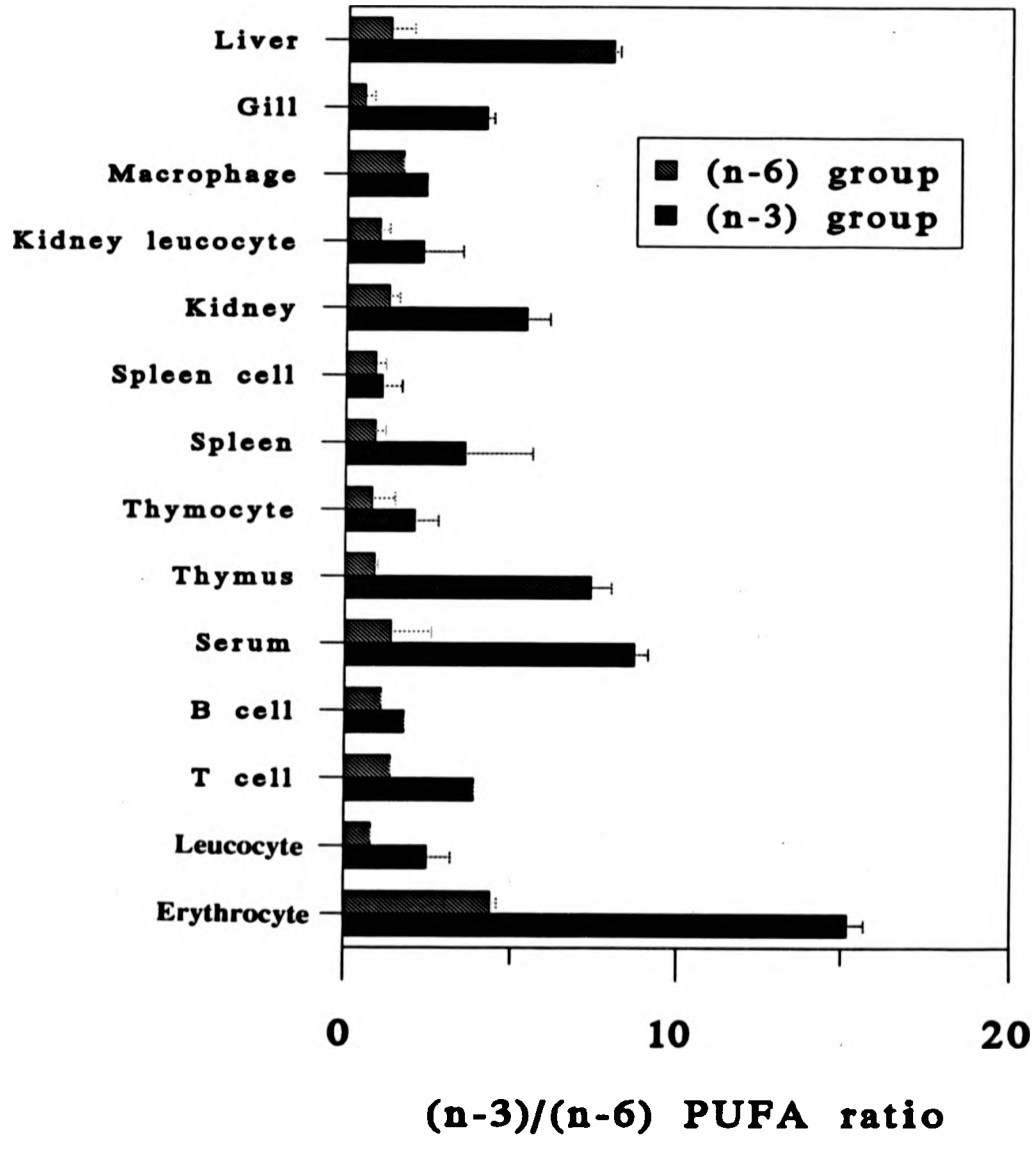


Figure 5.9

A comparison of the (n-3)/(n-6) PUFA ratios between tissues and their leucocytes from Atlantic salmon maintained on high (n-3) or (n-6) PUFA diets.

5.4 IMMUNE RESPONSE

5.4.1 SERUM PROTEIN MEASUREMENTS

5.4.1.1 Protein concentrations

Serum samples were collected from 4 fish for each of the dietary groups in trial 2 and 3 (see Section 3.2.3 for serum isolation), and total serum concentrations were measured according to Section 3.4.4, the values of which can be found in Table 5.33. Water temperature was 12 °C and 14.2 °C respectively. No difference was found between the total serum concentrations of (n-3) and (n-6) dietary groups in trial 2 and 3, and the values shown in Table 5.33 fell within the normal range established by other workers (Alexander and Ingram, 1980; Sandnes *et al.*, 1988).

5.4.1.2 Serum complement levels

Serum complement levels were determined by the direct haemolysis of erythrocytes as outlined in Section 3.4.5, for four Atlantic salmon from each of the dietary groups on day 145 of trial 2. Water temperature was 12°C. Serum complement activity is shown in Figure 5.10 and CH_{50} units are given in Table 5.33. Differences in complement activity due to dietary PUFA were not evident.

5.4.1.3 Kidney and serum lysozyme activities

Lysozyme activity in the kidney and serum of Atlantic salmon from dietary trial 2, on day 147, was measured by the plate method described in Section 3.4.6, with three fish from each dietary group being sampled. Both samples and standards were tested in

Table 5.33

Serum protein measurements of Atlantic salmon in trial 2, maintained on high (n-3) and (n-6) PUFA diets.

Serum Proteins	Diet	
	(n-3) PUFA	(n-6) PUFA
Total Serum Protein ($mgml^{-1}$)		
trial 2	49.8 ±16.7	57.8 ±8.0
trial 3	55.5 ±5.9	52.5 ±1.3
Serum complement activity (CH_{50} units)	26.4 ±2.2	25.2 ±4.1
Lysozyme (μgml^{-1})		
kidney	39.9 ±12.3	36.8 ±7.1
serum	3.7 ±7.1	4.7 ±1.4

Results expressed as mean ±S.D. n=4 for total serum protein and serum complement activity, n=3 for lysozyme

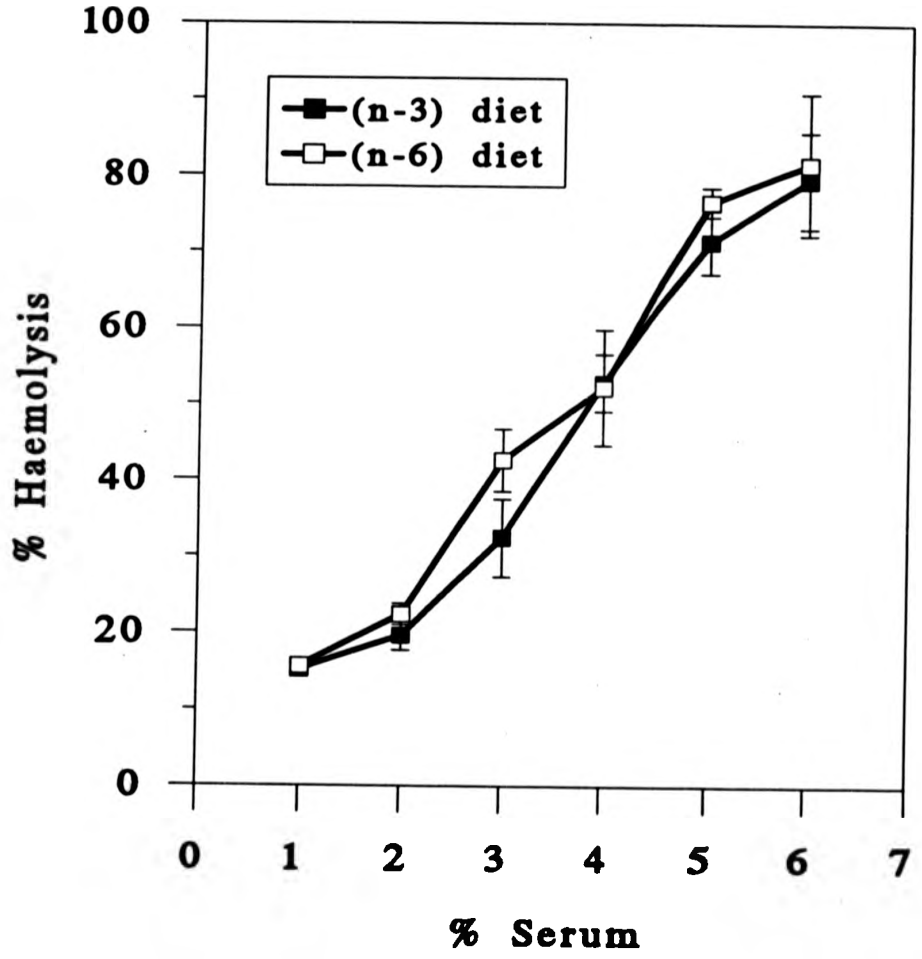


Figure 5.10 Serum complement activity for Atlantic salmon from each of the dietary groups of trial 2 on day 145.

Results expressed as mean ±S.D for three fish.

triplicate. Measured values of lysozymes activity shown in Table 5.33 were independent of dietary PUFA.

5.4.1.4 Anti-trypsin activity

Ellis (1990b) suggested the inhibition of trypsin activity as the most convenient method of measuring antiproteases. This was carried out as described in Section 3.4.7 for three Atlantic salmon in triplicate for each dietary group from trial 2 on day 146. Anti-trypsin activity was expressed as protease units, and inhibition of trypsin by α_1 antiproteases in the sera of the sampled salmon are illustrated in Figure 5.11. As the volume of test serum increased, greater inhibition of trypsin occurred. There was however, no difference in trypsin inhibition between the dietary groups.

5.4.2 MACROPHAGE ACTIVITY

5.4.2.1 Bacterial engulfment and killing

The engulfment of *Aeromonas salmonicida* by headkidney macrophages from Atlantic salmon from dietary trial 3 was carried out on day 112. The phagocytic activity of macrophages from the (n-3) and (n-6) PUFA dietary group of trial 3 are presented in Table 5.34 showing the results of examining 200 macrophages for phagocytosis in 4 fish from each of the dietary groups. There was no significant difference between these groups with respect to the Ph.I. value. Approximately 20.0 % of macrophages did not engulf bacteria, 55% engulfed 1-10 bacteria and 25% engulfed more than 10 bacteria. Killing of phagocytosed bacteria by headkidney macrophages from dietary trial 2 Atlantic salmon, on day 149, was carried out for 3 fish from each dietary group.

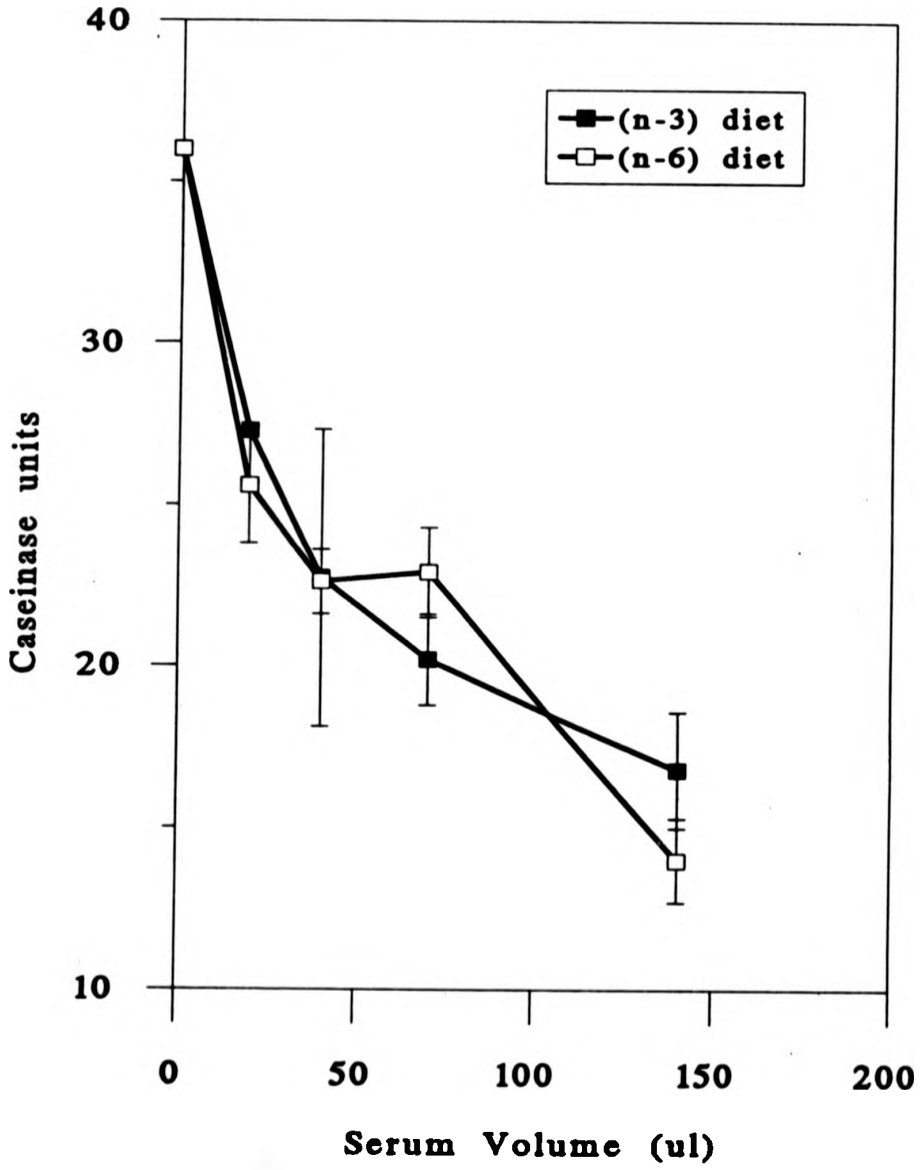


Figure 5.11. Inhibition of trypsin by α_1 antiprotease in the salmon sera from each of the dietary groups of trial 2 on day 146.

Results expressed as mean ±S.D for three fish.

Bactericidal killing of engulfed *Aeromonas salmonicida* by macrophages from Atlantic salmon maintained in trial 2 is presented in Table 5.35 and was found to be independent of dietary PUFA.

5.4.2.2 Respiratory burst enzymes

Bactericidal activity of respiratory burst enzymes in headkidney macrophages of Atlantic salmon from dietary trial 2 on day 149, were examined by means of the microtitre plate assays discussed in Section 3.4.9. Wells were set up in triplicate for four fish from each dietary group. These activities are shown in Figure 5.12 by the reduction of NBT by intracellular O_2^- and the oxidation of phenol red by H_2O_2, and in Figure 5.13 by the reduction of ferricytochrome C. The addition of SOD to the NBT and ferricytochrome C assays resulted in a lowering of absorbance, while its presence during the oxidation of phenol red resulted in increased absorbances. Although respiratory burst activity was generally higher for the (n-6) dietary group in each of the three assays measured, differences between the dietary groups were not significant.

Table 5.34

Engulfment of *Aeromonas salmonicida* by macrophages from Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets in trial 3

Phagocytic activity	Diet	
	(n-3) PUFA	(n-6) PUFA
Phagocytic Index	6.4 ±1.4	5.6 ±0.2
% macrophages showing no engulfing	20.6 ±3.5	20.0 ±1.8
% macrophages showing engulfing	79.4 ±3.5	80.0 ±1.9
1-10 engulfed bacteria (%)	54.9 ±4.2	55.3 ±6.2
>10 engulfed bacteria (%)	24.5 ±3.6	24.7 ±7.5

Values are expressed as mean ±SD, 200 macrophages were examined for 4 fish from each of the dietary groups.

Table 5.35

Bactericidal killing of engulfed *Aeromonas salmonicida* by macrophages from Atlantic salmon maintained in trial 2

	% Surviving Bacteria	
No. Bacteria well^{-1}	(n-3) PUFA diets	(n-6) PUFA diets
1 x10^{5} ml^{-1}	76.4 ±13.6	73.8 ±13.6
1 x10^{4} ml^{-1}	31.8 ±5.6	31.4 ±7.3
1 x10^{3} ml^{-1}	1.6 ±1.4	1.3 ±1.4

Values are expressed as mean ±SD. Sample no. = 3.

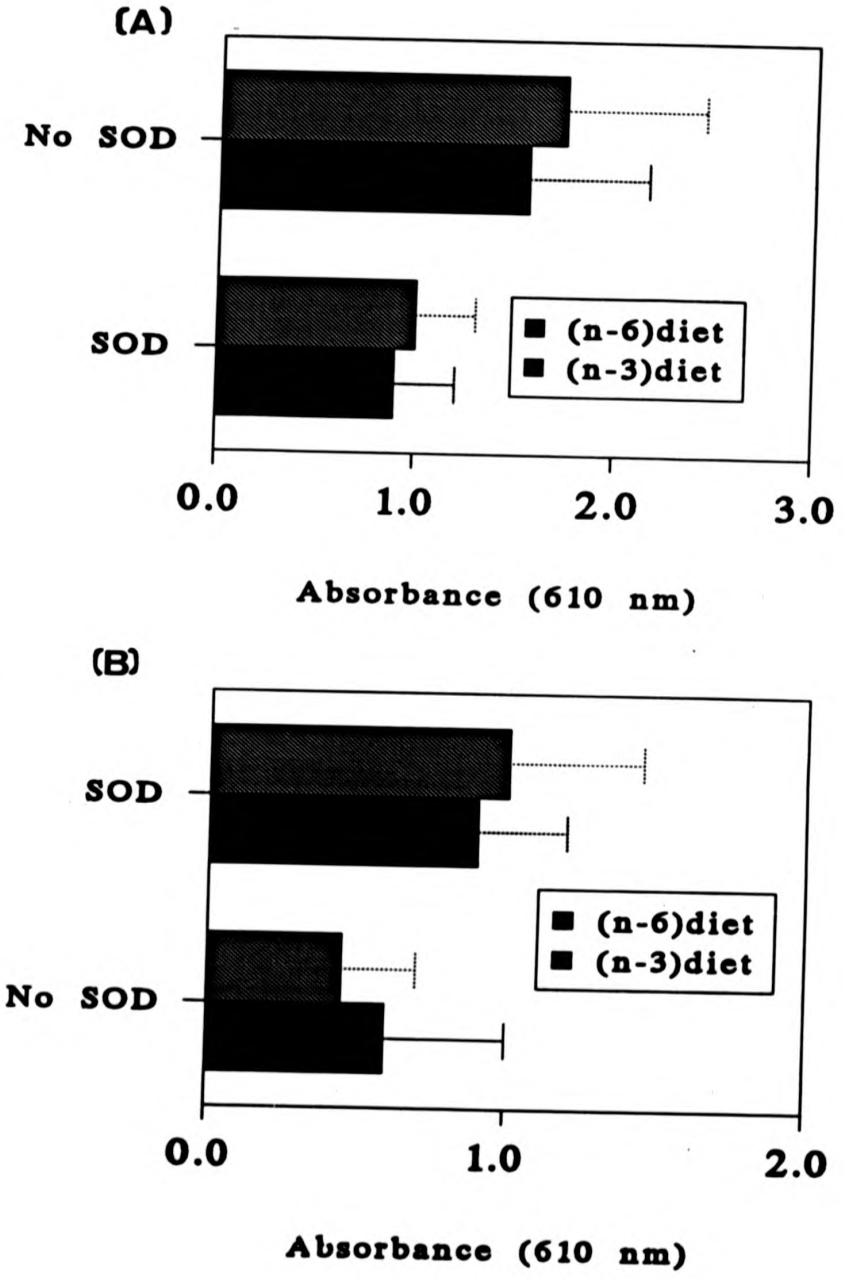


Figure 5.12

The reduction of (a) NBT and (b) phenol red by headkidney macrophages in Atlantic salmon in trial 2 on day 149. Results expressed as mean ±S.D for three fish/ 10^5 cells. SOD =Superoxide dismutase

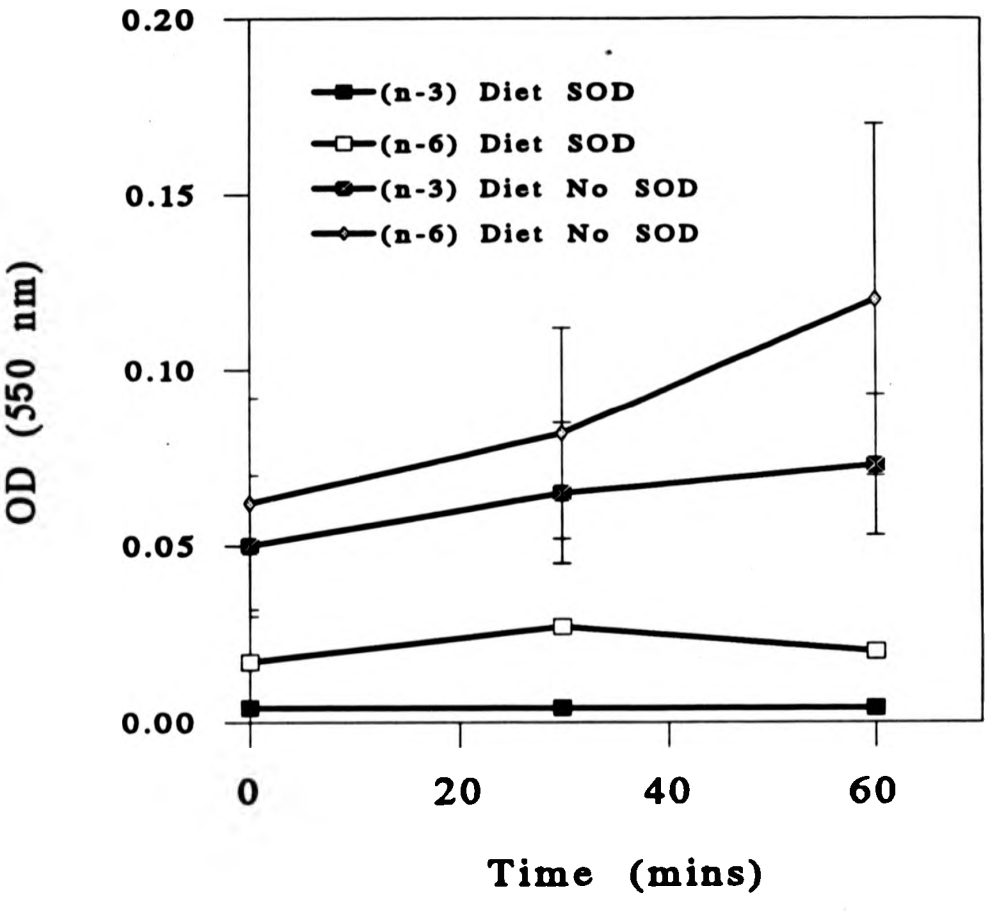


Figure 5.13

The reduction of ferricytochrome C by headkidney macrophages in Atlantic salmon from trial 2 on day 149. Results expressed as mean ±S.D for three fish/ 10^5 cells. SOD-Superoxide dismutase

5.4.2.3 Cytokine activity

(a) Macrophage activation factor (MAF) production:-

The production of MAF by headkidney leucocytes from groups of dietary trial 2 salmon was measured by assessing the activation of control headkidney macrophages cultured in the presence of MAF containing supernatants, by the NBT reduction assay reported in Section 3.4.9 (b). Differences due to dietary PUFA, either in the production of MAF or in its activity were not in evidence, as shown by the absorbances in Figure 5.14.

(b) Migration inhibition factor (MIF):-

MIF activity was measured by the migration of headkidney macrophages (see Section 3.4.13 for methodology), for fish maintained on high (n-3) and (n-6) PUFA diets in trial 2, the results of which are presented in Table 5.36. The migration by headkidney macrophages from a capillary tube is illustrated in Plate 5.2. There was no significant difference in the migration of headkidney macrophages from the two dietary groups.

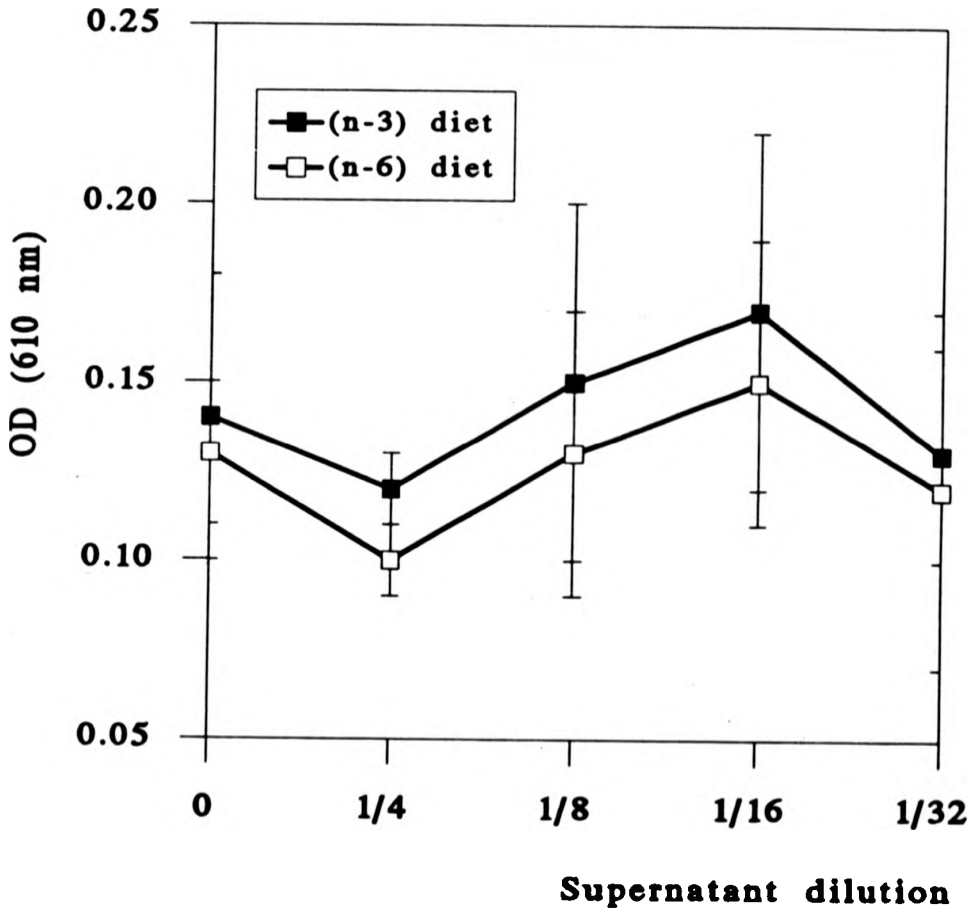


Figure 5.14 Determination of MAF production as measured by the NBT reduction by headkidney macrophages from dietary salmon of trial 2.

Results expressed as mean ±S.D for three fish/ 10^5 cells.

Table 5.36

Activity of Macrophage Inhibition Factor (MIF) in Atlantic salmon, vaccinated with *Aeromonas salmonicida*, maintained on high (n-3) and (n-6) PUFA diets in trial 2.

	migrated cell radius (mm)	
	(n-3) PUFA diet	(n-6) PUFA diet
Test	0.3 ±0.2	0.6 ±0.4
Control	0.2 ±0.0	0.3 ±0.1

Results expressed as mean (mm migration) ±S.D. (n=4) for triplicate samples

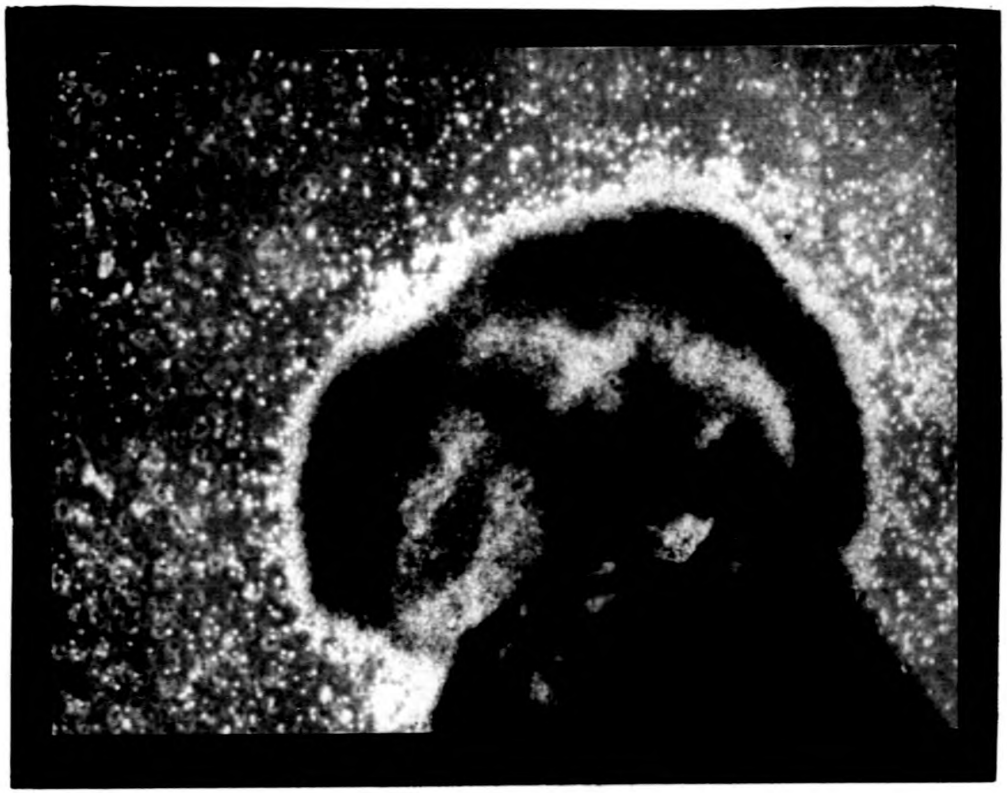


Plate 5.2 Headkidney macrophages migrating from a capillary tube in the absence of macrophage inhibition factor (MIF).

5.4.3 LEUCOCYTE ACTIVITY

5.4.3.1 Antigen proliferation assays

Antigen stimulation of headkidney leucocytes with *V. anguillarum* (1 $x10^8$ ml^{-1}) was carried out on six triplicate samples, from Atlantic salmon which had been maintained on high (n-3) and (n-6) PUFA diets or on a commercial diet in trial 1, and had been vaccinated with *V. anguillarum* for four weeks at the time of assaying.

Triplicate samples of headkidney leucocytes from eight fish were stimulated with *A. salmonicida* (1 $x10^7$ ml^{-1}). These Atlantic salmon had been maintained on high (n-3) and (n-6) PUFA diets in trial 2 and had been vaccinated with the bacterium for four weeks.

Stimulation indices for both assays are given in Table 5.37. Although antigen stimulation was notably different between vaccinated and nonvaccinated fish in trial 1 with *V. anguillarum*, antigen stimulation was independent of diet. There was no difference in stimulation in trial 2 with *A. salmonicida*.

5.4.3.2 Plaque forming cells

Plaque forming cells were enumerated in the kidney and spleen of three salmon from each dietary group of trial 2. The numbers of plaques counted for each organ are shown in Table 5.38. The (n-3) dietary PUFA group produced a significantly higher level of antibody producing cells, in both the kidney and the spleen, compared to the (n-6) PUFA group ($p < 0.05$).

Table 5.37

Leucocyte proliferation of Atlantic salmon maintained on high (n-3) and (n-6) PUFA diets

Trial 1

Antigen	Stimulation Index		
	commercial diet	(n-3) PUFA diet	(n-6) PUFA diet
V. anguillarum			
vaccinated fish	5.0 ±1.4	4.8 ±0.6	6.4 ±1.9
unvaccinated fish	1.2 ±0.1	-	-

Results expressed as mean ±S.D., (n=6) for each sample.

Trial 2

Antigen	Stimulation Index	
	(n-3) PUFA diet	(n-6) PUFA diet
A. salmonicida		
vaccinated fish	1.2 ±0.6	1.4 ±0.6
unvaccinated fish	1.1 ±0.2	1.2 ±0.5

Results expressed as mean ±S.D., (n=8) for each sample

Table 5.38

Enumeration of plaque forming cells present in kidney and spleen of trial 2 dietary fish after immunization with *Aeromonas salmonicida*.

	Diet	
1 x 10^6 cells	(n-3) PUFA	(n-6) PUFA
Kidney		
specific	780 ±63[a]	500 ±55[b]
nonspecific	71 ±3	92 ±7
Spleen		
specific	88 ±15[c]	43 ±17[d]
nonspecific	21 ±9	21 ±7

Results expressed as mean ±S.D. (n=4) for each sample.
Significant differences by ANOVA ($p < 0.05$), are indicated by a different superscript letter.

5.4.4 VACCINATION AND CHALLENGE

5.4.4.1 Introduction

Vaccination and challenge were used as *in vivo* indicators of the influence of different dietary (n-3)/(n-6) fatty acid ratios on innate and adaptive immunity in Atlantic salmon over the course of the three dietary trials. The effectiveness of the vaccination was described in terms of the RPS value for each vaccination. Parameters of the three dietary trials are discussed in Section 3.6.

The pathological symptoms which occurred in challenged fish due to vibriosis by *Vibrio anguillarum* were haemorrhaging around the mouth, on the gills and on the body, which in some cases turned into necrotic lesions at the site of injection. In furunculosis, caused by *Aeromonas salmonicida,* haemorrhaging at the base of the fins, lost of appetite and skin darkened occurred. Necrotic lesions on the fish's side also appeared. Plate 5.3 displays these symptoms.

5.4.4.2 Antibody titre

Antibody titres for trial 1 and 2 are given in Table 5.39, where values are expressed as antibody titre (-Log2+1) ±SD, measured both by ELISA and by agglutination. Antibody levels from vaccinated fish in trials 1 and 2 were monitored at two week intervals after vaccination. Antibody titres of sera from fish vaccinated against *V. anguillarum* in trial 1, measured by ELISA, are illustrated in Figure 5.15(a). Antibody titres for the commercial group in trial 1 were significantly higher than in the (n-6) dietary PUFA group for the first 6 weeks. There was no significant difference between the two

(A)

(B)

Plate 5.3 Challenged fish infected with (a) vibriosis (*Vibrio anguillarum)* interal organs pale with serious abdominal fluid and (b) furunculosis (*Aeromonas salmonicida*) haemorrage round the vent (=HV) and large "furuncule" lesion on flank (=F).

Table 5.39

Antibody titres

(a) Trial 1 Antibody titre from fish vaccinated with *V. anguillarum*

Weeks	Vaccination	Antibody Titre (-Log2+1)		
		commercial diet	(n-3) PUFA diet	(n-6) PUFA diet
2	1°	10.7 ±0.5^{a}	11.0 ±0.0^{a}	9.3 ±0.5^{b}
4	1°	13.0 ±0.0^{a}	12.3 ±0.9^{ab}	11.7 ±0.5^{b}
6	2°	15.0 ±0.0^{a}	14.3 ±0.9^{ab}	13.7 ±0.5^{b}
8	2°	14.7 ±0.5	15.3 ±0.5	14.7 ±0.5

Values are expressed as antibody titre ±SD for 3 fish measured by ELISA. Significant differences in antibody titre, by ANOVA ($p < 0.05$), are indicated by a different superscript letter.

(b) Trial 2 Antibody titre from fish vaccinated with *A. salmonicida*

Weeks	(n-3) PUFA diet		(n-6) PUFA diet	
	ELISA	Agglutination	ELISA	Agglutination
0	10.2 ±0.4^{c}	5.0 ±0.8^{a}	11.2 ±1.9^{c}	7.8 ±0.8^{b}
2	10.8 ±0.4^{c}	6.2 ±0.0^{a}	10.5 ±1.1^{c}	7.8 ±0.8^{b}
4	8.8 ±0.4^{b}	7.0 ±0.8^{a}	11.2 ±1.3^{c}	7.8 ±0.4^{a}
6	8.8 ±0.4^{b}	6.2 ±0.8^{a}	9.2 ±1.3^{ab}	7.2 ±0.8^{a}
8	10.2 ±0.8^{b}	7.8 ±0.4^{a}	9.2 ±1.6^{ab}	5.0 ±0.7^{c}
10	9.0 ±0.7^{c}	8.0 ±0.0^{a}	10.8 ±0.4^{d}	7.0 ±0.0^{b}
12	10.2 ±0.8^{c}	8.0 ±0.0^{a}	9.5 ±0.5^{c}	6.8 ±0.4^{b}

Values are expressed as antibody titre ±SD for 3 fish measured by ELISA or by agglutination. Significant differences in antibody titre, by ANOVA ($p < 0.05$), are indicated by a different superscript letter.

experimental groups, except at week 2.

Antibody titres of sera (determined by ELISA) from trial 2 fish, vaccinated with *A. salmonicida*, are shown in Figure 5.15(b) and appear to be independent of dietary PUFA. Figure 5.16 compares antibody titres obtained by ELISA with those obtained by agglutination from fish vaccinated against *A. salmonicida* in trial 2. Overall, the results of ELISA and agglutination would suggest that antibody levels in the (n-6) dietary PUFA group from trial 2 were higher than the (n-3) dietary PUFA group for the first four weeks after vaccination.

It was believed that the fish from trial 2 were asymptomatic carriers of *A. salmonicida* because of positive antibody titres to the bacterium being obtained prior to vaccination. Prevaccination bleeds were found to be positive against *A. salmonicida* by agglutination, ELISA and IFAT. Fish were stress tested to see if they developed furunculosis, but no symptoms of the disease developed. Western blots of the antisera directed against *A. salmonicida* were also carried out. Plate 5.4 (lane 1) illustrates the gel against which the Western blot was directed. The blot from fish sera prior to vaccination identified the same bands as those by the vaccinated fish, suggesting the fish had been previously exposed to *A. salmonicida*. Lanes 2, 3, and 4 of the gel in plate 5.4 represents sera from nonimmunised fish and fish from (n-3) and (n-6) dietary PUFA groups vaccinated with *A. salmonicida* respectively, and no visible difference was detected between them. Lane 6 shows purified salmon IgM (as described in Section 3.5), with bands of molecular weights corresponding to those reported by Pilstrom and Petersson, (1991).

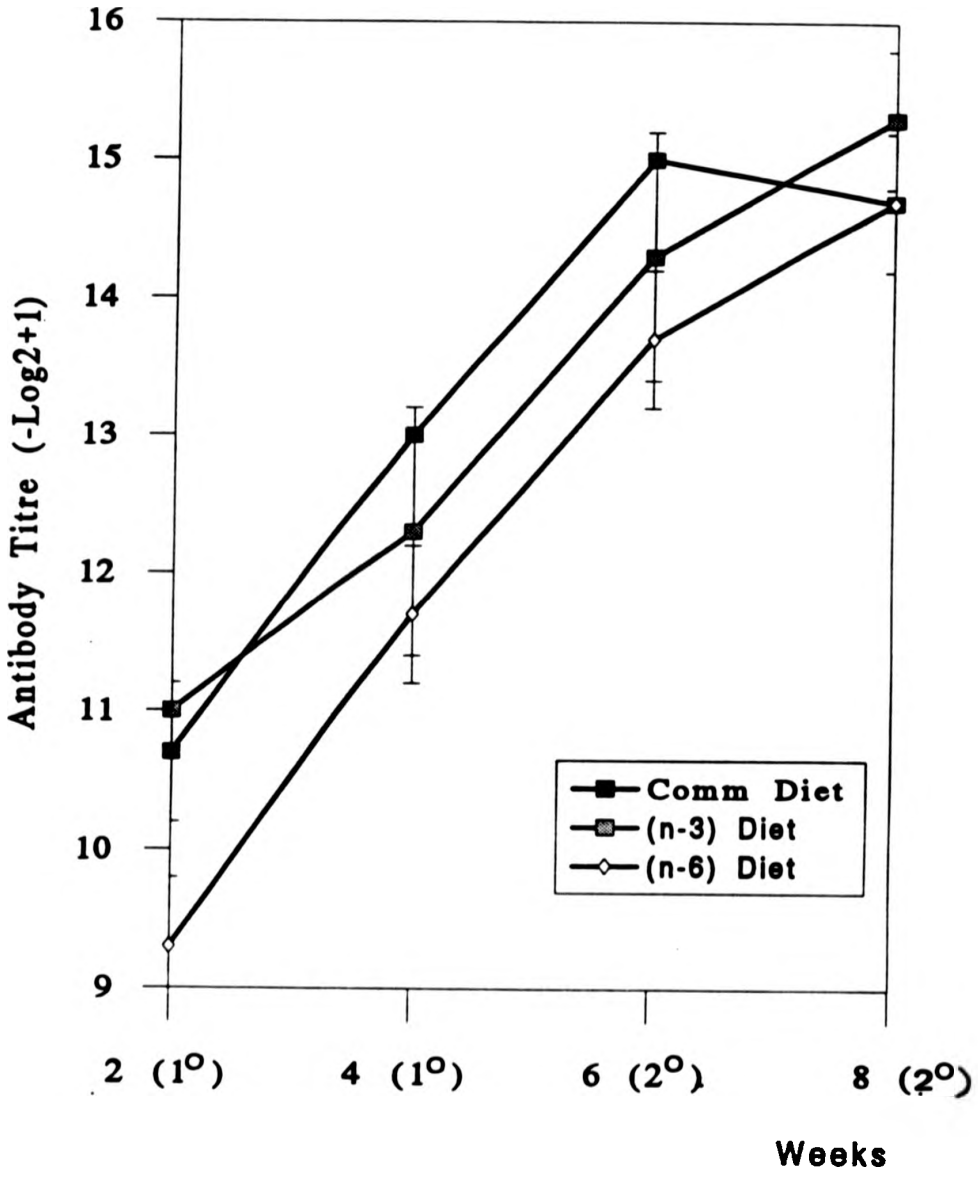


Figure 5.15 (a)

Antibody titres measured by ELISA from fish vaccinated with *Vibrio anguillarum* vaccine in trial 1 after a primary (1°) and secondary (2°) vaccination

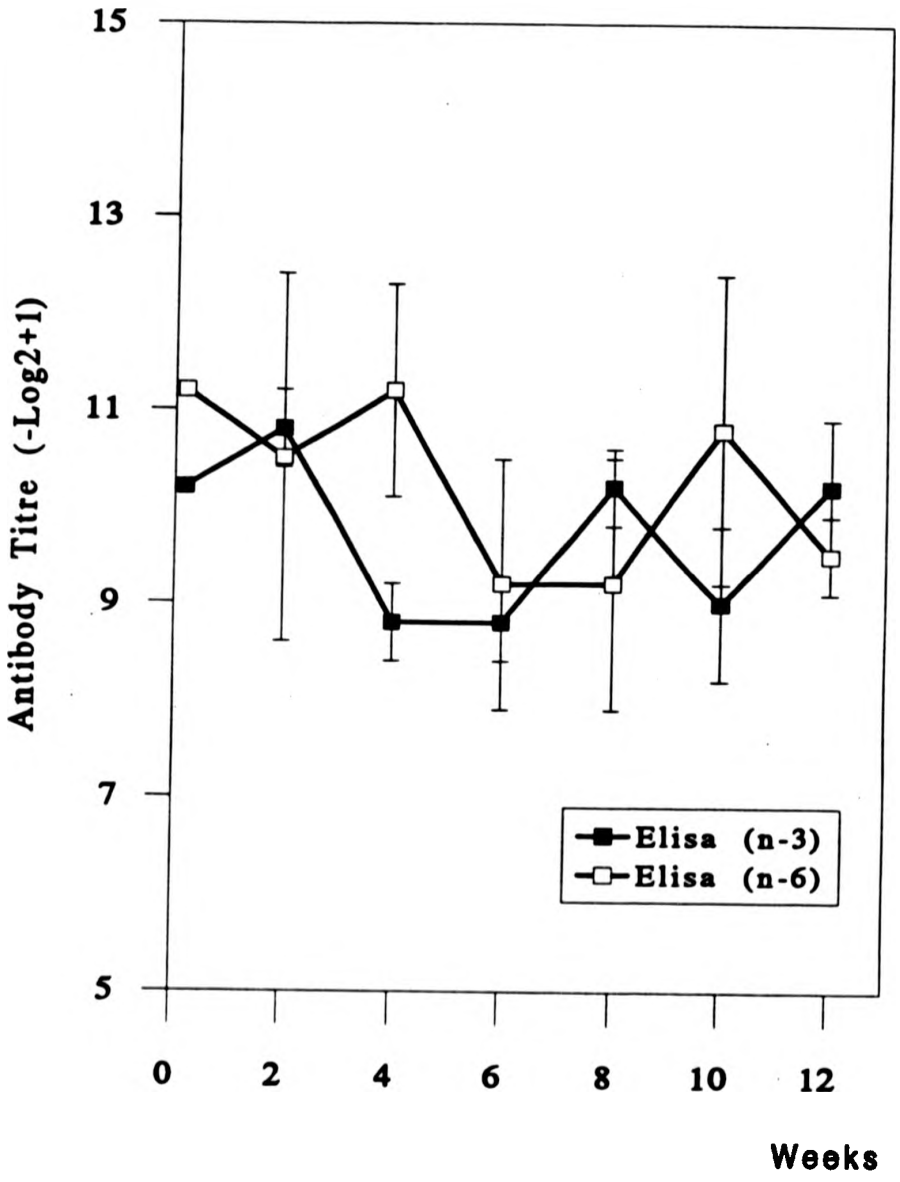


Figure 5.15 (b)

Antibody titres measured by ELISA from fish vaccinated with *Aeromonas salmonicida* vaccine in trial 2

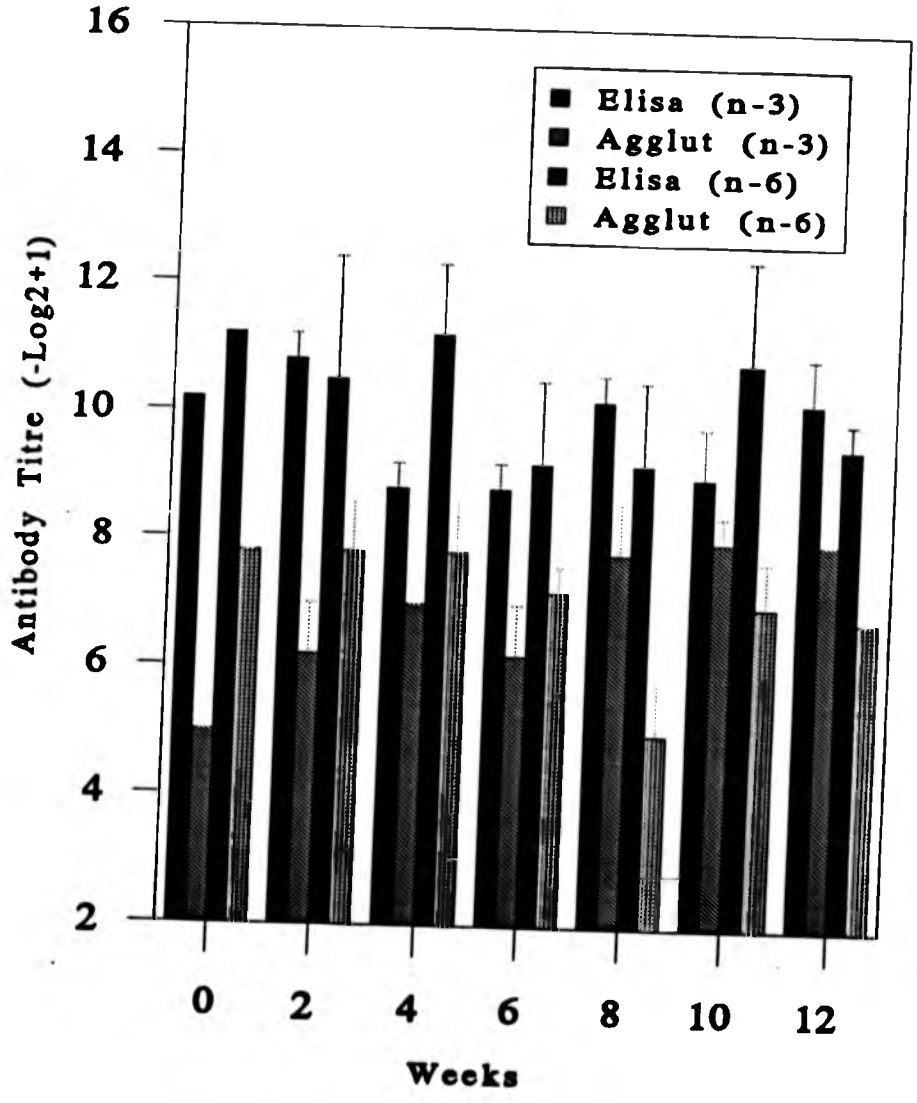


Figure 5.16

A comparison of antibody titres measured by ELISA or by agglutination for fish vaccinated against *A. salmonicida* in trial 2.

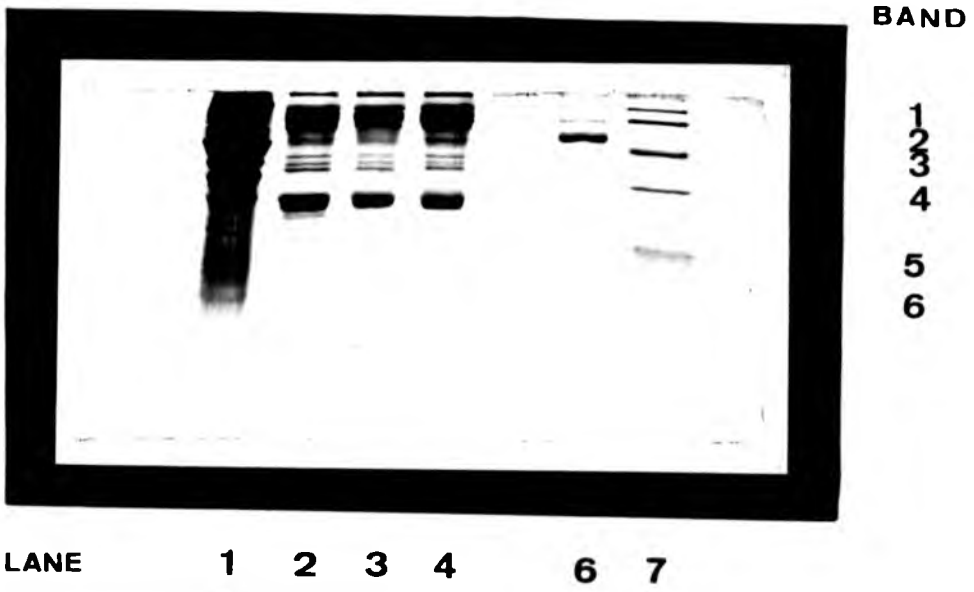


Plate 5.4

12.5 % SDS-page electrophoresis of *A. salmonicida*, nonimmunized and immunized fish sera and rabbit anti-salmon IgM

Lane 1 -Heat killed *A. salmonicida*, to which the western blot was directed.
Lane 2 -Nonimmunized fish sera
Lane 3 -Sera of (n-3) PUFA dietary group fish, vaccinated with *A. salmonicida*.
Lane 4 -Sera of (n-6) PUFA dietary group fish, vaccinated with *A. salmonicida*.
Lane 6 -Sodium sulphate precipitated/DE-52 purified salmon IgM.
Lane 7 -Low molecular weight markers (Biorad) Band 1 -106,000 KD;
Band 2 -80,000 KD; Band 3 -49,500 KD; Band 4 -32,500 KD;
Band 5 -27,500 KD; and Band 6-18,500.

5.4.4.3 Mortalities

Percentage mortalities and RPS values for the vaccinations described in Section 5.4.5.1 are presented in Table 5.40 for trial 1 and Table 5.41 for trial 2. In trial 1 the (n-6) PUFA group had the highest mortalities for both the 21 day period of the first challenge and the ten day period of the second challenge. In trial 2 the (n-6) PUFA group had the highest mortalities in the non-vaccinated population over the 21 days of the challenge, while the vaccinated population of this group had the lowest mortalities. The criteria for RPS values, outlined in Section 3.6.1 were however not met in both challenges. In the primary challenge of trial 1 three of the four mentioned criteria were not met (i.e. insufficient control fish died, the mortality rate was greater in some of the vaccinated groups than in the control group and duplicate samples of fish were not used). RPS values were determined using the non-vaccinated commercial group as the control for all three dietary groups and in the primary challenge only 24 % of them had died by day 21, less than in the vaccinated commercial group (36 %).

The level of protection obtained against *A. salmonicida* in trial 2 was higher in the (n-6) PUFA dietary group, but the RPS values reported in this trial depended on which control group was used for their determination. If the same dietary group was used as the control for the (n-6) PUFA group, higher RPS values were obtained than when the unvaccinated (n-3) PUFA group was used as the control (values shown in Table 5.41 in parenthesis). Cumulative mortalities of challenged fish in trial 1 are illustrated in Figure 5.17, trial 2 in Figure 5.18, and trial 3 in Figure 5.19. For each trial the (n-6) dietary PUFA group succumbed to the infection first, and had the largest number of deaths per group. The only significant difference in the death rate between the two

Table 5.40

Relative Percent Survival values after challenge in salmon in trial 1 vaccinated with *Vibrio anguillarum*

Dietary group	% Mortalities		RPS (%)
	vaccinated	Nonvaccinated	
1° challenge (day 10):			
Commercial	3.0	6.0	50
(n-3) PUFA	0.0	-	100
(n-6) PUFA	33.0	-	-
1° challenge (day 21):			
Commercial	36.0	24.0	-
(n-3) PUFA	7.0	-	70.8
(n-6) PUFA	37.0	-	-
2° challenge (day 10):			
Commercial	78.0	97.0	20
(n-3) PUFA	79.0	-	18.6
(n-6) PUFA	91.0	-	6.2

1° challenge n=33 in commercial group, n=30 in (n-3) PUFA group and n=31 in (n-6) PUFA group

2° challenge n=50 in commercial group, n=32 in (n-3) PUFA group and n=53 in (n-6) PUFA group

- not done

Table 5.41

Relative Percent Survival values after challenge in salmon in trial 2 vaccinated with *Aeromonas salmonicida*

Dietary group	% Mortalities		RPS (%)
	vaccinated	Nonvaccinated	
1° challenge (day 10):			
(n-3) PUFA	16.1	40.9	60.6
(n-6) PUFA	7.4	70.0	89.4 (55.3)
1° challenge (day 21):			
(n-3) PUFA	48.4	50.0	3.2
(n-6) PUFA	33.3	90.0	63.0 (33.4)

n=31 in (n-3) PUFA group and n=27 in (n-6) PUFA group

RPS values in parenthesis determined from nonvaccinated (n-3) PUFA group as control

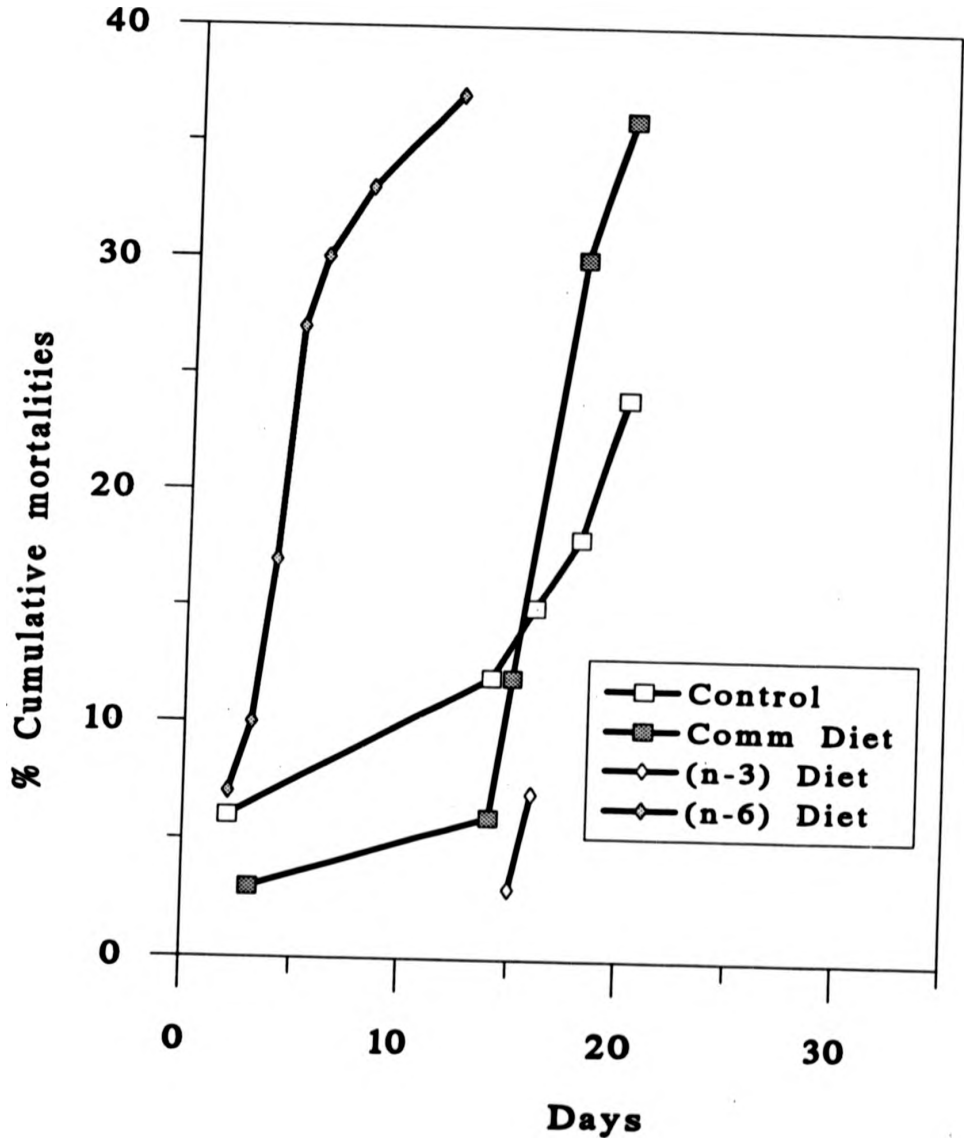


Figure 5.17 (a)

Cumulative mortalities of fish challenged with *Vibrio anguillarum* in trial 1 after a primary vaccination

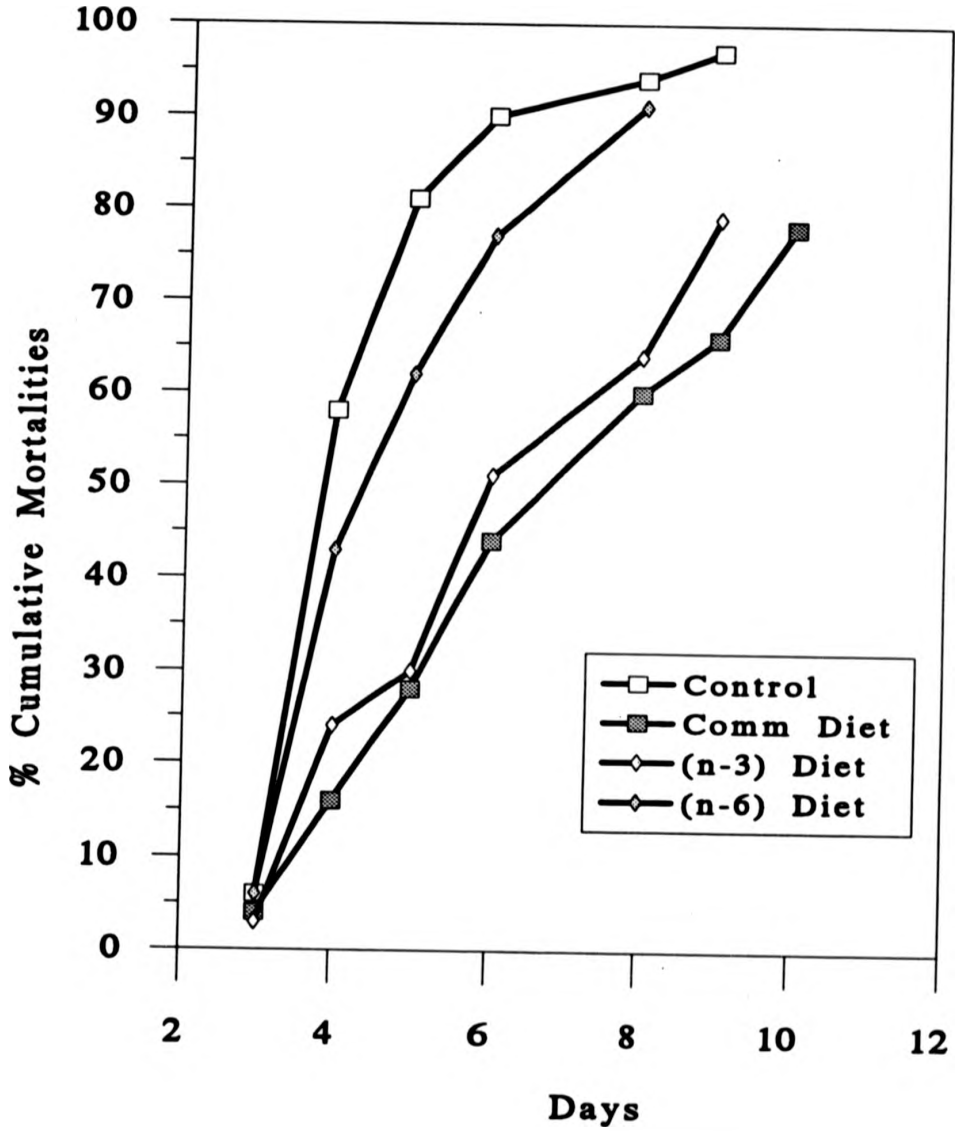


Figure 5.17 (b)

Cumulative mortalities of fish challenged with *Vibrio anguillarum* in trial 1 after a secondary vaccination.

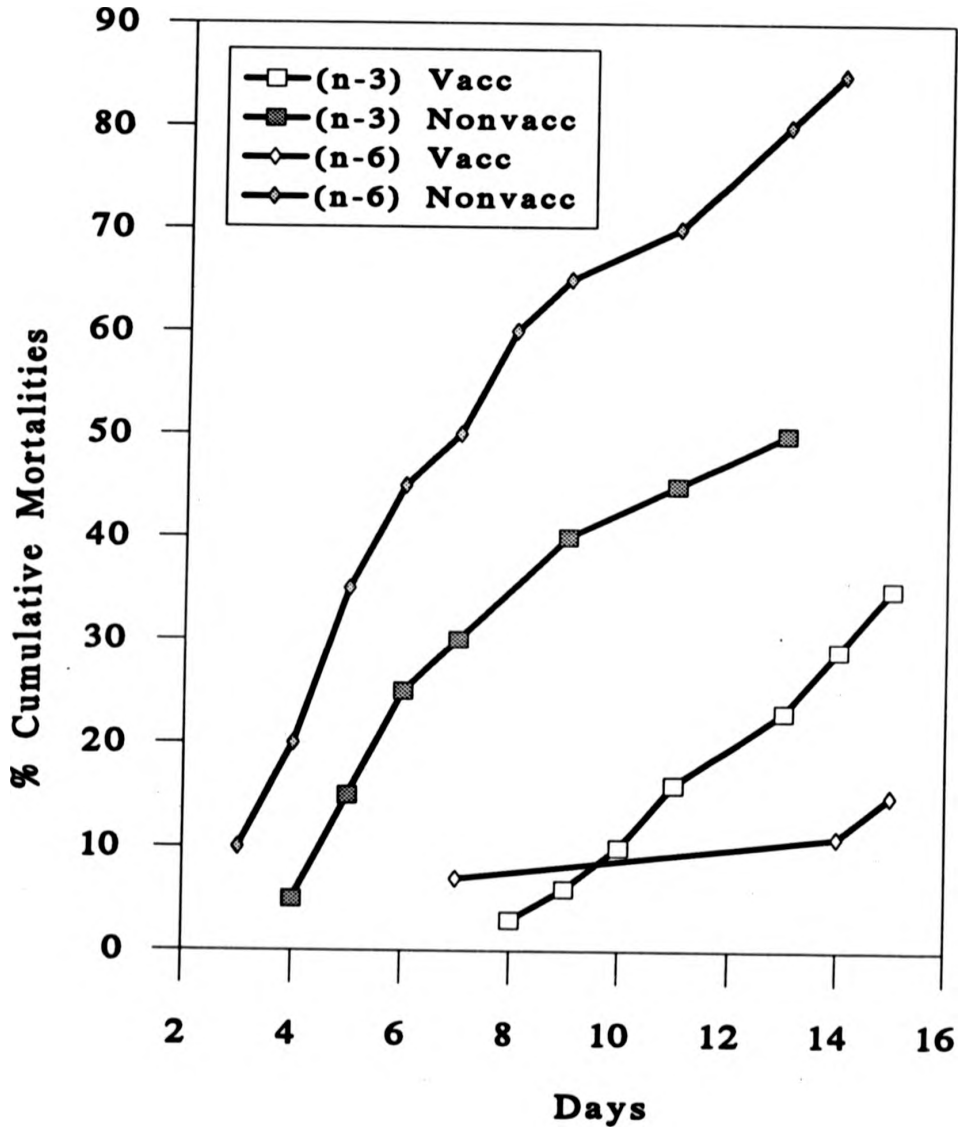


Figure 5.18

Cumulative mortalities of fish challenged with *Aeromonas salmonicida* in trial 2.

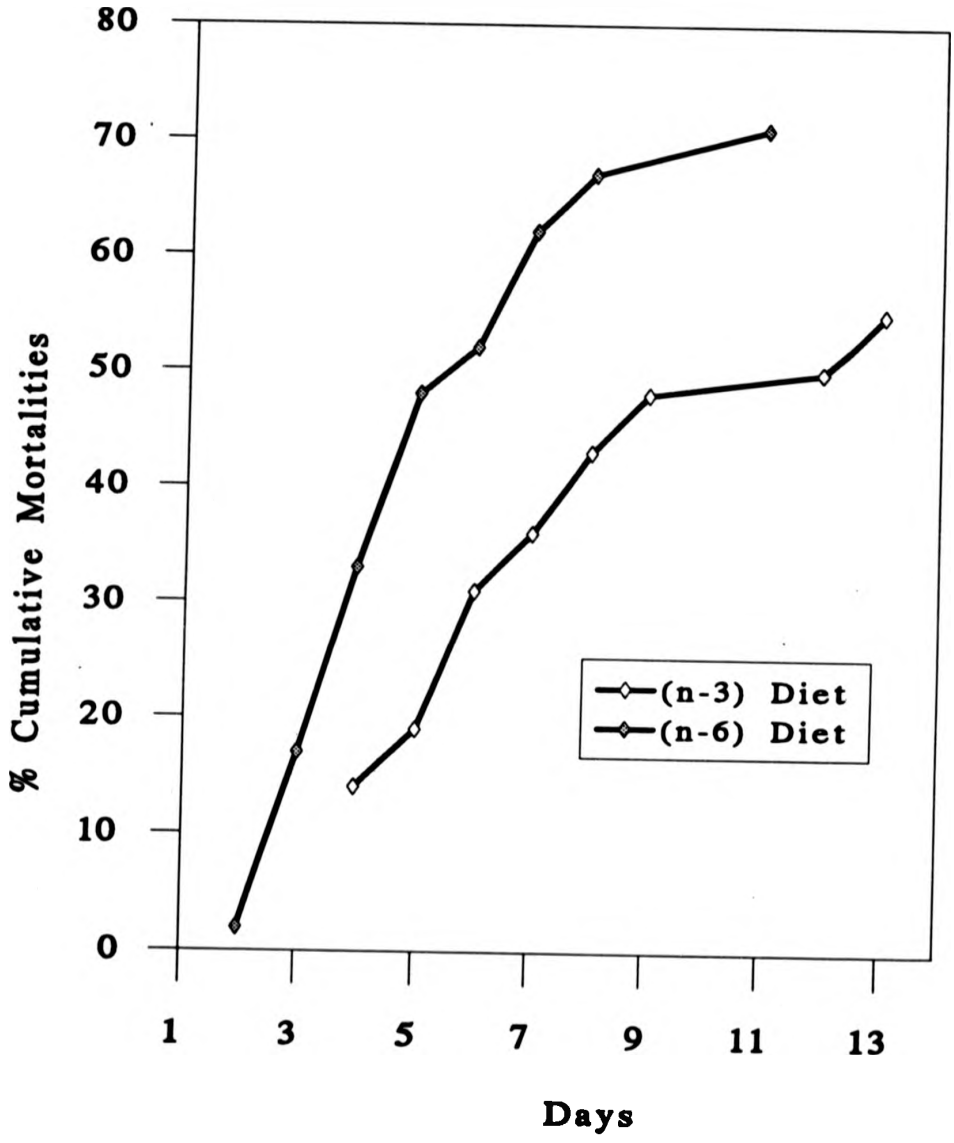


Figure 5.19

Cumulative mortalities of fish challenged with *Vibrio anguillarum* in trial 3

groups however, was with trial 3, ($p < 0.01$) determined by a log rank test.

5.5 CHAPTER SUMMARY

5.5.1 Physiological parameters

The effects of different (n-3)/(n-6) PUFA ratios on the physiology of Atlantic salmon parr were examined in the three consecutive dietary trials outlined in Chapter 3. As water temperature increased, there was an increased feeding rate in all three trials. A significant difference in the weight gained between the experimental populations of fish was observed only in the second trial. This in turn coincided with significant differences in condition factor, hepatosomatic index and their splenomatic index. There were no significant differences in blood cell numbers, differential leucocyte counts or haematocrit values between dietary groups.

5.5.2 Lipid composition

A time course to verify dietary manipulation of peripheral blood leucocytes, erythrocytes and serum showed increased 18:2(n-6) fatty acid levels in the total lipid of serum and leucocytes from fish maintained on a low (n-3)/(n-6) PUFA ratio diet, 4 weeks from the start of the trial. Leucocytes steadily incorporated 18:2 (n-6) into their lipid until a (n-3)/(n-6) PUFA ratio of 0.8 was maintained after 16 weeks. Erythrocytes, by comparison, did not show dietary manipulation until 16 weeks, at which point the 18:2(n-6) fatty acid level was observed to plateau.

Lipid class composition of lipids from leucocytes, erythrocytes and serum were all found to be independent of diet, but the fatty acid composition of lipid was influenced

by dietary PUFA. Leucocytes were found to contain greater proportions of dietary 18:2(n-6) in their lipid than erythrocytes (13.0 % compared to 6.7 %), but because erythrocytes had a much higher (n-3) PUFA content than the leucocytes, they had a also higher (n-3)/(n-6) PUFA ratio. Dietary lipid composition had little effect on (n-3) PUFA levels of erythrocyte lipid between the dietary groups. Saturated fatty acids and (n-3) PUFA, but not monoenoic fatty acids were displaced in response to dietary manipulation of the leucocyte lipid.

Larger differences were observed between the dietary groups in 18:2(n-6) fatty acid levels of immunocompetent tissue lipids, than was found in lipids of their constituent leucocytes. Phospholipids of tissues from the (n-3) PUFA dietary group possessed higher (n-3)/(n-6) PUFA ratios than those of their leucocytes due to a higher (n-3) PUFA content, while the (n-3)/(n-6) PUFA ratios were found to be similar between the tissues and leucocytes of the (n-6) PUFA dietary group. This was apparently because tissues of the (n-6) dietary group generally incorporated much more 18:2(n-6) dietary fatty acid into lipids than their leucocytes.

Similarities in fatty acid profiles of individual lipid classes of erythrocytes, leucocytes, serum and immunocompetent tissues were found throughout the study. The PC fraction had characteristically the highest level of 16:0 and high levels of 18:1, while PS and PI were rich in 16:0, 18:0 and 18:1. The PC fraction had the highest 18:1 level of all the lipid classes examined, while the PI lipid class contained a typically high level of 20:4(n-6). The lowest level of saturation was found in the PE fraction which was compensated for by a high level of (n-3) PUFA unsaturation. Similar patterns were

found between the dietary groups for the fatty acid patterns of individual phospholipid classes. The influence of dietary fatty acid was most evident in the PC and PE fractions.

5.5.3 Immune response

No apparent difference was found between the groups of fish on the different (n-3)/(n-6) PUFA ratio diets, in respect to serum protein concentrations, complement activity, kidney and serum lysozyme activity and anti-protease activity. There was also no difference in macrophage activity;- phagocytic activity, bacterial killing, oxidative burst and cytokine production. While no difference in antigen stimulation was found between the groups, there was a significantly higher number of responding B cells in the kidney and spleens of fish on high (n-3)/(n-6) PUFA ratio diets, measured by a plaque forming assay.

The effect of the different (n-3)/(n-6) PUFA ratios on the protective vaccination of the groups of fish on the different diets was inconclusive. Evidence for the fish on the low (n-3)/(n-6) PUFA ratio diets being more susceptible to disease comes firstly from the fish being more prone to fungal infection throughout the trial (Table 5.1) and secondly this group tended to die first and in larger numbers when challenged with bacteria. There was only a significant difference in the last trial when non-vaccinated fish were challenged with *Vibrio anguillarum*.

CHAPTER 6 EFFECTS OF EXOGENOUS FATTY ACIDS ON ATLANTIC SALMON LEUCOCYTES *IN VITRO*

6.1 INTRODUCTION

Cultured cells utilize lipids supplied from serum supplements as their main source of lipid (Spector *et al*, 1981). Fish cell culture has in the main been adapted from procedures used for mammalian cell culture. As a result, foetal calf serum (FCS) is routinely used as a serum supplement for fish cell culture, although alternative supplements have been used by some research groups. With channel catfish leucocytes, FCS was found to be inhibitory in mitogen stimulation assays, and a combination of 10 % human plasma and 5 % channel catfish sera is employed for optimal activity (Faulmann *et al.*, 1983). Autologous or homologous rainbow trout plasma increased trout leucocyte responses to LPS stimulation when compared with FCS (De Koning and Kaattari, 1991).

It is also possible to modify the fatty acid composition of cells by culturing them with fatty acids complexed to bovine serum albumin (BSA) (Spector *et al*, 1981). Tocher *et al* (1988) have shown that fatty acid profiles of established fish cell lines reflect the lipid composition of their sera supplement, and that fish cells which are cultured in medium supplemented with mammalian sera tend to be deficient in (n-3) PUFA. This may distort the activity of the cell and render them unsuitable as an experimental model for cellular function studies.

The aims of the present study were:- (a) to examine the influence of FCS and of

exogenous fatty acids on the lipid composition of fish leucocytes, (b) to determine the uptake of ^{14}C labelled exogenous fatty acids at 4°C and 15°C over a timed period, by leucocytes in the presence of mitogens and by leucocytes from different immunocompetent tissues, (c) to measure the uptake of ^{14}C labelled exogenous fatty acids into the lipid classes of Atlantic salmon leucocytes at 4°C and 15°C in the presence and absence of mitogens and, (d) to examine the influence of exogenous fatty acids on immune function.

Fatty acids used in the following incorporation studies were: 18:1(n-9), 18:2(n-6), 18:3(n-3), 20:4(n-6), 20:5(n-3) and 22:6(n-3). Radiolabelled fatty acids used were [1-^{14}C]18:1(n-9), [1-^{14}C]18:2(n-6), [1-^{14}C]18:3(n-3), [1-^{14}C]20:4(n-6) and [1-^{14}C]20:5(n-3) which had a specific activity of 52.7 mCi/mmol, 54.6 mCi/mmol, 53.9 mCi/mmol, 54 mCi/mmol, and 58 mCi/mmol, respectively. Purity of the fatty acids was reported as around 97% by high performance liquid chromatography (manufacturer's data). The above fatty acids were solubilized for use as tissue culture supplements by binding them to BSA (See 3.3.4).

Leucocytes were isolated from blood and immunocompetent tissues according to Section 3.2.4. The cells were resuspended in L-15 medium (with pen/strep and glutamine) to which had been added either FCS and/or FA complexes bound to BSA. The concentration at which the fatty acids were supplemented are detailed with each experiment as μM or nmoles added.

6.2 EFFECTS OF EXOGENOUS FATTY ACIDS ON THE LIPID COMPOSITION OF ATLANTIC SALMON LEUCOCYTES *IN VITRO*

6.2.1 Optimising the concentration of exogenous fatty acids in fish leucocyte cultures.

Admissible concentrations of BSA bound fatty acids were first established for the survival of Atlantic salmon leucocytes in culture. Leucocytes from the headkidney of three Atlantic salmon parr were isolated and resuspended at $1x10^6$ ml^{-1} in L-15 medium (with pen/strep, glutamine and 0.5% FCS) to which had been added either 18:1(n-9), 18:2(n-6), 18:3(n-3), 20:4(n-6), 20:5(n-3) or 22:6(n-3) bound to BSA at a final concentration of 10 µM - 100 µM. Cells were dispensed at 0.1 ml $well^{-1}$ into a 96 well round bottom microtitre plate. The plate was incubated at 15°C in a humidified chamber and the cells examined daily for 7 days. As in Section 3.3.1, the optimum fatty acid concentration was selected on the basis of cell appearance rather than on viability counts. Table 6.1 shows the survival of Atlantic salmon headkidney leucocytes with different concentrations of fatty acid/BSA solutions over the course of seven days. Atlantic salmon headkidney leucocytes could be maintained in culture for up to seven days at higher concentrations of exogenous 18:3(n-3) and 20:5(n-3) (100 µM) than 18:1(n-9), 18:2(n-6), 20:4(n-6) and 22:6(n-3) (10 µM). On the basis of the results obtained, it was decided to supplement leucocyte cultures with 10 µM fatty acids, unless otherwise stated. Incubations with fatty acids greater than 20 µM resulted in a visible accumulation of lipid within the cells in the form of cytoplasmic lipid droplets.

Table 6.1

Survival of Atlantic salmon headkidney leucocytes with different concentrations of fatty acid/BSA solutions for 7 days.

		fatty acids					
	µM	18:1	18:2	18:3	20:4	20:5	22:6
day 1	100	-	-	+++	-	+++	-
	50	+	-	+++	+	+++	-
	25	+	+	+++	++	+++	-
	10	+++	+++	+++	+++	+++	+++
day 2	100	-	-	+++	-	++	-
	50	-	-	+++	-	++	-
	25	+	+	+++	++	++	-
	10	+++	+++	+++	+++	+++	+++
day 3	100	-	-	+++	-	++	-
	50	±	-	+++	-	++	-
	25	+	++	+++	++	++	-
	10	+++	++	+++	+++	+++	++
day 5	100	-	-	+++	-	++	-
	50	±	-	+++	+	++	-
	25	++	+	++	++	++	-
	10	+++	+++	++	+++	++	++
day 7	100	-	-	+++	-	++	-
	50	-	-	++	-	++	-
	25	+	+	++	-	++	-
	10	+++	++	++	++	++	+

Cells examined under inverted microscope and cell survival scored accordingly: +++ healthy cells; ++ cells beginning to appear granular; + visible cell death; ± few surviving cells; - all cells dead and lysed. (Mean of 3 fish).

6.2.2 The influence of FCS and exogenous fatty acids bound to BSA on the lipid composition of rainbow trout leucocytes *in vitro*

(a) Culturing with FCS:-

The fatty acid compositions of rainbow trout leucocytes were determined after culturing *in vitro* with 10 % foetal calf serum. Peripheral blood leucocytes were isolated from three rainbow trout weighing approximately 250 g which had been maintained at 10 °C. Cells were plated out in 24 well plates at a concentration of 1 $x10^7$ $well^{-1}$ in L-15 with additives and 10% FCS. They were incubated at 15°C for 6 days in a humidified chamber, after which they were harvested on days 0, 3 and 6 by washing three times with HBSS and their total lipid content analysed as described in Section 3.8. The fatty acid composition of the leucocytes and the supernatant in which they were cultured are shown in Table 6.2.

The above experiment was repeated using peripheral blood leucocytes from a 400 g rainbow trout which had been maintained at 7°C. Cells were dispensed in a 24 well plate at a concentration of 5 $x10^6$ $well^{-1}$ in the presence of either 1 % or 5 % FCS, or 1 % fish serum, and then incubated at 15°C for 6 days in a humidified chamber. Cells and supernatant were harvested at days 0, 2, 4 and 6 and their fatty acid compositions analysed. These are shown in Table 6.3.

In the first experiment, the lipid profile of the trout leucocytes was not influenced by FCS. It is interesting to note that the fatty acid composition of the baseline cells at day zero closely resembled the fatty acid profile of the FCS (i.e. day 0 supernatant, Table

6.2). The only notable difference between the cells and the supernatant at day 0 was that the proportion of 22:6(n-3) fatty acid was higher in the cells and this level did not decrease upon incubation of cells with the FCS. The supernatant lipid, however, contained slightly higher levels of 20:4(n-6) than the cells and increased over the course of the incubation (Table 6.2). This increased 20:4(n-6) level resulted in an higher overall (n-6) PUFA level in lipid of the supernatant than the cells. In keeping with this observation, the (n-3)/(n-6) ratio was higher in the lipid of cells than that of supernatant.

The lipid composition of trout leucocytes in the second experiment also appeared to be unaffected by the supplemented FCS when present at either 1 % or 5 % (Table 6.3). Differences in values were observed between this and the previous experiment, but as cell samples came from only one fish the significance of the variation could not be determined. As in the previous experiment, there was no change in the level of (n-6) PUFA in leucocyte lipid over the course of the incubation.

The lipid of fish serum is high in (n-3) PUFA, especially 20:5(n-3) and 22:6(n-3) (Table 4.3). Trout leucocytes did not incorporate these high levels of (n-3) PUFA into their lipid when cultured with supplemented fish serum (Table 6.3c), suggesting that these cells have a preference for lower levels of (n-3) PUFA's in their cellular lipids or are incapable of fatty acid uptake. The cells at day zero contained lower levels of (n-3) PUFA than found in other fish tissues. Liver was included in the analysis as a comparison to the leucocytes and it can be seen from Table 6.3 that liver contains 3 times as much (n-3) PUFA (47.4 %) as the leucocytes.

Table 6.2

Fatty acid composition of rainbow trout leucocytes and supernatant after culturing *in vitro* with 10 % foetal calf serum

Fatty acids	Cells			Supernatant		
(% Total FA)	day 0	day 3	day 6	day 0	day 3	day 6
14:0	0.9 ±0.5	0.8 ±0.1	0.6 ±0.1	0.9 ±0.2	0.7 ±0.3	1.2 ±0.6
15:0	1.3 ±0.3	1.0 ±0.2	0.9 ±0.2	0.8 ±0.2	1.1 ±0.1	0.9 ±0.2
16:0	31.3 ±3.1	30.5 ±1.8	30.0 ±1.4	28.6 ±1.5	29.6 ±1.5	25.0 ±0.8
17:0	0.9 ±0.3	0.4 ±0.3	0.7 ±0.1	1.2 ±0.0	1.2 ±0.1	1.3 ±0.0
18:0	15.0 ±1.3	12.9 ±2.0	14.3 ±1.5	13.1 ±0.5	14.1 ±0.5	13.8 ±0.9
22:0	0.3 ±0.4	1.0 ±0.4	1.0 ±0.1	0.8 ±0.1	1.0 ±0.1	0.8 ±0.1
Σ saturates	**50.4 ±5.6**	**47.3 ±4.0**	**48.2 ±3.2**	**45.9 ±2.2**	**48.4 ±2.1**	**43.6 ±1.2**
16:1	5.7 ±1.9	4.7 ±0.9	4.4 ±1.1	5.4 ±0.5	5.4 ±0.4	4.4 ±1.6
18:1	18.4 ±1.9	22.0 ±1.0	20.5 ±1.1	23.9 ±0.6	21.9 ±0.4	23.4 ±0.7
20:1	1.5 ±0.7	2.5 ±0.6	2.0 ±1.0	2.2 ±1.4	1.9 ±1.0	2.0 ±0.5
22:1	0.8 ±0.4	1.4 ±0.4	1.7 ±0.5	1.3 ±1.1	1.1 ±0.5	1.5 ±0.7
24:1	1.1 ±0.1	0.9 ±0.4	1.0 ±0.1	0.9 ±0.1	0.9 ±0.1	0.5 ±0.2
Σ monoenes	**28.0 ±2.5**	**32.4 ±2.3**	**30.0 ±3.7**	**34.2 ±3.2**	**31.9 ±1.3**	**32.0 ±2.0**
18:2	5.6 ±2.3	3.4 ±0.2	3.8 ±0.6	4.6 ±0.5	4.5 ±0.7	4.6 ±0.2
18:3	1.4 ±0.5	0.2 ±0.2	2.0 ±0.2	2.0 ±0.5	2.8 ±0.7	2.8 ±1.7
20:3	-	0.6 ±0.9	-	0.7 ±0.0	0.6 ±0.0	1.1 ±0.1
20:4	1.7 ±0.5	2.0 ±0.4	2.0 ±0.5	2.7 ±0.1	2.8 ±0.2	3.7 ±0.3
22:5	0.4 ±0.1	0.2 ±0.2	-	0.1 ±0.2	0.8 ±0.0	2.2 ±0.2
Σ (n-6) PUFA	**10.0 ±3.7**	**7.4 ±0.7**	**9.6 ±1.7**	**11.5 ±1.2**	**12.6 ±0.7**	**16.1 ±2.2**
16:3	1.0 ±0.2	0.9 ±0.2	0.9 ±0.1	0.9 ±0.1	1.0 ±0.0	1.0 ±0.1
18:3	0.3 ±0.3	0.2 ±0.2	0.3 ±0.2	0.2 ±0.2	0.4 ±0.1	1.0 ±0.7
18:4	-	0.1 ±0.2	0.6 ±0.2	0.5 ±0.2	1.3 ±0.7	0.3 ±0.2
20:5	2.2 ±1.1	1.9 ±1.6	1.9 ±0.7	1.2 ±0.0	0.1 ±0.2	0.7 ±0.1
22:5	0.4 ±0.1	0.3 ±0.3	0.6 ±0.1	1.1 ±0.1	0.8 ±0.1	1.6 ±0.1
22:6	7.6 ±3.2	9.3 ±3.6	7.8 ±3.6	3.9 ±0.5	2.6 ±0.3	2.2 ±0.2
Σ (n-3)	**11.6 ±4.3**	**13.0 ±5.5**	**12.1 ±4.6**	**8.4 ±0.7**	**7.1 ±1.1**	**7.3 ±0.9**
(n-3)/(n-6)	**1.2 ±0.8**	**1.8 ±0.9**	**1.3 ±0.7**	**0.7 ±0.1**	**0.7 ±0.1**	**0.4 ±0.1**

Values are expressed as the mean percent of total fatty acids for three fish ±SD. Σ saturates includes 20:0; Σ monoenes includes 14:1; Σ (n-6) PUFA includes 16:2; 20:2, 22:2; Σ (n-3) include 20:4; where one or more of the six values is < 0.8

Table 6.3 (a)

The fatty acid composition of rainbow trout leucocytes and supernatant after culturing *in vitro* with 5 % foetal calf serum

Fatty acids		Cells				Supernatant			
(% Total FA)	Liver	D=0	D=2	D=4	D=6	D=0	D=2	D=4	D=6
14:0	1.7	4.4	4.4	5.2	2.5	1.6	3.7	2.1	ND
15:0	0.3	1.9	2.2	3.0	2.6	1.0	1.6	1.3	
16:0	17.5	27.9	25.2	26.6	22.9	20.0	27.9	22.3	
17:0	0.3	0.8	0.9	-	1.4	1.1	1.2	1.2	
18:0	5.0	8.6	7.8	10.4	7.4	8.5	11.7	9.2	
20:0	0.2	1.1	0.6	1.0	1.5	0.5	0.7	0.6	
22:0	1.1	1.1	0.9	2.8	4.9	2.1	-	2.0	
Σ saturates	**26.2**	**45.8**	**42.0**	**48.9**	**43.1**	**34.8**	**46.7**	**38.8**	
14:1	-	1.1	1.2	2.8	6.4	0.5	1.4	1.1	
16:1	2.7	5.7	6.0	9.1	5.2	6.7	6.3	7.0	
18:1	11.1	15.7	13.1	15.5	11.0	22.4	23.7	21.2	
20:1	2.4	2.9	1.9	1.6	2.4	3.1	2.1	2.6	
22:1	-	3.2	2.2	3.7	3.1	-	1.3	2.9	
24:1	2.9	2.0	2.2	-	4.5	5.9	0.8	5.1	
Σ monoenes	**19.1**	**30.6**	**26.8**	**32.7**	**32.6**	**37.9**	**35.6**	**40.0**	
16:2	0.2	0.3	0.4	-	-	2.3	1.1	-	
18:2	1.8	2.3	2.2	2.5	3.5	4.7	4.5	4.3	
18:3	0.2	0.4	0.3	-	1.1	1.0	-	-	
20:2	0.3	0.4	-	-	2.6	0.3	-	-	
20:4	3.6	2.3	1.7	1.6	1.4	4.4	3.9	3.7	
22:2	0.5	0.5	1.1	1.2	0.8	0.8	-	-	
Σ (n-6) PUFA	**7.3**	**7.3**	**5.7**	**5.1**	**10.3**	**14.5**	**10.8**	**8.9**	
16:3	0.2	1.0	0.5	1.3	1.1	0.8	1.2	1.0	
20:5	7.3	3.3	4.8	5.0	3.3	2.0	1.3	3.0	
22:5	2.6	0.7	1.2	-	1.6	2.8	1.1	2.1	
22:6	34.9	10.1	17.4	7.0	5.1	5.9	2.8	5.1	
Σ (n-3)	**47.4**	**16.3**	**25.6**	**13.3**	**13.9**	**12.8**	**7.0**	**12.4**	
(n-3)/(n-6)	**6.5**	**2.2**	**4.5**	**2.6**	**1.3**	**0.9**	**0.6**	**1.4**	

Abbreviations: ND-not done due to insufficient sample. Values are expressed as the percent of total fatty acids for one fish. Σ(n-6) PUFA includes 20:3; 22:5; Σ(n-3) include 18:3; 18:4; 20:4, where one or more of the values eight is < 0.8 D=day

Table 6.3 (b)

The fatty acid composition of rainbow trout leucocytes and supernatant after culturing *in vitro* with 1 % foetal calf serum

Fatty acids		Cells				Supernatant			
(% Total FA)	Liver	D=0	D=2	D=4	D=6	D=0	D=2	D=4	D=6
14:0	1.7	4.4	4.7	2.9	0.9	1.6	1.7	2.2	ND
15:0	0.3	1.9	1.9	1.6	2.8	1.0	1.1	1.4	
16:0	17.5	27.9	23.8	24.6	26.3	20.0	23.8	20.8	
17:0	0.3	0.8	1.2	0.9	0.4	1.1	1.0	1.2	
18:0	5.0	8.6	7.0	9.7	6.5	8.5	9.3	5.9	
20:0	0.2	1.1	0.7	1.4	0.5	0.5	0.5	0.4	
22:0	1.1	1.1	0.7	4.3	1.8	2.1	0.6	0.6	
Σ saturates	**26.2**	**45.8**	**40.1**	**45.3**	**39.2**	**34.8**	**38.1**	**32.4**	
14:1	-	1.1	0.8	3.1	1.0	0.5	0.7	0.9	
16:1	2.7	5.7	10.3	8.9	10.9	6.0	9.8	7.9	
18:1	11.1	15.7	26.0	14.7	14.9	22.4	35.3	35.6	
20:1	2.4	2.9	0.4	1.5	1.2	3.1	0.5	1.2	
22:1	-	3.2	0.9	1.5	2.7	-	1.0	1.4	
24:1	2.9	2.0	1.4	3.1	1.0	5.9	-	1.7	
Σ monoenes	**19.1**	**30.6**	**39.8**	**34.9**	**31.7**	**37.9**	**47.3**	**48.8**	
16:2	0.2	0.3	0.6	-	0.7	2.3	0.7	0.8	
18:2	1.8	2.3	5.2	5.7	4.0	4.7	3.7	4.6	
20:4	3.6	2.3	0.9	1.7	0.5	4.4	1.0	0.8	
22:2	0.5	0.5	-	-	1.4	0.8	0.5	1.1	
Σ (n-6) PUFA	**7.3**	**7.3**	**8.1**	**8.0**	**7.6**	**14.5**	**7.2**	**8.9**	
16:3	0.2	1.0	0.8	0.9	0.8	0.8	1.0	1.0	
18:4	0.3	0.3	0.9	0.8	1.6	0.3	-	0.7	
20:4	1.3	0.5	0.7	1.4	0.6	0.5	0.3	0.3	
20:5	7.3	3.3	2.9	2.4	10.2	2.0	2.0	2.4	
22:5	2.6	0.7	0.7	-	0.8	2.8	0.5	0.9	
22:6	34.9	10.1	4.6	6.3	5.4	5.9	2.9	3.4	
Σ (n-3)	**47.4**	**16.3**	**12.0**	**11.8**	**21.5**	**12.8**	**7.4**	**9.9**	
(n-3)/(n-6)	**6.5**	**2.2**	**1.5**	**1.5**	**2.8**	**0.9**	**1.0**	**1.1**	

Abbreviations: ND - Not done due to insufficient sample. Values are expressed as percent of total fatty acids for one fish. Σ (n-6) PUFA includes 20:2, 20:3, 22:5; Σ (n-3) include 16:4, 18:2, 18:3, where one or more of the eight values is < 0.8. D=day

Table 6.3 (c)

The fatty acid composition of rainbow trout leucocytes and supernatant after culturing *in vitro* with 1 % fish serum.

Fatty acids		Cells				Supernatant			
(% Total FA)	Liver	D=0	D=2	D=4	D=6	D=0	D=2	D=4	D=6
14:0	1.7	4.4	ND	1.3	3.4	1.3	1.5	2.4	1.2
15:0	0.3	1.9		1.4	2.9	0.3	1.3	0.6	0.5
16:0	17.5	27.9		27.4	25.1	20.4	22.1	22.0	27.8
17:0	0.3	0.8		0.9	1.1	0.6	0.7	0.4	0.2
18:0	5.0	8.6		7.8	7.4	3.4	5.3	4.8	4.6
20:0	0.2	1.1		-	1.4	-	0.2	0.3	0.1
22:0	1.1	1.1		-	1.0	-	0.2	0.5	0.3
Σ saturates	**26.2**	**45.8**		**38.8**	**42.3**	**26.0**	**31.3**	**31.0**	**34.7**
14:1	-	1.1		0.7	1.1	-	0.9	0.6	1.2
16:1	2.7	5.7		8.8	9.7	3.1	6.2	5.6	1.8
18:1	11.1	15.7		23.9	13.3	10.3	13.9	15.5	5.8
20:1	2.4	2.9		1.2	2.0	2.5	2.5	0.3	1.6
22:1	-	3.2		1.2	2.0	2.2	1.5	0.5	2.3
24:1	2.9	2.0		1.7	2.2	2.3	1.1	1.6	1.9
Σ monoenes	**19.1**	**30.6**		**37.5**	**30.3**	**20.4**	**26.1**	**24.1**	**14.7**
18:2	1.8	2.3		5.0	4.5	0.4	0.7	3.7	1.1
18:3	0.2	0.4		0.5	0.9	-	2.2	0.1	0.1
20:4	3.6	2.3		0.7	1.5	4.8	1.8	1.9	2.6
Σ (n-6) PUFA	**7.3**	**7.3**		**7.2**	**8.5**	**6.6**	**5.9**	**7.4**	**5.1**
18:3	0.6	0.4		1.3	0.3	0.4	0.5	1.0	0.2
18:4	0.3	0.3		1.2	0.7	0.4	0.3	1.5	0.1
20:4	1.3	0.5		0.7	1.5	2.1	0.5	1.0	0.5
20:5	7.3	3.3		5.0	3.8	9.4	7.3	9.0	8.2
22:5	2.6	0.7		1.3	0.7	2.1	1.8	2.7	1.7
22:6	34.9	10.1		5.5	10.8	31.9	25.4	21.6	34.4
Σ (n-3)	**47.4**	**16.3**		**16.5**	**19.0**	**46.8**	**36.7**	**37.5**	**45.5**
(n-3)/(n-6)	**6.5**	**2.2**	-	**2.3**	**2.2**	**7.1**	**6.2**	**5.1**	**8.9**

Abbreviations: ND - Not done due to insufficient sample. Values are expressed as percent of total fatty acids for one fish. Σ (n-6) PUFA includes 16:2; 20:2; 20:3; 22:2; 22:5; Σ (n-3) include 16:3, 16:4; where one or more of the eight values is < 0.8. D=day

(b) Culturing with FA/BSA complexes:-

The lipid class composition of Atlantic salmon headkidney leucocytes was examined after culturing *in vitro* at 15°C in the presence of different supplemented fatty acid/BSA complexes. Leucocytes, isolated from three fish weighing approximately 30 g which had been maintained at 7 °C, were plated out into 24 well plates at a concentration of 5 $x10^6$ $well^{-1}$ in L-15 with additives, 1 % FCS and 10 μM of 18:1(n-9), 18:2(n-6), 18:3(n-3), 20:4(n-6), 20:5(n-3) or 22:6(n-3). Plates were incubated at 15°C for 7 days in a humidified chamber and cells harvested on day 7. Lipid analysis was preformed as previously described in Section 3.8.2. Lipid class analyses of the sampled leucocytes are presented in Table 6.4 and the fatty acid compositions are shown in Table 6.5.

The lipid class composition of the sampled Atlantic salmon headkidney leucocytes suggested that supplementing the culture medium with 18:1(n-9) fatty acids increased the level of polar lipid within the cellular lipid due to increases in all of the polar lipid classes, particularly the PC and PE fractions which accounted for 29.7 % and 23.5 % of the total lipid, respectively. There was considerable variation within the three fish sampled, presumably due to the small lipid yields obtained from 5 $x10^6$ cells per sample. The fatty acid composition of the leucocytes presented in Table 6.5 also showed large variations between cell samples. However, no significant differences were found between cells incubated with the various fatty acids.

Table 6.4

The lipid class composition of Atlantic salmon leucocytes after culturing *in vitro* with different supplemented fatty acids at 15°C in the presence of fatty acid / BSA complexes.

Lipid class (% Total lipid)	Fatty Acid						
	18:1(n-9)	18:2(n-6)	18:3(n-3)	20:4(n-4)	20:5(n-3)	22:6(n-3)	FCS(10%)
Total polar lipid	73.3 ±12.0	53.7 ±7.5	51.6 ±18.0	57.3 ±3.8	62.1 ±3.5	53.8 ±14.1	52.7 ±13.4
Total neutral lipid	26.7 ±12.0	46.3 ±7.5	48.4 ±18.0	42.7 ±3.8	37.9 ±3.5	46.2 ±14.1	47.3 ±13.4
Polar lipid:							
LPC	-	-	-	-	-	-	-
SM	4.0 ±0.1	-	3.4 ±0.9	2.2 ±1.6	2.5 ±0.5	3.1 ±1.0	3.0 ±0.8
PC	29.7 ±2.4	25.7 ±6.4	23.1 ±7.1	20.5 ±3.7	23.8 ±2.3	22.3 ±4.3	20.6 ±3.4
PS	5.3 ±2.0	3.8 ±0.4	1.6 ±1.2	5.7 ±3.0	2.7 ±0.9	3.5 ±1.5	2.7 ±2.1
PI	7.0 ±3.1	5.9 ±1.1	3.9 ±3.4	2.7 ±0.7	3.0 ±1.3	2.8 ±0.7	3.9 ±0.6
Cardiolipin	3.9 ±2.0	0.9 ±0.8	2.0 ±2.1	0.6 ±0.6	1.3 ±0.4	3.5 ±5.0	3.8 ±2.7
PE	23.5 ±4.0	13.5 ±5.0	15.3 ±4.9	18.4 ±1.6	19.6 ±0.5	14.9 ±10.7	16.0 ±4.1
Sulphatides	-	0.5 ±0.7	1.1 ±0.8	2.0 ±2.9	1.1 ±1.0	1.3 ±1.0	1.2 ±1.0
Cerebrosides	-	3.5 ±2.1	1.2 ±1.3	5.1 ±3.8	8.0 ±1.5	2.3 ±2.3	1.8 ±1.3
Neutral lipid:							
Cholesterol	11.1 ±7.0	21.0 ±1.4	17.8 ±2.5	17.1 ±3.1	14.1 ±5.6	18.0 ±3.8	20.7 ±7.1
Free fatty acids	0.6 ±0.8	4.2 ±0.4	6.0 ±2.4	4.5 ±0.5	4.5 ±1.3	11.9 ±5.1	8.9 ±4.2
Triacylglycerols	0.6 ±0.9	12.6 ±7.2	8.9 ±6.2	9.8 ±3.3	7.6 ±2.3	6.0 ±2.4	12.3 ±3.0
Sterol esters	14.4±4.4	8.5 ±2.0	15.6 ±11.2	11.3 ±5.6	11.8 ±2.2	10.4 ±6.1	5.4 ±3.4
Unknowns	-	-	-	-	-	-	-

Abbreviations: LPC-lysophosphatidylcholine, SM-Sphingomyelin, PC-phosphatidylcholine, PS-phosphatidylserine, PI-phosphatidylinositol, PE-phosphatidylethanolamine.

Values are expressed as the mean percent of total lipid ±SD for three fish.

Table 6.5

The fatty acid composition of Atlantic salmon leucocytes after culturing *in vitro* with different supplemented fatty acids at 15°C

Fatty acids	Fatty Acid						
(% Total FA)	18:1(n-9)	18:2(n-6)	18:3(n-3)	20:4(n-6)	20:5(n-3)	22:6(n-3)	FCS(10%)
14:0	0.6 ±0.1	0.8 ±0.2	0.8 ±0.6	0.8 ±0.2	0.7 ±0.1	0.4 ±0.3	0.7 ±0.5
15:0	1.2 ±0.2	0.9 ±0.4	1.2 ±0.4	1.3 ±0.6	2.1 ±1.5	0.2 ±0.2	0.4 ±0.3
16:0	22.6 ±1.9	23.4 ±0.7	26.4±12.5	24.1 ±1.3	24.8 ±0.9	28.1 ±1.9	25.4 ±4.3
17:0	0.4 ±0.1	0.4 ±0.3	0.4 ±0.1	0.9 ±0.1	0.6 ±0.5	0.3 ±0.2	0.5 ±0.4
18:0	8.5 ±1.7	10.8 ±1.0	8.9 ±6.6	11.7 ±0.9	12.1 ±0.3	14.5 ±1.3	12.7 ±2.7
20:0	0.4 ±0.1	0.5 ±0.1	5.6 ±2.4	0.7 ±0.1	0.7 ±0.2	0.8 ±0.3	1.3 ±0.5
22:0	0.4 ±0.1	0.5 ±0.4	0.5 ±0.2	0.7 ±0.5	0.6 ±0.1	0.2 ±0.3	0.8 ±0.4
24:0	0.8 ±0.2	0.9 ±0.2	0.8 ±0.0	1.5 ±0.3	1.2 ±0.3	1.1 ±0.1	0.8 ±0.1
Σ saturates	**34.9 ±2.0**	**38.2 ±2.6**	**44.7 ±15.8**	**41.6 ±2.3**	**42.8 ±1.8**	**45.5 ±3.0**	**42.7 ±7.3**
14:1	0.7 ±0.2	0.7 ±0.4	0.9 ±0.6	1.1 ±0.6	0.9 ±0.3	0.1 ±0.1	0.4 ±0.3
16:1	3.1 ±0.2	4.3 ±0.5	9.9 ±7.7	3.8 ±0.2	4.3 ±1.3	4.4 ±0.8	3.8 ±2.3
18:1	17.5 ±1.0	15.4 ±0.7	14.3 ±0.9	16.2 ±0.2	14.9 ±0.7	17.6 ±0.8	15.7 ±1.9
20:1	1.5 ±0.6	1.5 ±0.5	1.4 ±0.9	1.3 ±0.4	1.1 ±0.2	1.0 ±0.2	1.3 ±0.5
22:1	0.6 ±0.3	0.8 ±0.8	0.4 ±0.3	0.2 ±0.3	0.8 ±0.4	0.2 ±0.2	0.7 ±0.4
24:1	1.7 ±0.2	2.7 ±1.4	1.9 ±0.7	3.6 ±1.6	0.7 ±0.7	1.6 ±1.1	2.3 ±0.6
Σ monoenes	**25.1 ±1.8**	**25.4 ±1.8**	**28.8 ±7.5**	**26.1 ±0.6**	**22.7 ±1.7**	**24.8 ±1.4**	**24.3 ±32.3**
18:2	4.8 ±1.3	6.8 ±1.9	5.1 ±2.3	7.8 ±4.0	6.9 ±0.7	5.9 ±1.1	4.4 ±1.4
20:3	1.6 ±1.2	0.8 ±0.5	0.5 ±0.5	-	0.4 ±0.0	0.1 ±0.1	0.3 ±0.3
20:4	3.0 ±0.2	2.7 ±0.3	2.1 ±1.1	3.1 ±1.1	2.6 ±0.6	2.8 ±1.2	2.5 ±1.1
Σ (n-6) PUFA	**11.2 ±0.2**	**11.3 ±2.3**	**9.3 ±4.5**	**11.9 ±2.9**	**11.3 ±0.7**	**9.7 ±1.1**	**8.1 ±0.1**
16:3	0.5 ±0.1	0.6 ±0.2	0.6 ±0.2	0.5 ±0.4	1.0 ±0.3	0.4 ±0.4	0.2 ±0.2
18:3	0.6 ±0.1	0.3 ±0.2	1.6 ±0.6	0.8 ±0.6	-	-	0.6 ±0.1
20:5	4.8 ±0.3	4.7 ±0.6	2.6 ±1.2	2.8 ±0.7	5.0 ±0.3	2.8 ±0.9	3.7 ±1.3
22:5	0.8 ±0.1	0.8 ±0.2	0.6 ±0.3	0.9 ±0.3	0.7 ±0.0	0.7 ±0.2	0.9 ±0.3
22:6	21.8 ±2.7	18.5 ±2.0	11.1 ±5.7	15.3 ±4.1	15.6 ±1.4	14.9 ±4.6	19.3 ±8.3
Σ (n-3)	**28.8 ±3.2**	**25.1 ±2.8**	**17.2 ±7.7**	**20.4 ±4.4**	**23.3 ±1.1**	**20.0 ±5.3**	**24.9 ±10.1**
(n-3)/(n-6)	**2.6 ±0.3**	**2.2 ±0.7**	**1.8 ±1.7**	**1.7 ±0.8**	**1.9 ±0.2**	**2.1 ±0.8**	**3.0 ±1.3**

Values are expressed as mean percent of total fatty acids for three fish ±SD. Σ (n-6) PUFA includes 16:2; 18:3; 20:2; 22:2; 22:5; Σ (n-3) include 18:4; 20:4; where one or more of the seven values is < 0.8.

6.3 UPTAKE OF EXOGENOUS ^{14}C-FATTY ACIDS BY ATLANTIC SALMON LEUCOCYTES *IN VITRO*

In the present section, the uptake of ^{14}C labelled exogenous fatty acids by Atlantic salmon leucocytes was examined by incubating the leucocytes *in vitro* with fatty acid at either 4°C or 15°C over a three day or seven day period. Fatty acid uptake by leucocytes in the presence of mitogens and by leucocytes from the blood, spleen, kidney and thymus was also determined.

Unlabelled fatty acids were mixed to include 2 μM of each of the following; 18:1(n-9), 18:2(n-6), 18:3(n-3), 20:4(n-6), and 20:5(n-3). The final concentration of 10 μM consisted of a mixture of each fatty acid with one being a test fatty acid. The test fatty acid (eg. 18:1(n-9)) included a portion of labelled fatty acid (eg. [1-^{14}C]18:1(n-9)), and had a maximum fatty acid concentration of 2 μM. The reason for adding a mixture of fatty acids to the culture medium was to prevent the cells becoming deficient in a particular fatty acid. The amounts of radioactive fatty acid included in each mixture is given in the experimental details. Cells were incubated with the fatty acids in 96 well microtitre plates (200 μl well^{-1}) in a humidified chamber, after which they were harvested onto harvester mats and the [1-^{14}C] fatty acid uptake measured according to Section 3.3.3. The amount of labelled fatty acid taken up by the cell was used to determine how much of the 2 μM solution was incorporated by the cell. The results are expressed in pmoles and include both labelled and unlabelled fatty acid taken up by the cells.

The uptake of exogenous fatty acids by Atlantic salmon and by rainbow trout leucocytes over three days after culturing *in vitro* with the fatty acids at 12°C is shown in Table 6.6. Values for the rainbow trout include a sampling after the first ten minutes. Uptake by rainbow trout over 7 days at 4°C and 15°C is given in Table 6.7.

The greatest incorporation of fatty acids by peripheral blood leucocytes of salmon and trout occurred within the first day of incubation (Table 6.6). Cells from trout generally took up more 18:1(n-9) and 20:4(n-6), followed by 20:5(n-5), 18:2(n-6) and 18:3(n-3). The salmon leucocytes did not appear to incorporated as high amounts of fatty acids as the trout cells, but this may be a consequence of the water temperature at which the salmon had been maintained ((15°C) compared with the trout (4°C)), rather than a species variation. The peak fatty acid incorporation occurred by trout leucocytes around day 2 when incubated at 15°C, but occurred around day 5 when incubated at 4°C (Table 6.7).

The incorporation of fatty acids by Atlantic salmon leucocytes after culturing *in vitro* for 5 days at 4°C and 15°C in the presence of mitogens and fatty acids is presented in Table 6.8. The addition of mitogens to the culture medium appeared to promote the uptake of fatty acids into the cell when they were cultured at 15°C, particularly 18:1(n-9). The reason for such an increase may be two fold. Firstly the cells require fatty acids for cell synthesis during cell division and secondly the fatty acids may have an intrinsic requirement in the mitogenic response. There was very little uptake at 4°C of any fatty acid substrate.

The uptake of ^{14}C fatty acids by Atlantic salmon leucocytes isolated from kidney, spleen, thymus or blood, after culturing *in vitro* for 5 days at 4°C and 15°C or 2 days at 12°C in the presence of ^{14}C substrates is shown in Tables 6.9 a and b respectively. Leucocytes from the kidney and the blood incorporated greater amounts of 20:4(n-6) and 18:1(n-9) fatty acid than those from the thymus and the spleen at all time points and temperatures examined. Leucocytes from all tissues displayed a preference for 20:4(n-6) and 18:1(n-9) fatty acids over the other three fatty acids examined. Incorporation of fatty acids into the various tissue leucocytes was highest on day two of the study at 12°C (Table 6.9b). The reason why very little difference was seen between cells incubated at 4°C and 15°C may be due to cells having acclimated before day 5 the only time point examined (Table 6.9a).

Table 6.6

Uptake of fatty acids (p moles) by Atlantic salmon leucocytes and rainbow trout after culturing *in vitro* for up to three days at 12°C

	Fatty acid				
	18:1(n-9)	18:2(n-6)	18:3(n-3)	20:4(n-6)	20:5(n-3)
Salmon:-					
day 1	334 ±172	440 ±146	226 ±106	518 ±242	298 ±154
day 2	466 ±218	540 ±254	306 ±164	844 ±258	392 ±186
day 3	508 ±232	518 ±184	356 ±166	732 ±274	402 ±188
Trout:-					
10 mins	48 ±12	236 ±80	320 ±24	364 ±8	378 ±38
day 1	1052 ±24	792 ±144	780 ±116	1464 ±128	510 ±2
day 2	1072 ±112	816 ±36	824 ±24	1444 ±36	814 ±18
day 3	1424 ±56	1128 ±84	860 ±20	1360 ±24	964 ±46

Values are expressed as mean pmoles fatty acid ±SD per well, taken up by peripheral blood leucocytes for three fish (5×10^5 cells $well^{-1}$). Salmon had been maintained at a water temperature of 15°C and weighed 35g. The amount of radiolabelled fatty acids in fatty acid mixtures added to the salmon cells were present in wells at; [1-^{14}C]18:1(n-9) 0.3 nmol, [1-^{14}C]18:2(n-6) 0.2 nmol, [1-^{14}C]18:3(n-3) 0.2 nmol, [1-^{14}C]20:4(n-6) 0.2nmol and [1-^{14}C]20:5(n-3) 0.3 nmol respectively. Trout had been maintained at a water temperature of 6°C and weighed 150g. The amount of radiolabelled fatty acids in fatty acid mixtures added to the trout cells were present in wells at; [1-^{14}C]18:1(n-9) 0.1 nmol, [1-^{14}C]18:2(n-6) 0.1 nmol, [1-^{14}C]18:3(n-3) 0.1 nmol, [1-^{14}C]20:4(n-6) 0.1 nmol and [1-^{14}C]20:5(n-3) 0.15 nmol respectively.

Table 6.7

Uptake of fatty acids (p moles) by rainbow trout leucocytes after culturing *in vitro* for up to 7 days, at 4°C and 15°C

	Fatty acid				
	18:1(n-9)	18:2(n-6)	18:3(n-3)	20:4(n-6)	20:5(n-3)
4°C					
7 hours	1224 ±272	394 ±108	332 ±108	466 ±66	346 ±120
day 1	1056 ±280	480 ±86	188 ±52	360 ±33	226 ±40
day 2	1064 ±104	412 ±74	320 ±52	614 ±80	680 ±140
day 3	1472 ±134	538 ±98	406 ±80	690 ±240	694 ±83
day 5	1472 ±80	766 ±86	572 ±126	1200 ±146	980 ±186
day 7	1408 ±152	594 ±120	452 ±102	960 ±174	814 ±320
15°C					
7 hours	1368 ±208	372 ±156	488 ±64	680 ±174	526 ±86
day 1	1048 ±40	488 ±144	288 ±56	466 ±134	466 ±352
day 2	1464 ±136	752 ±136	568 ±112	786 ±94	694 ±86
day 3	1120 ±32	616 ±46	472 ±80	680 ±80	540 ±34
day 5	1056 ±48	412 ±40	432 ±32	654 ±80	374 ±54
day 7	1008 ±48	544 ±80	464 ±62	640 ±120	566 ±74

Values are expressed as mean pmol fatty acid taken up by headkidney leucocytes per well for three fish ±SD. Cells were plated out at 2 x10^5 $well^{-1}$. Fish had been maintained at a water temperature of 8°C and weighed 30g. The amount of radiolabelled fatty acids in fatty acid mixtures were present in wells at; [1-^{14}C]18:1(n-9) 0.05 nmol, [1-^{14}C]18:2(n-6) 0.07 nmol, [1-^{14}C]18:3(n-3) 0.07 nmol, [1-^{14}C]20:4(n-6) 0.03 nmol and [1-^{14}C]20:5(n-3) 0.06 nmol respectively.

Table 6.8

Effects of the presence of mitogens on uptake of fatty acids (p moles) by Atlantic salmon leucocytes after culturing *in vitro* for 5 days, at 4°C and 15°C.

	Fatty acids				
	18:1(n-9)	18:2(n-6)	18:3(n-3)	20:4(n-6)	20:5(n-3)
15°C					
No mitogen	1624 ±504	400 ±6	512 ±28	1534 ±346	584 ±8
LPS	2480 ±56	538 ±54	720 ±152	1334 ±186	796 ±32
Con A	4472 ±1064	680 ±86	720 ±152	1480 ±80	960 ±180
4°C					
No mitogen	880 ±192	326 ±10	280 ±8	666 ±20	548 ±80
LPS	904 ±8	518 ±62	512 ±132	1092 ±80	392 ±40
Con A	592 ±72	430 ±14	548 ±144	866 ±134	608 ±80

Values are expressed as mean pmol fatty acid taken up by peripheral blood leucocytes ±SD for three fish. Fish had been maintained at a water temperature of 12°C and weighed 30g. Cells were plated out at 1 $x10^5$ $well^{-1}$. The amount of radiolabelled fatty acids in fatty acid mixtures were present in wells at; [1-^{14}C]18:1(n-9) 0.05 nmol, [1-^{14}C]18:2(n-6) 0.15 nmol, [1-^{14}C]18:3(n-3) 0.1 nmol, [1-^{14}C]20:4(n-6) 0.03 nmol and [1-^{14}C]20:5(n-3) 0.1 nmol respectively.

Con A was used at a concentration of 25 μg ml^{-1} and LPS at 100 μg ml^{-1}

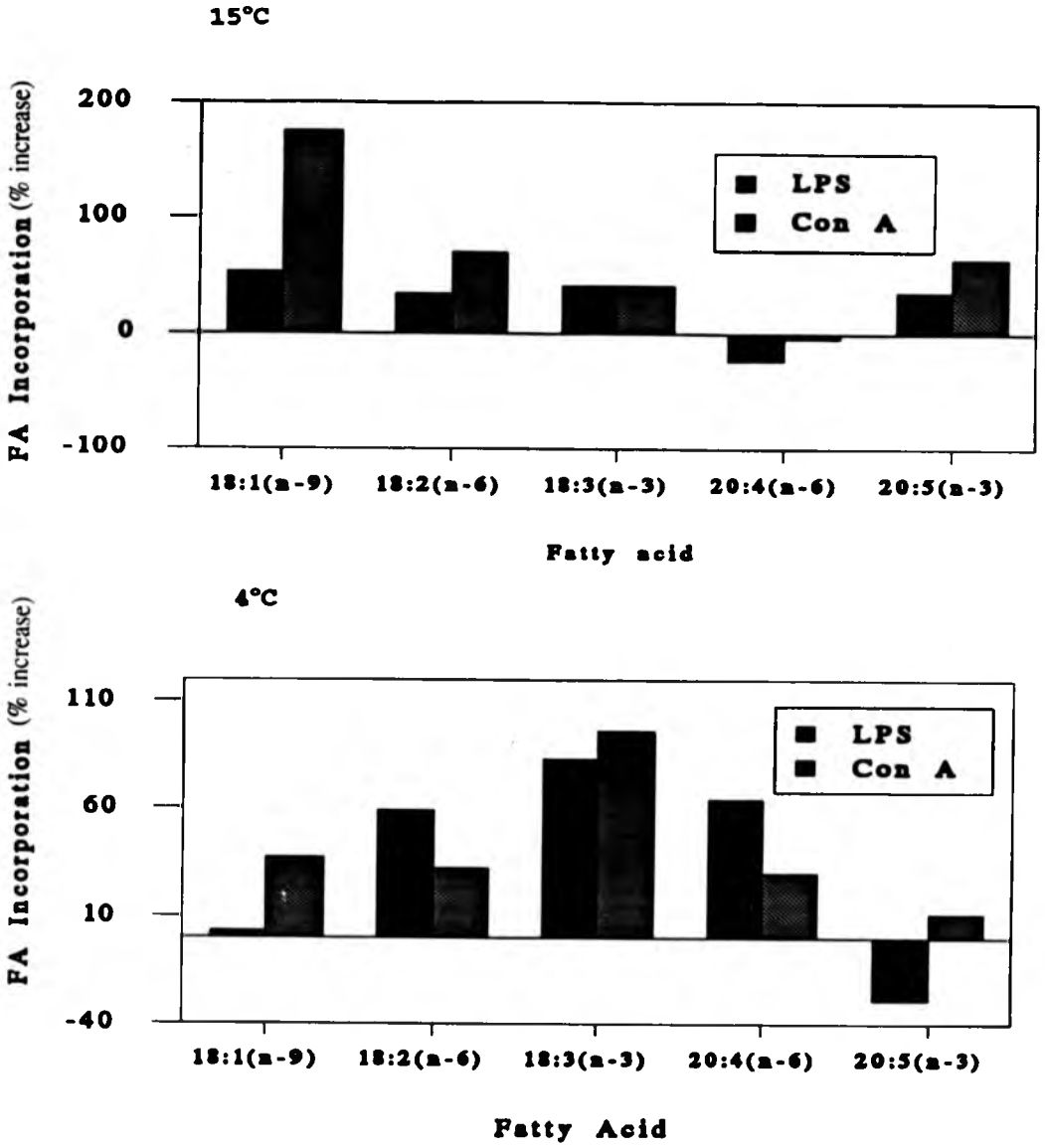


Figure 6.1

Effects of the presence of mitogens on fatty acid incorporated by Atlantic salmon leucocytes after *in vitro* culturing for 5 days, at 4°C and 15°C.

Fatty acid uptake in the absence of mitogen was taken to be 100 % incorporation

Table 6.9 (a)

Uptake of fatty acids (p moles) by Atlantic salmon leucocytes isolated from kidney, spleen, thymus or blood, after culturing *in vitro* for 5 days, at 4°C and 15°C

	Fatty acids				
	18:1(n-9)	18:2(n-6)	18:3(n-3)	20:4(n-6)	20:5(n-3)
4°C					
Spleen	616 ±40	176 ±2	236 ±36	173 ±20	184 ±48
Thymus	672 ±124	240 ±24	220 ±32	120 ±27	220 ±100
Kidney	840 ±88	334 ±56	376 ±48	440 ±40	512 ±40
Blood	848 ±72	208 ±2	224 ±96	213 ±40	268 ±44
15°C					
Spleen	640 ±112	166 ±6	184 ±48	334 ±14	332 ±76
Thymus	640 ±16	190 ±2	156 ±32	240 ±14	184 ±12
Kidney	872 ±40	352 ±4	380 ±76	786 ±94	688 ±40
Blood	832 ±144	198 ±6	204 ±16	400 ±66	328 ±84

Values are expressed as mean pmol fatty acid ±SD per well, taken up by leucocytes for three fish. Cells were plated out at 1 $x10^5$ $well^{-1}$. Fish had been maintained at a water temperature of 12°C and weighed 30g. The amount of radiolabelled fatty acids in fatty acid mixtures were present in wells at; [1-^{14}C]18:1(n-9) 0.05 nmol, [1-^{14}C]18:2(n-6) 0.15 nmol, [1-^{14}C]18:3(n-3) 0.1 nmol, [1-^{14}C]20:4(n-6) 0.03 nmol and [1-^{14}C]20:5(n-3) 0.1 nmol respectively.

Table 6.9 (b)

Uptake of fatty acids (p moles) by Atlantic salmon leucocytes isolated from kidney, spleen, thymus or blood, after culturing *in vitro* for 2 days at 12°C

	Fatty acids				
	18:1(n-9)	18:2(n-6)	18:3(n-3)	20:4(n-6)	20:5(n-3)
7 hrs					
Spleen	680 ±54	560 ±10	400 ±14	660 ±1	240 ±10
Kidney	706 ±26	510 ±10	454 ±14	820 ±1	420 ±10
Thymus	640 ±54	540 ±80	200 ±14	640 ±2	340 ±60
Blood	666 ±0	640 ±10	426 ±66	600 ±2	320 ±30
Day 1					
Spleen	746 ±54	560 ±40	346 ±54	1220 ±80	540 ±60
Kidney	1120 ±94	900 ±40	626 ±40	2080 ±80	1070 ±70
Thymus	534 ±106	480 ±100	320 ±56	920 ±220	470 ±110
Blood	866 ±80	680 ±70	614 ±26	1320 ±200	640 ±120
Day 2					
Spleen	946 ±94	750 ±70	254 ±80	820 ±80	540 ±70
Kidney	1400 ±346	1070 ±80	786 ±66	2080 ±80	1070 ±70
Thymus	746 ±80	600 ±30	440 ±66	1400 ±320	670 ±130
Blood	1146 ±106	950 ±80	746 ±66	1640 ±80	890 ±120

Values are expressed as mean pmol fatty acid ±SD per well, taken up by leucocytes for three fish. Cells were plated out at 1 $x10^5$ $well^{-1}$. Fish had been maintained at a water temperature of 6.5°C and weighed 30g. The amount of radiolabelled fatty acids in fatty acid mixtures were present in wells at; [1-^{14}C]18:1(n-9) 0.03 nmol, [1-^{14}C]18:2(n-6) 0.04 nmol, [1-^{14}C]18:3(n-3) 0.03 nmol, [1-^{14}C]20:4(n-6) 0.02 nmol and [1-^{14}C]20:5(n-3) 0.04 nmol respectively.

The uptake of exogenous fatty acids into the lipid classes of Atlantic salmon leucocytes in the presence and absence of mitogens was examined by incubating the headkidney leucocytes at 4°C and 15°C with the fatty acid mixtures described at the beginning of Section 6.3. These contained a 10 μM solution of fatty acids, i.e. a 2 μM solution of each fatty acid (18:1(n-9); 18:2(n-6); 18:3(n-3); 20:4(n-6); 20:5(n-3)) in which a portion of the test fatty acid was ^{14}C labelled. The amount of each labelled fatty acid added to the wells was [1-^{14}C]18:1(n-9) 0.22 nmol, [1-^{14}C]18:2(n-6) 0.43 nmol, [1-^{14}C]18:3(n-3) 0.22 nmol, [1-^{14}C]20:4(n-6) 0.08 nmol and [1-^{14}C]20:5(n-3) 0.27 nmol respectively. Headkidney and peripheral blood leucocytes were isolated from three 35 g Atlantic salmon which had been maintained at a water temperature of 12°C. They were then pooled from each fish and plated out in to 24 well plates at a concentration of 0.5 x10^7 cells well^{-1}. Con A was used at a concentration of 25 μg ml^{-1} and LPS at 100 μg ml^{-1}. Cells were incubated in a humidified atmosphere for 5 days before harvesting for lipid analysis. Methods for lipid analysis and autoradiography were discussed previously in Section 3.8.3. Autoradiographic development of ^{14}C labelled total lipid extracts of Atlantic salmon leucocytes after culturing *in vitro* for 5 days, at 4°C and 15°C, in the presence of ^{14}C labelled fatty acids and mitogens are illustrated in Plates 6.1 (a and b). Results in Table 6.10 are expressed as pmol fatty acids taken up by the cells (0.5 x10^7 cells well^{-1}).

No conclusions could be drawn as to the effects of mitogen on fatty acid uptake although certain trends were apparent in the distribution of incorporated fatty acids in lipid classes. Thus, PC was heavily labelled by all ^{14}C fatty acids in lipid classes at 4°C and 15°C, particularly the former in the presence of LPS. TAG was also heavily

Table 6.10 (a)

Effect of LPS on the uptake of ^{14}C-fatty acids (p moles) into the lipid classes of Atlantic salmon leucocytes after culturing *in vitro* for 5 days, at 4°C and 15°C

	Fatty acids									
	18:1(n-9)		18:2(n-6)		18:3(n-3)		20:4(n-6)		20:5(n-3)	
4°C	-	+	-	+	-	+	-	+	-	+
SM	168	95	51	112	36	91	100	100	85	56
PC	1618	1727	674	1046	818	1227	450	488	1596	963
PS	95	136	49	70	64	132	175	150	81	233
PI	300	118	74	60	59	64	800	400	226	137
Cardiolipin	150	82	91	153	132	174	125	88	93	37
PE	445	291	326	419	164	818	250	212	296	137
Cerebrosides	291	123	93	5	104	118	262	188	189	144
MAG	864	91	98	114	191	81	575	300	481	85
DAG	864	91	165	91	195	59	762	300	359	63
FFA	2182	318	720	395	818	273	2125	575	1759	159
TAG	2909	132	350	140	327	50	3750	2875	556	63
15°C										
SM	364	27	121	46	86	27	300	75	41	26
PC	304	118	112	256	1545	100	1250	175	667	89
PS	291	23	5	33	164	27	350	75	107	15
PI	314	23	105	40	118	18	788	62	89	11
Cardiolipin	273	27	84	28	100	18	912	112	130	22
PE	727	50	279	98	404	36	662	88	115	78
Cerebrosides	323	45	123	74	263	32	500	138	126	70
MAG	682	36	219	98	245	32	525	125	104	52
DAG	682	36	302	65	209	36	650	75	204	56
FFA	2045	363	837	56	909	27	2250	62	704	329
TAG	1045	262	581	256	204	114	1875	800	518	267

In the presence (+) and absence of LPS (100 μg ml^{-1})(-)
Values are expressed as mean as pmol fatty acid taken up by lipid classes for one fish.

Table 6.10 (b)

Effect of Con A on the uptake of ^{14}C-fatty acids (p moles) into lipid classes of Atlantic salmon leucocytes after culturing *in vitro* for 5 days, at 15°C

	Fatty acids									
	18:1(n-9)		18:2(n-6)		18:3(n-3)		20:4(n-6)		20:5(n-3)	
15°C	-	+	-	+	-	+	-	+	-	+
SM	364	218	121	44	86	64	300	188	41	74
PC	304	3768	112	581	1545	500	1250	362	667	630
PS	291	14	5	81	164	141	350	312	107	267
PI	314	286	105	65	118	77	788	388	89	130
Cardiolipin	273	114	84	28	100	86	912	400	130	89
PE	727	499	279	232	404	209	662	212	115	104
Cerebrosides	323	182	123	49	263	109	500	725	126	204
MAG	682	286	219	81	245	91	525	262	104	244
DAG	682	586	302	100	209	109	650	550	204	100
FFA	2045	2102	837	326	909	268	2250	712	704	170
TAG	1045	427	581	193	204	118	1875	3250	518	207

In the presence (+) and absence of Con A (25 μg ml^{-1}) (-)
Values are expressed as mean as nmol fatty acid taken up by lipid classes for one fish.

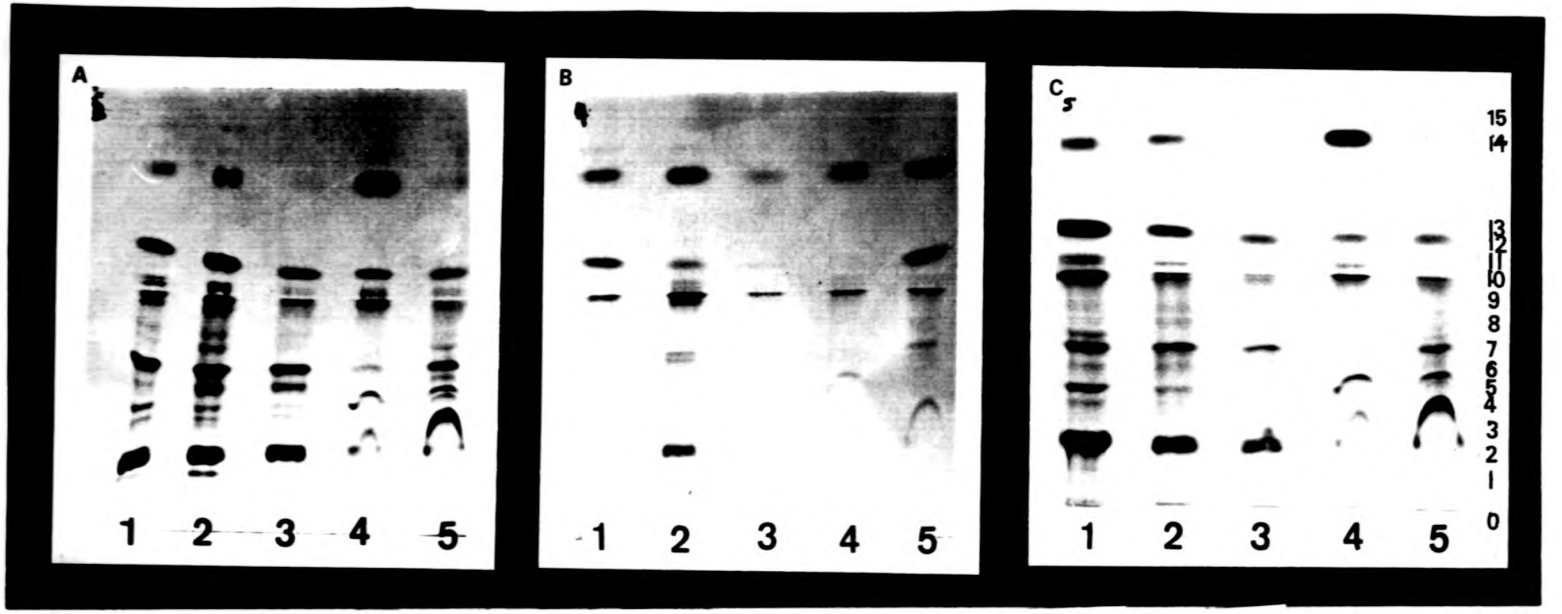
Band

15-sterol esters
14-triacylglycerol
13-free fatty acids
12-diacylglycerol
11-monoacylglycerol
10-solvent front
9-cerebrosides
8-sulphatides
7-phosphatidylethanolamine
6-cardiolipin
5-phosphatidylinositol
4-phosphatidylserine
3-phosphatidylcholine
2-sphingomyelin
1-lysophosphatidylcholine
0-origin

Lane

1-18:1(n-9)
2-18:2(n-6)
3-18:3(n-3)
4-20:4(n-6)
5-20:5(n-3)

Plate 6.1 (a) Autoradiographic development of ^{14}C labelled total lipid extracts of Atlantic salmon leucocytes after culturing *in vitro* for 5 days, at 4°C and 15°C, in the presence of ^{14}C labelled fatty acids (a) 4°C, (b)15°C



Band

0-origin
1-sphingomyelin
2-phosphatidylcholine
3-phosphatidylserine
4-phosphatidylinositol
5-cardiolipin
6-phosphatidylethanolamine
7-sulphatides
8-cerebrosides
9-solvent front
14-sterol esters
13-triacylglycerol
12-free fatty acids
11-diacylglycerol
10-monoacylglycerol

Lane

1-18:1(n-9)
2-18:2(n-6)
3-18:3(n-3)
4-20:4(n-6)
5-20:5(n-3)

Plate 6.1 (b)

Autoradiographic development of ^{14}C labelled total lipid extracts of Atlantic salmon leucocytes after culturing *in vitro* for 5 days, at 4°C and 15°C, in the presence of ^{14}C labelled fatty acids and mitogens (a) 4°C in the presence of LPS, (b) 15°C in the presence of LPS, (c) 15°C in the presence of Con A.

labelled by 18:1(n-9) and 20:4(n-6).

6.4 EFFECTS OF EXOGENOUS FATTY ACIDS ON THE IMMUNE RESPONSE OF ATLANTIC SALMON LEUCOCYTES *IN VITRO*

As well as examining the incorporation of fatty acids into Atlantic salmon leucocytes, an attempt was made to demonstrate that the addition of certain exogenous fatty acids, supplemented in the culture medium, could either enhance or suppress the immune response of the cells. Several immunological assays were investigated, but inconsistent responses by the salmon leucocytes meant that any differences due to a particular fatty acid could not be confirmed.

Immunological assays used in the investigation of exogenous fatty acids on the Atlantic salmon immune response included mitogen and antigen induced proliferation. Bly *et al.* (1990) showed that Con A responses of catfish leucocytes were enhanced by 18:1(n-9). It was therefore anticipated that exogenous fatty acids could increase or decrease the mitogenic response of Atlantic salmon leucocytes. Unfortunately, stimulation indices obtained were general below 2, and in the case of Con A, suppression was sometimes observed (i.e. stimulation indices below 1). Other assays carried out on leucocytes and macrophages previously incubated with fatty acid/BSA complexes for three days included *in vitro* vaccination, (measuring the response by PFCs and antibody production), phagocytosis and respiratory burst enzyme activity, but results were inconclusive and these studies require further investigation. It was observed that incubation of macrophages with different fatty acids affected their spreading and adherence.

6.5 CHAPTER SUMMARY

In summary it was shown in Chapter 6 that:-

(a) Atlantic salmon headkidney leucocytes could be maintained in culture for up to seven days at higher concentrations of exogenous 18:3(n-3) and 20:5(n-3) than 18:1(n-9), 18:2(n-6), 20:4(n-6) and 22:6(n-3). On the basis of these results, fatty acids were supplemented to cultures at 10 μM. Incubations with fatty acids greater than 20 μM resulted in a visible accumulation of lipid within the cells in the form of cytoplasmic lipid droplets.

(b) Trout leucocyte lipid had a very similar composition to FCS prior to culturing with the serum and were therefore not influenced by the fatty acid composition of the serum. Although fish serum contained high levels of 20:5(n-3) and 22:6(n-3), the fish leucocytes did not take these fatty acids up into their lipids.

(c) Greatest fatty acid incorporation by the lipid of fish peripheral blood leucocytes occurred within the first day of incubation. Cells generally took up more 18:1(n-9) and 20:4(n-6), followed by 20:5(n-5), 18:2(n-6) and 18:3(n-3).

(d) Fatty acid incorporation by rainbow trout leucocytes peaked around day 2 when incubated at 15°C, but around day 5 when incubated at 4°C.

(e) The addition of mitogens to the culture medium appeared to promote an uptake of fatty acids into peripheral blood leucocytes when they are cultured at 15°C, particularly

18:1(n-9).

(f) Leucocytes isolated from kidney and blood of salmon incorporated greater amounts of 20:4(n-6) and 18:1(n-9) fatty acid than those from the thymus and the spleen, but leucocytes from all of these tissues displayed a preference for 20:4(n-6) and 18:1(n-9) fatty acids over the other three fatty acids.

(g) No conclusions could be drawn as to the effects of mitogen on fatty acid uptake into individual lipid classes, although certain trends were apparent in the distribution of incorporated fatty acids in lipid classes. Thus, PC was heavily labelled by all ^{14}C fatty acids in lipid classes at 4°C and 15°C, particularly the former in the presence of LPS. TAG was also heavily labelled by 18:1(n-9) and 20:4(n-6).

CHAPTER 7 GENERAL DISCUSSION

7.1 LIPID COMPOSITION OF IMMUNOCOMPETENT CELLS AND TISSUES OF ATLANTIC SALMON

Little information is available on the lipid composition of immunocompetent cells and tissues in Atlantic salmon, see below, although during the course of this project a few reports have been published on closely related aspects or component cells, (Pettitt, et al., 1989a; Pettitt and Rowley, 1990; Bell *et al.*, 1991a; Bell *et al.*, in press).

The extent to which lipid analysis could be carried out in the above studies was limited by the availability of lipid sample. The main source of leucocytes was the blood of salmon parr, with a 25 g fish yielding 0.2 - 0.3 ml of blood with a cell count of around 2×10^7 ml^{-1}. The weight of lipid obtained from the number of leucocytes analysed routinely was less than one mg. Problems, such as increased amounts of artifacts from solvent residues, were incurred in the analysis of these very small lipid samples, but all measurements of fatty acid composition were corrected for such impurities. The lipid content of the Atlantic salmon erythrocytes in this study was found to be 0.92 ±0.6 $\mu g/10^6$ cells from blood with a cell count of 1.0 - 1.4 $x10^9$ cells ml^{-1}. The lipid content of the erythrocyte membranes for several fish species, measured by Bolis and Fange (1979) ranged from 1.9 - 3.1 $mgml^{-1}$, but the cited values were not related to cell number. The lipid of mature mammalian erythrocytes is found mainly in the plasma membrane where it accounts for around 50 % of the membrane mass (Shohet, 1977) and the quantity of lipid extracted from whole mammalian erythrocytes ranges from 0.26 - 0.48 $\mu g/10^6$ cells, depending on the species (Nelson, 1972). Fish erythrocytes differ

from those of mammals in being both larger and nucleated. The extent to which this morphological difference influences the lipid profile of the erythrocyte is unknown, but may explain why larger lipid yields were obtained with salmon erythrocytes. The population of Atlantic salmon erythrocytes used for lipid analysis presumably consisted of a mixture of cells at different stages of development, including juvenile, developing, mature and post mature cells. The extent to which erythrocyte maturity influenced their lipid composition is again unknown.

The lipid compositions of Atlantic salmon and mammalian erythrocytes differ both in the total amount of lipids, and the percentages of the component lipid classes. In mammals, the lipid comprises almost equal proportions of phospholipids and unesterified cholesterol (Sweeley and Dawson, 1969; Quinn, 1976), with the phospholipid classes PC, PE and SM each contributing around 25 % of the total phospholipid and PS 15 % (Shohet, 1977). The phospholipids of the Atlantic salmon made up around 70 - 75 % of the total erythrocyte lipid, while the neutral lipids, consisting mainly of cholesterol, represented the remainder. The values presented in this study are similar to those cited by Bolis and Fange (1979), who examined lipid classes from a variety of fish erythrocyte membranes. Erythrocyte lipid in the Atlantic salmon, as shown in this study, contained much higher proportions of PC and lower proportions of SM than those of the mammalian erythrocyte, confirming the findings of Quinn (1976).

The fatty acids in the lipids of erythrocytes from Atlantic salmon contained longer chain polyunsaturated fatty acids than those of mammals, with over half of the fatty acids

being either C20 and C22, although there was also a high presence of the fatty acid 16:0. Human erythrocytes, by contrast, have a predominance of 16:0, 18:0, 18:1(n-9), 18:2(n-6) and 20:2(n-6) fatty acids in their membrane lipids (De Gier and Van Deenan, 1964). The presence of high (n-3) PUFA in the lipid of salmon erythrocytes, as explained in Chapter 2, is directly related to their availability in dietary lipid, but may also be involved in the cells ability to adapt to environmental changes such as temperature or salinity. The fatty acid composition of fish erythrocyte lipid in relation to temperature is discussed below, though Boris and Fange (1979) showed that there was no correlation between the fatty acid composition of fish erythrocytes and water salinity.

There are marked differences between the fatty acid content of erythrocyte lipid classes of the Atlantic salmon observed in the present study and those reported for the marine cod (Lie *et al.*, 1989b), particularly in the level of PC. The PI portion for both species was typically higher in 18:0 and 20:4(n-6) fatty acids than the other lipid classes, but the PI of cod erythrocyte contained firstly, 20:5(n-3) fatty acid levels which were higher than 20:4(n-6) levels, and secondly, much higher 20:5(n-3) levels than were found in the PI fraction of salmon erythrocyte lipid. The fatty acid 20:4(n-6) is usually cited as being present in much higher levels in the PI fraction than 20:5(n-3) in freshwater fish (Henderson and Tocher, 1987). The fatty acids 20:4(n-6) and 20:5(n-3) are both important precursors of eicosanoid production. The higher levels of 20:5(n-3) which were observed in the PI of cod erythrocyte lipid, compared to the 20:4(n-6) levels, suggests that the 20:5(n-3) fatty acid may produce the more predominant eicosanoid series in cod, since this phospholipid has been implicated as a source of PUFA for

eicosanoid formation (Berridge, 1984). Dimethyl acetals, which are derived from plasmalogen forms of PE, were also found in cod and salmon erythrocyte lipid.

Leucocytes in the present study, isolated from fish peripheral blood, were mixed populations of thrombocytes, lymphocytes and polymorphonuclear cells, but attempts to separate these populations would have resulted in lipid samples too small to analyze. Thrombocytes made up around 35 % of the peripheral blood leucocyte count (Table 5.3).

The percentage contribution of phospholipids to the total lipid of salmon leucocytes (around 35 %) corresponds well with values found for human lymphocytes, although the total lipid content of the fish leucocytes was notably higher than that of mammalian leucocytes (0.4 mg/ 1 $x10^7$ cells in salmon vs. 0.1 mg/ 1 $x10^7$ mammalian cells) (Gottfried, 1972). Salmon leucocytes also contained 20 - 40 times more lipid than salmon erythrocytes. The dominant lipid classes in the Atlantic salmon erythrocytes were PC, PE and cholesterol, while the PS and PI fractions were higher than those found in the salmon leucocyte lipid. The lipid of the salmon leucocytes, on the other hand, bore similarities to mammalian lymphocytes, with higher levels of glycolipids, free fatty acids, TAGs and sterol esters (Gottfried, 1972). Mammalian leucocytes resemble other mammalian cells in their lipid class profiles, except for large proportions of glycolipids (up to 18 %) (Gottfried, 1972). These are believed to play a role related to immunological function (Gottfried, 1972).

A good correlation exists between the levels of free fatty acids and cholesterol within

the lymphoid cells (Kigoshi *et al.*, 1976), and this may suggest that cholesterol esters are the main source of free fatty acids in these cells, although cholesterol esters are less likely to undergo hydrolysis than the TAGs (Meade and Mertin, 1978), which comprise around 20 % of the total lipid of mammalian leucocytes (Gottfried, 1972). TAG levels contributed up to 25 % of the total lipid of Atlantic salmon leucocytes, whereas the sterol ester fraction represented up to 35 % of the total lipid. However, while the proportions of TAG in the lipid of Atlantic salmon leucocytes in this study agree with those of mammals, variations in these levels were obtained over the course of the work. In most animals, lipid energy reserves are stored within the cell as TAG. The proportions of the TAG in fish lipids have been observed to decrease when fish are starved, for example when fish are maintained at a low water temperature and have a lower food consumption (Henderson and Tocher, 1987), and this may also explain the variations observed in the TAG levels of leucocyte lipid. Another answer may be that the leucocytes utilize the energy stores during cellular function, or free fatty acids may be released from the TAG fraction for use in cellular function. This in turn may explain why high free fatty acid levels are observed in extracts of leucocyte lipid. Free fatty acids may simply have been cleaved from the TAG of leucocyte lipid during the storage and extraction procedure, as a result of more active lipases than seen in erythrocytes. If the fatty acids do have a role in cell activity, as speculated by Hwang (1989), it would be expected to find a greater quantity and/or more active lipases in the cells as a result. Although reports by Resch *et al.*, (1971) suggest that lymphocytes are not rich in phospholipases and nothing is known of their content of neutral lipases, leucocytes may nevertheless require greater precautions than were used to prevent lipase activity during lipid extraction. In this study, both erythrocytes and leucocytes were stored and

extracted under the same conditions.

The levels of free fatty acids in mammalian leucocytes are thought to have a number of possible roles; (1) they may exert a direct cytotoxic effect during the immune response, (2) they may act as signals for macrophage and lymphocyte interactions or (3) they may serve as precursors of eicosanoid production, especially by macrophages (Hwang, 1989). The phospholipids of mammalian lymphocytes contain 20 % 20:4(n-6) in their total lipid, the main eicosanoid precursor in mammals (Stossel *et al*, 1974). In salmon leucocyte lipid 20:4(n-6) contributed only 2.5 % to the total fatty acid content and 20:5(n-3), another eicosanoid precursor, comprised 3.6 %.

Of the total leucocyte lipid, ≈30 % was phospholipid and ≈70 % was neutral lipid, in agreement with the findings of Pettitt and Rowley, (1990). Neutral lipids in fish generally have a greater saturated fatty acid content compared to the phospholipids, but contain a lower percentage of PUFA (Henderson and Tocher, 1987). In keeping with this pattern, the lipid of salmon leucocytes had a higher neutral lipid fraction than erythrocytes and the leucocytes had an overall lower level of PUFA in their lipids.

In both the lipid of erythrocytes and leucocytes from salmon, PE contained the highest overall degree of unsaturation, but because it also had a higher (n-6) PUFA level than that of the PC lipid class, its (n-3)/(n-6) ratio was lower than the reported PC fraction. This shows that care should be taken when interpreting (n-3/n-6) ratios between samples.

Lipid yields obtained for the Atlantic salmon serum (21.8 mg ml^{-1}) were in close agreement with those obtained for rainbow trout (Leger *et al.*, 1981). Fish serum has a high lipid content, around six times higher than that documented for man (Delattre, 1976). Analysis of serum lipid classes in the salmon showed them to be high in proportions of PC, TAG and sterol esters, but low in proportions of PE and PS. Erythrocytes do not syntheses their own lipid, but derive it from the plasma (see below). Although the fatty acid profiles from the lipid of erythrocytes and serum of Atlantic salmon were very similar, the lipid of erythrocytes contained lower 18:1(n-9) fatty acid levels, higher 20:4(n-6) levels and much higher levels of 22:6(n-3) fatty acids, showing perhaps a preferential uptake of the latter two fatty acids.

The present study demonstrated that Atlantic salmon macrophages contained higher proportions of polar lipid than headkidney lymphocytes, due to high levels of PC and PE lipid classes. Macrophages contain a large quantity of endoplasmic reticulum and most of the membranes of phagocytic vesicles are actually derived from the plasma membrane (Meade and Mertin, 1978). The contribution of polar lipid in these cells may have been due simply to a greater membrane content and thus phospholipid content. The total phospholipid fraction of mammalian macrophages contains 20 % of its fatty acids as 20:4(n-6) (Mason *et al*, 1972). Total lipid from Atlantic salmon macrophages was analysed rather than the phospholipids in this study, but it was apparent that it did not contain such high proportions of 20:4(n-6), only 0.7 % (equivalent to only 1.7 % in total lipid). The total lipid of these cells contained 2.8 % 20:5(n-3) PUFA (equivalent to 4.8 % in total lipid). Only small differences are found in the lipid composition of mammalian granulocytes, macrophages and lymphocytes (Gottfried,

1972). The fatty acid profiles of the salmon total headkidney leucocyte lipid were similar, with little difference in the fatty acid profiles of T and B cells. Likewise, channel catfish T and B cells do not differ in their phospholipid fatty acid composition (Bly *et al.*, 1986b), in contrast to the phospholipids of mouse T cells which are richer in 20:1 fatty acid than B-cells. These in turn contain more 16:0, 16:1 and 18:2(n-6) than the T-lymphocytes (Buttke *et al.*, 1985).

It was not surprising to find that there was little difference in the lipid composition between trout and Atlantic salmon erythrocytes or leucocytes, especially since they had been maintained at the same water temperature and on similar diets. The diet of the trout contained slightly more (n-6) PUFA and slightly less (n-3) PUFA. While the lipid class composition of their erythrocytes was very similar overall, the lipid of leucocytes from the salmon did contain more sterol esters and the PE levels in trout leucocyte lipid were higher than in the salmon, resulting in an overall higher phospholipid level in the former species. The fatty acid composition of erythrocytes from the two species were again very similar, but the lipid of trout leucocytes contained more monoenoic fatty acids and less saturated fatty acids.

Results obtained from the lipid analysis of rainbow trout leucocytes in the present study do not correspond to those of Pettitt and Rowley, (1990), who found that the neutral lipid of rainbow trout leucocytes was composed of 25 % TAG, 15 % cholesterol and 52 % sterol esters, while PC, PE and PS all contributed 30 % to the total phospholipids. Differences in analytical techniques between the two studies may explain the variation in the results. They had previously reported the levels of 20:4(n-6), 20:5(n-3) and

22:6(n-3) as 6 %, 5 % and 40 % respectively in the total lipid of rainbow trout leucocytes (Pettitt and Rowley, 1989). Corresponding values of these fatty acids in the total lipid of rainbow trout leucocytes from the present study were 4 %, 3 % and 16 % for 20:4(n-6), 20:5(n-3) and 22:6(n-3). The analysis of Pettit and Rowley (1989) therefore showed rainbow trout leucocytes to have much higher levels of 22:6(n-3) in their lipid than found in the current study. It is unlikely that the 22:6(n-3) levels in their study were the result of erythrocyte contamination, since erythrocytes were present at $\leq$ 1 % of the leucocyte population.

The trout and African catfish used in the present study had both been maintained on the same diet, but had been held at different temperatures. African catfish erythrocytes contained more 18:0, 18:1(n-9), 18:2(n-6) and more 20:4(n-6) in their total lipid than the trout erythrocytes. They also had a significantly higher level of 20:5(n-3), but a much lower 22:6(n-3) level than the trout erythrocyte lipid. Differences in the erythrocyte fatty acids indicate that the cells of the two species select different fatty acids, either by lipid uptake from the plasma or during cell formation. Likewise, the lipid of African catfish leucocytes contained more 16:0, 18:2(n-6), 20:5(n-3) and less 18:1(n-9) and 22:6(n-3) than found in the total lipid of trout leucocytes. Some of these observed differences between the two species may have been related to differences in temperature.

The study of the lipid composition of erythrocytes and leucocytes from Atlantic salmon, African catfish and rabbit emphasized the differences between mammalian and fish erythrocytes described above. The lipid content of the rabbit erythrocytes was typical

of other mammalian erythrocyte values, containing much higher SM levels than the fish erythrocytes, equal quantities of SM, PC and PE lipid classes and a high cholesterol level (Shohet, 1977). The lipid classes of the leucocytes were generally similar between fish and rabbit except that rabbit leucocytes contained very high cholesterol levels, even higher than documented for mammalian leucocytes and it was suggested that this may have been an experimental irregularity. Both fish species contained higher sterol ester levels in their leucocyte lipid than the rabbit.

Rabbit erythrocytes contained higher levels of SFA than those of the fish, due to their high 16:0 and 18:0 fatty acid contents, and contained high 18:2(n-6) levels - a reflection of dietary fatty acids. These cells contained very little (n-3) PUFA in their lipid, albeit there was a moderate level of 18:3(n-3) fatty acid in the diet. High levels of 18:2(n-6) may have inhibited the desaturation of 18:3(n-3) by Δ^6 desaturase. It has been suggested that the 18:3(n-3) content of lipid in the diets of rats has to be greater than 15 % to inhibit the conversion of 18:2(n-6) to arachidonic acid (20:4(n-6)) (Kramer, 1980). The lipid of catfish erythrocytes had higher monoenes and higher (n-6) PUFA than found in the equivalent salmon cells, and the level of 20:5(n-3) fatty acid was higher than that of the 22:6(n-3) fatty acid.

The fatty acid profiles for salmon and catfish leucocyte lipids were very similar, even with the fish having been maintained at different water temperatures. Catfish leucocyte lipid had a fatty acid profile more typical of fish than mammals, with high (n-3) PUFA levels, which therefore may suggest that the fatty acid profile of the leucocytes was not entirely temperature-dependent since the fish had been cultured at a water temperature

of 27°C. The level of 20:5(n-3) fatty acid was higher in catfish leucocyte lipid that of salmon. The leucocytes of the cold-water salmon and warm-water catfish, in contrast to their erythrocytes, contained similar levels of 22:6(n-3), suggesting that either the lipid composition of leucocytes, or the 22:6(n-3) fatty acid level in the lipid, is not susceptible to temperature adaptation.

In comparison with the warm water African catfish, it is unclear why salmon should contain higher TAG levels in their neutral lipid, higher 18:1(n-9) in their phospholipids and also have a significantly higher level of (n-6) PUFA in their leucocytes compared with their erythrocytes. Nor is it clear why the erythrocytes from cold water salmon and rainbow trout should contain such high levels of 22:6(n-3) compared to the warm water African catfish, in the latter case 22:6(n-3) possibly plays a role in homeoviscous adaptation. However, the erythrocytes of the two species had similar levels of PC and PE lipid classes. As explained in Chapter 2, these are important moieties in homeoviscous adaptation. Different fatty acid roles must be related to the function of the membrane phospholipids, perhaps by conserving a correct balance between the SFAs, monoenes, and PUFA and thus maintaining the correct membrane fluidity for cellular function. Bly *et al.*, (1988) noted that erythrocytes are involved with gaseous exchange, a process relatively independent of membrane fluidity, whereas lymphocytes, involved in the immune response, would need a dynamic membrane for rapid signalling and active transport. The increased levels of 22:6(n-3) at lower temperatures is not related to eicosanoid production since it has been shown that the 22:6(n-3) in rainbow trout leucocyte lipid is not a substrate for leukotriene synthesis (Pettitt and Rowley, 1989).

Bly *et al*, (1986b) showed increased saturation in the fatty acid composition of channel catfish thrombocytes, erythrocytes and T and B cells when the fish were acclimated to 27 °C from 22 °C. The authors concluded that temperature had a more dramatic effect on the fatty acid profiles of lymphocytes, compared to erythrocytes and thrombocytes. The present study comparing the lipid composition of erythrocytes and leucocytes in Atlantic salmon suggested that temperature acclimation in these fish did not involve large changes in membrane lipid composition or, alternatively, that a longer period than 35 days was required before such changes are observed. Bly and Clem (1988) found that T cells and thrombocytes took 3-5 weeks to acclimate, B cells took only 1-3 weeks, whereas red blood cells from channel catfish had not completed homeoviscous adaptation after the 8 week duration of the experiment, and they suggested therefore that erythrocyte function was independent of membrane fluidity, or that homeoviscous adaptation was achieved by changes to only new cell populations. It was shown in the present study that no change occurred in the fatty acid profiles of lipids from Atlantic salmon erythrocytes until 8-16 weeks after being maintained on the high (n-6) PUFA diet, and it is suggested that (n-6) PUFA incorporation occurs during the development of the erythrocyte (see below). Lie *et al.*, (1989b) similarly showed in cod which had been acclimated over 4 weeks to 8, 12 and 16°C and maintained at these temperatures for 9 weeks, that both the proportions of SFAs and the monoenoic fatty acids of their erythrocyte phospholipids declined with decreasing temperature, and that PUFA levels increased. The effects of temperature on the lipid composition of cod erythrocytes, as observed by Lie *et al.*, (1989b), are consistent with the response of other fish tissues to environmental temperature (Cossins, 1977; Selivonchick *et al.*, 1977; Hazel 1979).

An increase, although not a significant one, was observed here in lipid content of the erythrocytes and leucocytes of salmon at the lower temperature. Lie *et al.*, (1989b) have shown that erythrocytes have a reduced average size with increased temperature, which may explain the increased erythrocyte lipid content at lower temperatures.

It is known that of all the phospholipid classes, PE and PC, which together comprise 60-80 % of the total phospholipid classes in the plasma membrane, show the greatest magnitude of change in response to temperature in fish (Hazel, 1979). In agreement with other studies on temperature adaptation by fish tissues (Dreidzic *et al.*, 1976; Selivonchick *et al.*, 1977; Hazel 1979; Hazel and Carpenter, 1985; Hazel and Landrey, 1988a), the PE fraction of Atlantic salmon erythrocytes was significantly higher at 2°C than 12°C. A converse response in PC levels was not observed, however, in leucocyte lipid from salmon maintained at the elevated water temperature.

Conservative estimates for the number of phospholipid molecular species in the membrane of erythrocytes range from 150 to 200 (Hazel and Williams, 1990). Although no differences were observed in the fatty acid composition of the fish erythrocytes at 2°C and 12°C in this study, there may have been a "restructuring" of the lipid species within the cell membranes, which was not apparent from the analysis carried out. A membrane composed of the molecular species 16:0/16:0-, 18:0/18:0-, and 18:1/18:1-PC can re-arrange to 16:0/18:0-, 16:0/18:1-, and 18:0/18:1-PC without altering the overall fatty acid composition of the membrane. An example cited by Hazel and Williams (1990), shows that substituting 18:1(n-9) for 16:0 at the *sn*-2 of PC to form 16:0/18:1-PC, reduces the gel/fluid phase transition temperature by 50°C. He also

suggests that retailoring of lipid species may possibly be the most widespread response in temperature adaptation.

Data comparing the lipid composition of leucocytes from Atlantic salmon fresh water parr to those of marine smolts, maintained on varying (n-3)/(n-6) dietary PUFA ratios, showed the lipid of leucocytes of the freshwater fish to contain higher neutral lipid levels, which perhaps reflects the higher saturated fatty acid level in these cells, and conversely, the lipid of leucocytes of marine fish to contain higher polar lipid levels. The higher proportions of neutral lipid in the leucocytes of the parr is a consequence of higher TAG levels. During smoltification, lipids are depleted from this fraction in muscle lipid by being mobilized as energy reserves, and an overall reduction in the lipid content of the fish results (Sheridan, *et al.*, 1983). Since the absolute amount of lipid was not determined for the leucocytes, the lower TAG levels which were observed in the lipid of marine smolt leucocytes could not be quantified. Percentages of elevated polar lipid levels seen in the leucocytes of marine salmon were compensated for by decreased neutral lipid percentages including the percentage of TAG. There was no difference, however, in the absolute amount of fatty acid components from the lipids of the different fish leucocytes, which might have been expected if the low TAG levels in the marine leucocytes were a result of lipid depletion.

In general, the lipids of marine fish are characterised by high (n-3) PUFA while their freshwater counterparts have high (n-6) PUFA (Sheridan, 1989). In this study, no significant difference could be seen in the unsaturated PUFA levels, or in the (n-3)/(n-6) PUFA ratio of fatty acid components of total leucocyte lipid from the parr and the

marine smolts on a comparable fish oil diet. Consequently, there was no increase in the degree of (n-3) PUFA unsaturation in relation to seawater adaptation within the marine leucocyte population of the present study in contrast to the situation seen in the liver, the dark muscle and the adipose tissue by other investigators (Sheridan et al, 1985a).

Comparing the fatty acid profiles of PC and PE lipid classes of peripheral blood leucocytes from Atlantic salmon parr in dietary trial 2 to those from another study by Bell *et al.*, (1991a), in which Atlantic salmon smolts were maintained on similar fish oil or sunflower oil diets, they showed that the smolt leucocytes contained higher levels of (n-3) PUFA and lower levels of monoenoic fatty acids in these lipid classes, than did the freshwater parr in the present study.

It is well established that the fatty acid composition of dietary lipid influences the fatty acid composition of the body lipids of fish (Watanabe, 1982). The salmon smolts fed the linolenic acid diet (Section 4.7) had higher levels of most (n-3) PUFA fatty acids in the lipid of their leucocytes, but also a higher 20:4(n-6) PUFA level when compared with the freshwater parr. Although the linolenic acid diet contained higher amounts of 18:2(n-6) than the smolt fish oil diet (diet 1), there was no difference in the 20:4(n-6) level of leucocyte lipid between the two groups fed these diets. This is in keeping with a preference for (n-3) fatty acid over (n-6) fatty acid substrates by the enzymes of desaturation and/or elongation. This situation has been observed before both in mammals and in fish (Henderson and Tocher, 1987). The influence of dietary fatty acid composition was illustrated by elevated amounts of (n-6) PUFA present in smolts fed on the sunflower supplemented diet, reflected by a decreased (n-3)/(n-6) PUFA ratio.

Freshwater fish in general have been shown to desaturate 18:2(n-6) and 18:3(n-3) by Δ^6 desaturase, followed by elongation of the product and desaturation by Δ^5 desaturase. The final products are either 20:4(n-6), or 22:6(n-3) after another cycle of elongation and desaturation by Δ^4 desaturase (Henderson and Tocher, 1987). It is believed that marine species generally lack Δ^5 desaturase activity and require highly unsaturated fatty acids in their diets. Bell *et al*, (1989, and in press) have shown that post smolt Atlantic salmon can metabolise 18:2(n-6) and 18:3(n-3) via this pathway, resulting in increased 20:4(n-6), 20:5(n-3) and 22:6(n-3) in their membrane lipids.

In the lipid of leucocytes from marine salmon maintained on a high (n-6) PUFA diet, monoenoic fatty acids were apparently displaced in preference to the (n-3) PUFA in compensation for increased (n-6) PUFA level within the cell lipid. The conversion of dietary 18:3(n-3) PUFA to 22:6(n-3) in the lipid of leucocytes from marine salmon, maintained on the linseed oil diet, showed apparent Δ^6, Δ^5 desaturase and Δ^4 desaturase activity. There must be a preference by the desaturases of Atlantic salmon for (n-3) PUFA since the salmon on the high (n-6) PUFA diet had higher levels of 22:6(n-3) in their total lipid than the marine fish maintained on the fish oil diet. Bell *et al*, (in press) showed that the phospholipids of leucocytes from Atlantic salmon smolts fed on a linseed oil diet contained higher 22:6(n-3) PUFA levels in PC, PE and PS fractions than was found in smolts maintained on a fish oil diet. Such results and those of the present study suggest that the longer chain PUFA, in part, inhibit Δ^6, Δ^5 and Δ^4 desaturase activity in smolt leucocytes (or liver if leucocytes assimilate PUFA previously modified in the liver).

Smolts tend to have higher proportions of PUFA in phospholipids of their total body lipid than parr, suggesting that the bioconversion of 18:2(n-6) and 18:3(n-3) to the PUFA derivatives is more active in smolts. However, in normal diets with sufficient dietary long chain PUFA the difference in the bioconversion of linoleate and linolenate to the PUFA derivatives between parr and smolts is masked (Sheridan *et al*, 1985a; Ogata and Takeshi, 1989).

7.2 DIETARY MANIPULATION OF THE LIPID COMPOSITION OF IMMUNOCOMPETENT CELLS AND TISSUES OF ATLANTIC SALMON

The study set out to compare the effects of diets with different (n-3)/(n-6) ratios on firstly, the lipid composition of Atlantic salmon peripheral blood cells and immunocompetent tissues and secondly, on their immune response. It was hoped initially to have included a diet with an (n-3)/(n-6) ratio which was higher than could be obtained from a commercial diet but, unfortunately, the commercially available diets of salmon parr already contained high levels of 20:5(n-3) and 22:6(n-3) PUFA in diets containing up to 22 % lipid (see Table 5.5). It was, therefore, difficult to prepare experimental diets with higher levels of 20:5(n-3) and 22:6(n-3) PUFA than could be obtained from a commercial diet, short of spraying the oils on to the pellets before feeding or using concentrates of PUFA. It was therefore decided to compare low and high (n-3)/(n-6) ratio diets, the low being in the form of diets supplemented with sunflower oil and the high in the form of diets supplemented with a standard, high quality, fish oil.

When examining the influence of dietary lipid on the lipid composition of Atlantic

salmon immunocompetent cells and tissues, important considerations are the physiological and environmental conditions of the fish. Since the dietary fatty acid composition influences smoltification (Ogata and Takeshi, 1989), and stress due to smoltification results in a decreased immune response, it is important to mention that the lipid composition of tissues and cells from freshwater parr in Chapter 4 and 5 may also have been influenced by smoltification. It has been previously shown that the lipid composition of freshwater smolts resembles marine smolt lipid patterns and is independent of diet (Lovern, 1934: Ogata, 1989). Some of the fish on the dietary trials in Chapter 5 took on the silver characteristics of smoltification in early spring, but reverted back to parr. These were presumably potential S1 in an S2 population.

Another important consideration is that when fish experience stress, as explained in Chapter 2, not only is their immune response suppressed, but a variety of metabolic processes, including lipid metabolism may be affected (Barton and Iwama, 1991).

Peroxidation of the highly unsaturated fatty acids found in fish oil can be problematic. Free radicals produced during the process have been shown to have toxic properties in biological systems (Southern, 1988). Of the fifteen vitamins required by salmon for optimal health, shown in Table 3.2, vitamin E (tocopherols), in association with selenium, appears to be part of a complex which controls free radicals produced by autooxidation of fatty acids in the presence of O_2. The greater the unsaturation of the cell membrane the greater is the requirement for vitamin E (Lucy, 1974). In this study, an antioxidant mixture was added to the oil before it was mixed with the other ingredients (see Table 3.2). The use of a synthetic antioxidant in respect to disease

resistance has not been investigated in fish, but it has been suggested that they do not completely satisfy the requirement of vitamin E by some animals (Tengerdy *et al.*, 1981). Diets were kept at -20°C until used, to prevent deterioration. Lipid peroxidation was not measured in the prepared diets.

While no difference was observed in the lipid or protein content of the leucocytes and erythrocytes from the dietary groups in the study, there was no doubt that the fatty acid composition of cells was influenced by the lipid composition of the diet (Table 5.7).

The life span of a human RBC is around 120 days, during which time, except for the first 1-2 days of the reticulocyte phase, the cell is unable to synthesis fatty acids *de novo*, resulting from what is believed to be a lack of acetyl-CoA carboxylase (Pittman and Mertin, 1966). A continuous restructuring of lipid, however, occurs within the cell through passive and active exchange pathways with plasma lipid. RBC cholesterol is rapidly exchanged with unesterified, but not esterified, plasma cholesterol. Likewise, there is a passive equilibrium between RBC, albumin-bound free fatty acids, and phosphatides, mainly PC and lyso-PC. Lyso-PC, originally from lysophosphatide obtained from the plasma, is also actively acylated with free fatty acids to produce PC. The production of PE from lyso-PE is similar to that of PC, but is also produced from the transacylation of PC to lyso-PE. Free fatty acids from the phosphatides return to the plasma after being cleaved from the membrane by phospholipases. (See Shohet, 1977 for lipid metabolic pathways). No reference can be found relating to similar lipid metabolism in salmon erythrocytes, which may differ since; (1) they are nucleated, (2) they have a different lipid composition to mammalian cells or (3) this aspect has not

been examined.

In mammalian erythrocytes, 20:5(n-3) is preferentially distributed in the plasma side of the biomembrane, whereas 22:6(n-3) is preferentially located in the cytoplasmic side (Popp-Snijders *et al.*, 1984). It is thought that the amount of 20:5(n-3) present in the erythrocyte membrane is determined by lipid exchange with plasma lipoproteins, while 22:6(n-3) is incorporated during the development of the erythrocyte. Thus, the level of 20:5(n-3) reflects the dietary lipid pool in the short term while the 22:6(n-3) level reflects longer term dietary intake (Von Schacky *et al.*, 1985).

There were no changes in the fatty acid profiles of lipids of Atlantic salmon erythrocytes examined during trial 2, from fish maintained on high (n-6) PUFA diets, until 8-16 weeks after commencing feeding. At this time the (n-6) PUFA level peaked and reached a plateau, suggesting that (n-6) PUFA were incorporated during the development of the erythrocyte, rather than from acclimation of existing cells with the dietary lipid (see Figures 5.5 and 5.6). As mentioned above mammalian erythrocytes have a continuous restructuring of lipid by exchange with plasma lipid. The (n-6) PUFA level of serum from the experimental fish had changed by the fourth week and there would have been a more rapid increase in the (n-6) PUFA content of the erythrocyte lipid than might have been expected after this time. The life span of the mammalian erythrocyte is around 17 weeks. In the case of the leucocytes 18:2(n-6) fatty acid was gradually incorporated into their lipids over the time course, peaking at 21 weeks. This suggests that a different mechanism of fatty acid uptake exists between the two cell types. The results of this study do not reflect the situation in mammalian

erythrocytes. The rate of change in lipid composition by dietary fats to a point where there is a steady state exchange between mammalian plasma and blood cell lipid composition, takes generally 5-12 days (Knapp *et al.*, 1986 and Von Schacky *et al.*, 1986; Kinsella, 1987).

The influence of dietary lipids on trout erythrocytes reflects both physical and morphological changes within the cell (Leray *et al.*, 1986). Increased saturated fatty acids in the membrane correlates with an increase in the osmotic haemolysis rate. Increased osmotic fragility of erythrocytes occurs in Hitra disease, resulting in haemolysis of the erythrocytes by the causative agent, *Vibrio salmonicida*. Salte *et al.* (1988), showed reduced membrane fragility and decreased mortalities due to Hitra, in fish fed a high (n-3) PUFA diet, thus emphasising the relationship between (n-3) PUFA, erythrocyte membrane composition and disease resistance.

Leucocytes are much less numerous than erythrocytes in blood, but the importance of their lipid metabolism to their cellular function is probably greater. They respond to specific signals and stimuli, which when applied *in vitro* allows their lipid composition and metabolism to be monitored. Leucocytes are composed of morphologically and functionally distinct populations of heterogeneous cells. Mature mammalian neutrophils have a life-span of 2 days (Wintrobe, 1974). Macrophages, on the other hand, spend around 30 hours as monocytes in the blood before migrating into the tissues and differentiating into macrophages. T cell lymphocytes by comparison can have a life-span of several years. Although the life of the different fish leucocyte populations has not been conclusively defined, differences in fatty acid metabolism by these various

populations may reflect differences in their life spans.

While lymphocytes are capable of *de novo* fatty acid synthesis (Liljeqvist, 1973), Buttke *et al.*, (1989) have reported that murine T cells are deficient in unsaturated fatty acid synthesis due to a lack of Δ^9 desaturase activity and are thus unable to convert stearic acid (18:0) to oleic acid (18:1(n-9)). Bly *et al.*, (1990) has found a corresponding deficiency in T cells in channel catfish. Both groups claim that the addition of exogenous 18:1(n-9) fatty acid is necessary for T leucocyte growth *in vitro*, and that immunosuppression due to low temperature can be overcome with the addition of 18:1(n-9) fatty acid (Bly *et al.*, 1990; Buttke *et al., 1991*). This, as discussed in Chapter 2, is due to the effects of decreased membrane saturation. The suggestion that T cells are phylogenetically conserved comes from evidence that virgin T cells, but not B cells, from both mice (Buttke *et al.*, 1991), and catfish (Bly and Clem, 1992), are immunosuppressed by low *in vitro* temperatures, and that this immunosuppression can be rescued by 18:1(n-9).

There are reports of Δ^9 desaturase activity in mammalian lymphocytes which had been stimulated with PHA. The cells, however, were incubated with 18:0 which had been experimentally bound on to FCS, and would have undoubtedly been exposed to 18:1(n-9) present in the FCS. This could explain the observed increase of 18:1(n-9) in the stimulated T cells (Anel *et al.*, 1990). Both dietary groups of the salmon leucocytes examined here contained more 18:1(n-9) than 18:0 and while the diets had low levels of 18:0, the levels of 18:1(n-9) were very similar between cells and diet. This suggests that if salmon T cells, like those of the catfish, are deficient in Δ^9 desaturase activity,

they must have incorporated 18:1(n-9) from their diet.

Catfish is equivalent to the 'experimental mouse' of the fish domain, exhibiting an excellent immune response in comparison to the salmon. The lipid composition of the leucocytes from the catfish and salmon examined here were very similar because they had both been maintained on a fish meal diet, high in (n-3) PUFA. The only real apparent difference between salmon and catfish leucocyte lipid, under these circumstances, is a higher 20:5(n-3) level in leucocyte lipid of catfish (7.7 % vs. 3.9 % in salmon leucocyte lipid). The disparity in their immune response may be reflected in the differences in lipid composition. It would be interesting to examine immune function in the two species when both fish had been fed a fish oil diet and compare them with catfish and salmon fed a high (n-6) PUFA diet. Catfish leucocytes may need more (n-6) PUFA in their lipids for optimal activity. The fatty acid profiles of catfish erythrocytes resemble their leucocyte lipid with high monoenes, higher total (n-6) PUFA compared to the salmon erythrocytes, low 22:6(n-3) and high 20:5(n-3). This may indicate that catfish have a less active Δ^4 desaturase activity.

Fish leucocytes contain high proportions of glycolipids in their total lipid compared with other fish cells, and as mentioned earlier these are believed to play some immunological role. In the past few years there has been increased interest in gangliosides in relation to the immune response (Muthering *et al.*, 1987). These are a group of heterogenous glycosphingolipid molecules found on the cell surface, and appear to be of specific types, attributed to different subpopulations of lymphocytes. For example, the major fatty acid components of the ganglioside molecules in stimulated T cells tend to be

either 16:0, 24:0 and 24:1 (Muthering *et al.*, 1987). No documented reports of ganglioside structures in fish lymphocytes could be found, but it is feasible that these molecules too may be under the influence of dietary PUFA.

The role of cholesterol in membrane fluidity, and its requirement in lymphocyte blastogenesis was discussed briefly in Chapter 2. Cholesterol levels in the lipid of erythrocytes and leucocytes from the dietary fish were completely unrelated to dietary PUFA composition.

Atlantic salmon serum from experimental fish reflected the dietary lipids more than any of the cells or tissues analysed, but had a much higher level of 22:6(n-3) fatty acid than the diet. This is in accordance with the known processes of lipid absorption and transport, found to operate in fish (Sargent *et al.*, (1989)

The main lymphoid organs in teleost fish are the thymus, head kidney, spleen and intestine, while leucocytes have also been isolated from the skin and gills (van Muiswinkel, 1992). The spleen of teleost fish is believed to be an erythropoietic and secondary lymphoid organ, the thymus is a more a primary lymphoid organ and the kidney is a mixture of both. The lipid composition of the immunocompetent tissues will be influenced by the number of leucocytes present in the tissue, which itself is governed by the age, disease status of the fish, temperature at time of analysis, stress factors etc (see below). The number of leucocytes per weight of tissue was not determined in this study. For such an analysis relating to age, see Tatner and Manning, (1983).

Leucocytes from all immunocompetent tissues had a high neutral lipid content. The peripheral blood leucocytes had higher 22:6(n-3) fatty acid levels in their lipid and incorporated more dietary 18:2(n-6) fatty acid than leucocytes from thymus and spleen. These high levels could possibly have been attributed to the presence of the thrombocytes, which contributed around 35 % of the total peripheral blood leucocyte count.

The structure of the thymus in fish parallels that of higher vertebrates in being a framework of reticulo-epithelial cells which provide support for the lymphocytes, with the network being encased in an epithelial capsule (Chilmonczyk, 1992). In mammals, it is believed that the epithelial cells produce 20:4(n-6) metabolites, which in turn have an essential role in the differentiation of thymocytes. The regulation of eicosanoid production by the epithelial cells is thought to be controlled by the thymocytes themselves, through cell-cell contact of the thymus epithelial cells and the thymocytes (Sun *et al.*, 1990). These finding imply that the differentiation of thymocytes may possibly be under the influence of dietary lipid.

Spleen tissue, the site of erythropoiesis, did not contain as much 22:6(n-3) in its total lipid as expected even though erythrocytes are rich in 22:6(n-3). The level of 22:6(n-3) fatty acid in spleen lipid might have been expected to increase at higher temperatures, as the spleen produced more erythrocytes to satisfy an increased oxygen demand. The leucocytes of both spleen and thymus contained very low levels of 22:6(n-3) in their lipid compared to the kidney and peripheral blood leucocytes.

The lipid of gills was found to be very rich in TAG, and also high in dietary 18:2(n-6), which was probably associated with the TAG fraction. Gills are metabolically active, and may rely on energy obtained from the TAG lipid stores for the osmoregulation of NaCl. The lipid of gills, which is associated with oxygen transport, is not high in 22:6(n-3), unlike that of the erythrocytes. Bell *et al*, (1991a), found that increases in the level of dietary 18:2(n-6) resulted in alterations in the phospholipid fatty acid composition of gills and leucocytes of Atlantic salmon smolts, correlating with a modified eicosanoid spectrum from these tissues.

Feeding fish a diet rich in 18:2(n-6) did not dramatically alter fatty acid profiles of Atlantic salmon erythrocytes and leucocytes. It is known that there are differences in the preference of substrates for the elongation and desaturation enzymes between species and the presence of one type of fatty acid may influence the metabolism of another fatty acid (Leger *et al*, 1981). Firstly, long chain PUFA can exert inhibition of Δ6 desaturase activity by negative feedback, thus preventing the desaturation of 18:2(n-6) or 18:3(n-3), and secondly an excess of (n-6) or (n-3) PUFA may inhibit metabolism of the other series. Inhibition of desaturation and elongation of 18:2(n-6) to 20:4(n-6) by excess 18:3(n-3) has been shown to occur in rats (Mohrhauer and Holman, 1963) and there may have been sufficient 18:3(n-3) in the high (n-6) PUFA diets for this purpose, resulting from the herring meal component. The sunflower oil diet has (n-3) PUFA from herring meal (4-5 % 22:6(n-3) and 3 % 20:5(n-3) of total fatty acids), and these long chain PUFA can inhibit 18:2(n-6) elongation and desaturation by inhibition of $^{6}\Delta$ desaturase (Christiansen *et al.*, 1991). Therefore dietary manipulation by (n-6) PUFA was complicated by the presence of (n-3) PUFA. There is still a lack of understanding

as to why cells do not simply incorporate only the dietary fatty acids which they require. Lipid selection is not under genetic control but while there is a preference for particular fatty acids in lipid classes, it is known that if fish are deprived of particular fatty acids they will elongate another series of fatty acids to compensate for this deficiency (Castell, 1972a). Specific fatty acids are selected in preference over other fatty acids into neutral and phospholipid classes. The fatty acids of the (n-3) series are selectively distributed among the lipid classes of mammals (see review by Simopoulos, 1991). Fatty acids 18:3(n-3) and 20:5(n-3) are found in triglycerides, cholesterol esters, and in membrane phospholipids (18:3(n-3) PUFA is found only in very small quantities in the latter); 22:6(n-3) is found mainly in the phospholipids, especially of the cerebral cortex, retina, testis and sperm. Despite extensive research it has not been discovered why 18:2(n-6) and 20:4(n-6) are found in membranes but not 18:3(n-3).

As the potential for dietary manipulation of the immune response in fish becomes evident, caution needs to be emphasised on the importance of experimental design. Several problems associated with studies on the effects of nutrition on the immune response of fish have been outlined above, and can cause experimental variations which are frequently hard to control and make it difficult to replicate experimental conditions. These include water temperature, seasonal influences, stress, and the genetic stock of the fish, to name but a few. Problems are also associated with comparing investigations between laboratories, since diet formulations, fish species or immunological assays are not consistent. Cold water fish have different dietary requirements to warm water fish, and the requirements also depend on whether the fish is a carnivore, herbivore or omnivore. These variations in turn effect their lipid content and the requirements for

vitamins associated with increased lipid levels. When fish experience stress, metabolic processes can affect lipid metabolism through the action of cortisol (Barton and Iwama, 1991) and many aspects of fish handling are known to cause stress (e.g. transport, anaesthesia, temperature, smolting, sexual maturation). This, itself, suppresses the immune response, and hence must be taken into account when interpreting the results of such studies. In addition, there is also a large variation in immunological responses between fish species, and between individual fish of the same species.

7.3 EFFECTS OF MAINTAINING ATLANTIC SALMON ON HIGH (N-3) AND (N-6) PUFA DIETS ON IMMUNE RESPONSE

Although the water temperature did not appear to have a great effect on the lipid composition of Atlantic salmon erythrocytes and leucocytes in the study, it did affect the amount of diet the fish ate. During starvation, for example through periods of low temperature, it has been shown that levels of 22:6(n-3) fatty acid increase as a result of those of monoenoic fatty acids decreasing as they are used for energy production (Jezierska et al 1982). Since fish were maintained at ambient water temperatures throughout the trials, the time of year will have affected the amount of diet the fish ate, and in turn, affected the lipid composition of their cells and tissues. The high (n-3) PUFA dietary group may have eaten more dietary rations because the experimental diet was formulated totally with ingredients from fish. The high (n-6) PUFA diet was supplemented with sunflower oil and fish maintained on this diet may not have found it as appetizing as the former diet. Laboratory prepared diets did not contain Finn stim, a chemical attractant added to the diets by food manufacturers (Ewos). In trial 1, the

growth rate of fish was statistically different between those maintained on the commercial diet, and those maintained on the experimental diets, and statistically different between experimental dietary groups in trial 2. The difference between the commercial and experimental groups in trial 1 was possibly due to the commercial group consuming more dietary rations since they were familiar with the diet and the diets contained Finn Stim. Trial 1 and 2 dietary (n-3) PUFA groups were heavier and had a higher condition factor than the (n-6) PUFA groups. Changes in condition based on length-weight data do not indicate the nutritional status of the fish and care must therefore be exercised when interpreting condition factors based solely on length-weight data since "total energy growth" can occur independently of changes in body weight (Boulger and Connolly, 1989).

The higher hepatosomatic index values for the (n-3) and (n-6) PUFA dietary groups in trial 1, and for the (n-6) PUFA dietary group in trial 2, reflected a higher lipid content in these livers. Salmonid fish fed diets low or deficient in EFAs usually develop signs of fatty livers which were swollen, pale and have a high lipid content (Henderson and Tocher, 1987). The (n-6) PUFA group in both trials was not deficient in EFAs since it had sufficient (n-3) EFA levels in its diet from the herring meal constituent. A higher spleen index (Sp.I.) can indicate an enlarged spleen possibly due to elevated leucocyte numbers in response to an infection. The (n-6) PUFA dietary group were more prone to fungal infections than the (n-3) PUFA group in trial 2, and this may explain the higher Sp.I. for this group in trial 2.

Haematological tests and analysis of serum constituents are useful in diagnosis of

disease in fish, but influences such as age, season, strain, nutritional status, environmental stress (e.g. crowding, water quality) and sexual maturation can vary haematological measurements (see Sandnes *et al.*, (1988)). The need to define normal ranges for fish haematology was addressed by Blaxhall and Daisley (1973), who suggested routine haematological screening methods to aid health assessment of diseased and stressed fish. They found the physiological normal ranges for fish were wider than those for humans. It is important to note that haematological values for fish are influenced by temperature (Lane, 1979; Houston, 1980; Dunn *et al*, 1989; Lie *et al*, 1989). Leucocyte numbers can also be reduced by cortisol secretion.

Values for the Atlantic salmon differential leucocyte counts were comparable to those established by other workers (Hardie *et al.*, 1991). Haematocrit values, used to check anaemia, ranged from 47.2 to 49.0 % in the experimental fish, which agreed with the normal range for salmon (Sandnes *et al.*, 1988)

Values for Atlantic salmon total serum protein concentrations (49.8 - 57.8 $mgml^{-1}$) were comparable to previous reports (Alexander and Ingram, 1980; Hardie *et al.*, 1991). Reports on the effects of diet on nonspecific immune activity by fish serum proteins are few (Blazer and Wolke, 1984a; Blazer *et al.*, 1984; Hardie *et al*, 1990, 1991). In the current study, some of the proteins present in the serum of the dietary fish were assessed and their activity was found to be independent of dietary lipid.

Fish serum has been shown to exhibit haemolytic (Ingram, 1980), and bactericidal (Kawakami *et al.*, 1984) activities by complement-like proteins, both via classical and

alternative pathways (see reviews by Ingram 1980, 1990). In a number of nutritional studies, diet has been shown to influence complement haemolysis. A decrease in this activity has been demonstrated in Vitamin E-deficient fish (Blazer and Wolke, 1984a; Hardie *et al*, 1990), and again in Vitamin C-deficient fish (Li and Lovell 1985; Hardie *et al*, 1991) who also noted increased haemolytic activity with elevated ascorbic acid levels. The CH_{50} unit used in this study is based on an arbitrary unit defined as the quantity of complement in the serum necessary for 50% lysis of RBCs sensitized with antibody. As mentioned in Section 5.4.2.2, the complement activity in this study was independent of dietary PUFA.

Lysozyme, one of the oxygen-independent processes of macrophage killing, splits the peptidoglycan in bacterial cell walls, particularly Gram positive bacteria, causing the cell to lyse (Chipman and Sharon, 1969). Lysosomes, containing the lysozyme, fuse with the phagosomes and degranulation from the lysosome into the phagosome results as the two vacuoles fuse together. Macrophages and granulocytes are believed to be the main producers of lysozyme, but it is also found in mucus and serum. In mammals, lysozyme has many functions such as bacteriolysis, opsonisation, antineoplastic activity, immune response potentiation (Lie and Syed, 1986). Its activity has been detected in fish with levels varying between species (Mock and Peters, 1990). Rainbow trout has a 20 times greater activity of the enzyme than Atlantic salmon. Distribution of lysozyme activity is associated with leucocyte-rich tissues, with the kidney having the highest levels of activity in Atlantic salmon, then the alimentary canal, spleen, skin mucus, serum, gills, liver and muscle respectively (Lie *et al.*, 1989a). The levels of lysozyme concentration increase during infection and injection of foreign material

(Fletcher and White, 1973; Siwicki and Studnicka, 1987).

Problems arose with the cited method of lysozyme determination in Section 3.4.6. Firstly, samples had to be diluted five times more than stated in the method of Ellis (1990b), to fall within the region of the calibration curve which was proportional to the $\log_2$. Secondly the optimal pH range for fish serum and kidney lysozyme is pH 5.5 - 6.0 (Mock and Peters, 1990). In contrast, the optimal pH for the hen egg white lysozyme activity is pH 7.5. Not only was the hen egg white lysozyme not at its optimal activity during the assay, fish lysozyme concentration was being determined by comparing it to an enzyme with a different activity, from a different species (A. Catcho, personnel communication). However, using the same method a similar range was obtained to that reported by Hardie *et al.*, (1990).

Of the few studies carried out on fish antiproteases, a protein comparable to mammalian α_1 antiprotease has been identified in cod (Hjelmeland, 1983). Ellis (1990b) suggested the inhibition of trypsin activity as the most convenient method of measuring antiproteases. Similar trends to those observed by Hjelmeland (1983) for cod were observed in this study for salmon, but inhibition of trypsin was not affected by the type of dietary PUFA.

Killing of organisms by macrophages and granulocytes is based on oxygen-independent and oxygen-dependent mechanisms and not all organisms are killed by the same method. All aspects of macrophage activity which were examined in relation to the influences of dietary PUFA showed dietary lipid to have no bearing on macrophage

activity in salmon. In previously reported studies on bacterial engulfment and killing by fish macrophages, diet has been shown to be beneficial to this activity. Such studies have shown a decreased phagocytic activity in Vitamin E-deficient fish (Blazer and Wolke, 1984a; Hardie *et al*, 1990), and again in Vitamin C-deficient fish (Li and Lovell, 1985). These vitamins are involved in preventing the oxidation of membrane PUFA *in vivo*. Sheldon and Blazer (1991) showed that dietary lipids influenced macrophage function in channel catfish, with enhanced intracellular killing correlated to increased levels of (n-3) PUFA. This occurred at both optimal and sub-optimal temperatures for channel catfish maintained on a menhaden diet which contained 7 % oil. In a previous study they showed no significant difference in the ability of macrophages to engulf bacteria from channel catfish which had been maintained on 10 % lipid diets of menhaden oil, soybean oil and beef tallow, but there was a difference in killing rates of 60.4, 45.6, and 44.4 % respectively (Blazer and Sheldon, 1991). These rates increased significantly after immunisation for menhaden oil and soybean oil fed fish to 73.4 and 56.1 % respectively, and at sub-optimal temperatures, immunization also enhanced killing by 17.8 % and 18.7 % respectively. In beef tallow fed fish, killing was only enhanced by 1.6 %. The current study had a much higher lipid level and therefore greater (n-3) PUFA level. The possible significance of this is discussed below.

Another microbicidal activity of the phagocytes is the oxygen dependent respiratory burst which is autonomous to the phagocytic process. Chung and Secombes (1988) demonstrated the production of $O_2^{\cdot}$, H_2O_2 and OH reactive oxygen moieties from the respiratory burst by PMA stimulated trout macrophages, and used them as indicators of macrophage activation in rainbow trout (Chung and Secombes, 1987). They examined

rainbow trout macrophages, activated *in vitro* with Con A and *in vivo* with *A. salmonicida*, for microbicidal activity by the oxygen-dependent production of $O_2^{\cdot}$, H_2O_2 and OH reactive oxygen moieties of the respiratory burst. They found increased $O_2^{\cdot}$ production, shown by elevated reduction of the redox dye NBT, but they did not find an increase in H_2O_2 production or bactericidal activity after 3 and 5 hours, in the Con A stimulated macrophages. The H_2O_2 production and bactericidal activity however, were increased in *A. salmonicida* activated macrophages. Results suggest that mitogens are unable to fully activate macrophages, since the macrophages did not have an increased killing capability. Secombes *et al.* (1988), measured extracellular $O_2^{\cdot}$ production using a ferricytochrome C reduction assay and showed that the reduction of ferricytochrome C was due to $O_2^{\cdot}$ since the addition of exogenous SOD converted $O_2^{\cdot}$ to H_2O_2. By inhibiting endogenous SOD with sodium nitroprusside, an increased reduction of the ferricytochrome C was observed.

Nutritionally-based decreases in phagocytic index due to depressed levels of myeloperoxidase and NADP-reduced oxidase have been reported for mammalian macrophages (MacFarlane and Path, 1977). Hardie *et al*, (1990, 1991) showed that the production of superoxide, by the reduction of NBT, was not influenced by dietary vitamins E or C. The present study showed fish maintained on the different experimental diets of high and low (n-3)/(n-6) ratios had similar respiratory burst enzyme activities.

High concentrations of lymphokine-containing supernatants sometimes have a suppressive effect upon NBT reduction (Graham and Secombes, 1990a). It was

confirmed that a 1/4 dilution of the supernatant in Section 5.4.3.4 was inhibitory, while there was a peaked response at 1/16 dilution. There was, however, no influence of dietary PUFA on the activity of macrophage activation factor.

While there is evidence that nutrition can influence the T-cell function of macrophage inhibition factor (MIF) activity by sensitised lymphocytes, as in the trial by Blazer and Wolke, (1984a), the MIF test in this study was inconclusive since control cells inhibited macrophage migration more than the immunized test cells. This may have been due to the control cells having had a previous exposure to *Aeromonas salmonicida* as suggested by IFAT, Western blot and antibody data, but not by the stress testing, as discussed in Section 5.4.5.

The role of various PUFAs in mammalian lymphocyte activation and the influence of diet were dealt with extensively in Chapter 2. Antigen stimulation, *in vitro*, in relation to dietary PUFA does not appear to have been studied previously in fish. Although results from trial 2 of the present study were inconclusive because of the small stimulation indices obtained with *A. salmonicida*, they, together with the results from trial 1, suggested that there was no dietary impact on this activity. Attempts to quantify mitogen stimulation in the presence of Con A and LPS were unsuccessful since very little cell proliferation occurred in response to the mitogen. In fact in some cases it appeared that Con A suppressed leucocyte stimulation. This may have been a result of inadequate culture conditions, (see below for the effects of exogenous fatty acids on mitogen stimulation).

The values obtained for PFCs in the present study are in agreement with those of other workers (Chiller *et al.*, 1969). Decreased B-cell function in the form of *in vitro* antibody responses, measured by the PFC assay, has been found in fish fed a α-tocopherol deficient diet (Blazer and Wolke, 1984a). B-cell function was found to be significantly enhanced by (n-3) PUFA in the current study. This finding does not reflect the situation *in vivo*, however, where there appeared to be no real difference in antibody production between the dietary groups.

The criteria described in section 3.6 required for a successful vaccination are hard to obtain in the laboratory because of the difficulty in achieving the required level of challenge. While it is more meaningful to assess vaccine efficacy by a challenge system resembling natural exposure, water borne challenges are often difficult to accomplish. It was decided to inject the *Vibrio anguillarum* to challenge the fish in the first trial, so as to obtain sufficient mortality levels. It is also important to note that laboratory-grown vaccines, as was the *V. anguillarum* vaccine used here, vary from batch to batch and make comparisons between laboratories difficult. The *A. salmonicida* vaccine was commercially prepared, and following advice from I. Bricknell (personnel communication), the challenge in trial 2 was successfully given by bath.

While several investigations can be found in the literature on the effect of diet on the antibody response to vaccination in fish, antibody titres do not always correlate with protection against the disease (Cossarini-Dunier, 1986). Antibody titres, determined by ELISA, do not show binding affinity or avidity of the antibody. One previous study showed antibody titres to be lowest in groups of Atlantic salmon fed with the highest

(n-3) PUFA diet (Erdal *et al.*, 1991). The likelihood of survival when fish from this group were challenged with *Yersinia ruckeri* was also reduced, but there was no positive correlation between the level of serum antibodies and the degree of protection induced after vaccination with the *Y. ruckeri*.

In trial 1, high antibody titres were demonstrated in fish after vaccination with *V. anguillarum* and a secondary boost served only to slightly increase these. The reason for such a vigorous response was possibly due to the high water temperature (15°C). High antibody titres from trial 1, (which did not offer a great deal of protection after the secondary challenge), and the confusion with antibodies found in the sera of fish prior to vaccination with *A. salmonicida* in trial 2, emphasises the need to put greater significance on the challenge results. Protection against disease in fish is probably due to many elements and is possibly more dependent on non-specific and cell-mediated protection, than on antibody-mediated protection.

Pre-immunization bleeds in trial 2 were positive, corresponding to the finding of I. Bricknell (Personnel communication), who suggested that positive preimmunization sera had to be diluted 1/64 to remove any positive reaction in a Western Blot. The preimmune serum from this study was still reacting with bacteria in IFAT at a 1/100 dilution. The fish, however, did not develop the disease under stress testing, and so were therefore not carriers of the disease. It was assumed that the most likely explanation for the antibody titres was that *A. salmonicida* possesses antigens common to other ubiquitous bacteria.

It was previously mentioned (Section 5.4.5) that the RPS values from trials 1 and 2 were inconclusive. Trial 2 gave ambiguous results in that the (n-6) PUFA group had higher protection from the vaccine when challenged with *A. salmonicida*, but the corresponding nonvaccinated group had a lower natural resistance to the disease compared with the (n-3) PUFA group. Although the (n-3) dietary PUFA group had a statistically better survival rate than the (n-6) dietary PUFA group in trial 3, the lack of replicate groups throughout trials 1-3 means that data from this study should only serve as indications of disease resistance. There had been a tendency for the (n-6) PUFA group to succumb to the bacterial infections more readily than the (n-3) PUFA group in the present study, but no definite conclusions can be drawn from this. The was no correlation between increased mortalities and the investigated parameters of the immune response such as respiratory burst enzymes, cell numbers and antibody titres.

The (n-6) dietary group were more prone to fungal infections reflected by the occasional mortalities which occurred throughout the trial. No further investigation of this was made apart from noting their occurrence. Ackman and Takeuchi (1986) suggested that low n-6/n-3 fatty acid ratios may be responsible for the fin erosion which they observed in hatchery-raised Atlantic salmon.

Dietary (n-3) PUFA, in diets containing 23 % lipid (14 % 20:5(n-3) and 10 % 22:6(n-3) as a percentage of total fatty acids), exerted an immunosuppressive effect on fish which had been challenged with *Yersinia ruckeri* (Erdal *et al.*, 1991). As a result of being immunocompromised, they possessed both significantly lower survival rates and antibody levels, and these authors claimed that these finding were possibly due to

oxidative stress.

The lipid requirements of juvenile Atlantic salmon parr are satisfied by high dietary (n-3) PUFA levels in commercial feeds, but as a result, the greater unsaturation in the membranes of fish cells means a greater need for α-tocopherol (Lucy, 1974). In fish fed α-tocopherol-deficient diets immunological parameters were significantly lower (Blazer and Wolke, 1984a; Hardie *et al.*, 1990), while increased dietary (n-3) PUFAs in juvenile Atlantic salmon, showed fish to be immunocompromised by the high levels of dietary (n-3) PUFAs (Erdal *et al.*, 1991), possibly due to oxidative stress as described above. Histological investigations indicated a higher incidence of degenerative changes in cardiac and skeletal muscle in fish fed fish oil supplemented diets (Erdal *et al.*, 1991). There may be an upper limit for long chain dietary (n-3) fatty acids that will not compromise fish health due to the lipid oxidation, but these still have to be determined. On the other hand, (n-6) PUFA may also be detrimental to fish. Bell *et al.*, (1991b) observed cardiac lesions in Atlantic salmon smolts maintained on a high (n-6) PUFA diet. It has been demonstrated that phospholipase A activity, responsible for cleaving free fatty acids from phospholipid moieties, increases in cardiomyocytes cultured *in vitro* in the presence of (n-6) PUFA (Nalbone *et al.*, 1990) and in heart tissue *in vivo* (Bell *et al.*, in press).

Antigen receptors and histocompatibility antigens are amongst the most important membrane proteins in relation to the lymphocytes immunological role, and their expression which is membrane dependent is in turn affected by dietary lipids. As explained earlier, the major precursor of eicosanoid production in mammals is 20:4(n-6)

and that great interest is currently being given to the effects of diet modulation on the production of eicosanoids from the (n-3) PUFA series. Fish, rich in (n-3) PUFA, may be expected to produce increased levels of eicosanoids derived from of the (n-3) PUFA series in preference to those derived from (n-6) PUFA. At present it is only surmised that PI is solely responsible for the release of free fatty acids for eicosanoid substrates. Nevertheless, the results of this study would suggest that PI fractions in the immunocompetent cells of salmon lipid resembles that of mammals in terms of its content of high 20:4(n-6) PUFA.

The immunoregulatory action of eicosanoids in mammals was discussed in Chapter 2. Eicosanoids are known to be produced by fish leucocytes using enzymatic pathways similar to those in mammals (Rowley *et al.*, 1987; Rowley, 1991). As pathways in fish continue to be elucidated, evidence suggests that 20:4(n-6) may be the predominate precursor of eicosanoid synthesis (Pettitt, *et al.*, 1989b; Pettitt, *et al.*, 1991; Rowley, *et al.*, 1991).

7.4 EFFECTS OF EXOGENOUS FATTY ACIDS ON ATLANTIC SALMON LEUCOCYTES *IN VITRO*

This study showed that when exogenous fatty acids were added to fish leucocyte cultures, the leucocytes could tolerate higher concentrations of 18:3(n-3) and 20:5(n-3), compared to the 18:1(n-9), 18:2(n-6) and 20:4(n-6). This may indicate that salmon leucocytes require higher concentrations of these two fatty acids, although this was not confirmed with the ^{14}C labelled 18:3(n-3) and 20:5(n-5). Bly *et al.*, (1990) found that channel catfish leucocytes were able to endure much higher concentration of exogenous

18:0 and 18:1(n-9) fatty acids (80-160 μM), while concentrations ≥ 240 μM were inhibitory to mitogen stimulation.

Lipid droplets formed in the salmon leucocytes at higher concentrations of all exogenous fatty acids. Tocher and Dick (1990b) have shown that the lipid content of fish cells is dependent on the concentration of fatty acid with which they have been cultured *in vitro* and demonstrated that epithelial Atlantic salmon cells cultured with 50 μM or greater supplemented fatty acid accumulated lipid droplets.

Leucocyte fatty acid composition was not influenced by culturing *in vitro* with FCS or fish serum, presumably because the lipid of cells had a similar (n-6) PUFA level to FCS and possibly the cells were already at their optimal limit of (n-3) PUFA content. It would be interesting to culture lymphocytes with serum from fish maintained on a high (n-6) PUFA diet. It is possible that the leucocytes are unable to incorporate fatty acids bound to BSA as efficiently as from serum lipoproteins.

The effects of exogenous fatty acids on the immune response of Atlantic salmon leucocytes *in vitro* showed a convincing incorporation of 18:1(n-9) and 20:4(n-6) into leucocytes, which was generally independent of temperature, species (trout vs. salmon), and tissue. Mammals have a bias for a higher (n-6) PUFA content in their lipid because of the influence of their diet, while PUFA in the organs and tissues of marine fish are mainly of the (n-3) series (see Figure 2.4). Therefore when mammalian cells are cultured with (n-3) PUFA any uptake of (n-3) is noticeable, because of the predominance of (n-6) PUFA. A rapid incorporation of 20:5(n-3) PUFA into the

plasma, platelets, neutrophils, monocytes, and T and B-lymphocytes of human subjects after 2 weeks of being maintained on a MaxEPA dietary supplement, suggests firstly that there is an exchange of 20:5(n-3) between the plasma and the phospholipid of these cells, and secondly that the human cells had a greater preference for (n-3) PUFA than the fish cells had for (n-6) PUFA (Gibney and Hunter, 1993).

Attempts to establish if exogenous fatty acids had any effect on immunological function were inconclusive. Problems with mitogen assays were basically due to the lack of stimulation. It was impossible to confirm that there was any effect by the fatty acids when the initial stimulation was so nominal. The addition of exogenous fatty acids did not enhance proliferation and ascorbic acid, cholesterol or fish serum were added, unsuccessfully, to try to improve stimulation. Other preliminary studies of lymphocyte function in the presence of fatty acids included antigen stimulation measured by proliferation, and *in vitro* antibody production in the presence of fatty acids. Again, these were inconclusive.

Macrophage studies, including respiratory burst assays and a bacterial killing assay, after culturing adherent monolayers with exogenous fatty acids, gave inconsistent results. There were variations in the degrees of adherence and spreading when macrophage monolayers were incubated with different fatty acids, and this may possibly explain the inconsistencies of the results. In the presence of 18:1(n-9) fatty acids, adherent macrophages spread well and threw out large lamellipods, while in the presence of 22:6(n-3) all macrophages became detached from the monolayer.

7.5 CONCLUSION AND FUTURE PROSPECTS

It is concluded that while there were few positive effects of polyunsaturated fatty acids on the immune response of Atlantic salmon parr under the conditions examined, the fatty acid composition of the cells and tissues examined were definitely influenced by their dietary fatty acids. Although fatty acid profiles of cells and tissues were influenced by their dietary lipid, they did not have identical fatty acid compositions to their diet. This was because the results of dietary manipulation on the fatty acid profiles of the cells and tissues were the consequence of complex interactions of enzymes which preferentially selected certain dietary fatty acids. The influence of endogenous fatty acid synthesis in fish cells and tissues on lipid metabolism has not been assessed over the course of the work, but this too possibly contributed to the lipid profiles which were obtained.

This study gave no indication of whether diet modulation of the immunocompetent cell lipid had a greater impact on eicosanoid production or on membrane function. The following deductions were, however, made. Leucocytes contained high levels of TAG, which may have been the initial store of dietary fatty acids, and which supplemented the phospholipids' requirements for fatty acids. It was shown with ^{14}C labelled fatty acids that the TAG fraction, as well as PI, incorporated large amounts of 20:4(n-6) from the exogenous fatty acid pool. Fish leucocytes contained higher levels of 20:5(n-3) than 20:4(n-6) in their total lipid, but it was consistently the 20:4(n-6) which was found to be incorporated into the PI fraction, both from dietary lipid and ^{14}C labelled fatty acids. It has not been confirmed that mammalian lymphocytes are capable of eicosanoid production, but it can be speculated that it is the macrophages which are responsible for

their production from unesterified fatty acids such as 20:4(n-6). Furthermore, it is feasible that fish lymphocytes can present macrophages with either 20:4(n-6) or 20:5(n-3) for the synthesis of eicosanoids. In fish leucocyte lipid 20:4(n-6) contributes 2.5 % to the total lipid content and 20:5(n-3) fatty acid, another eicosanoid precursor, contributes 3.6 %. Macrophage lipid contained slightly less of both of these fatty acids in their lipid. The 20:4 / 20:5 ratio for leucocytes from the dietary studies showed (n-3) PUFA dietary groups to have only slightly lower ratios than the (n-6) PUFA groups. The percentage of 20:4(n-6) fatty acid in fish leucocytes was much less than is found in mammalian leucocytes, but the mammalian cells contain 4 times less lipid. In terms of absolute amounts of 20:4(n-6) per cell, the fish and mammalian leucocytes may actually be similar.

Although eicosanoids are known to be produced by fish leucocytes using enzymatic pathways similar to those in mammals, an analogous situation to that described in Chapter 2 for mammals, where immunosuppression results from an imbalance in their levels, still has to be ascertained for fish. While the effects of different (n-3)/(n-6) PUFA ratios on the immune response of Atlantic salmon remains unresolved, this work has suggested that the lymphoid system of fish is predisposed to (n-6) PUFA. This conclusion is based on the fact that (a) the fatty acid profiles of fish leucocytes contained less total (n-3) PUFA and more total (n-6) PUFA than the erythrocytes and tissues, and (b) the incorporation of ^{14}C 20:4(n-6) into the leucocytes, particularly the TAG and PI fractions. This may suggest that 20:4(n-6) forms the predominant eicosanoid series in salmon. The preferential incorporation of 18:1(n-9) into the fish leucocytes undoubtedly must play some role in maintaining membrane structure, and in

fulfilling the inability of T-lymphocytes to elongate 18:0.

In hindsight, it would have been useful to have compared the fatty acid profiles of neutral lipid from the erythrocytes and leucocytes. The (n-6) series of PUFA is preferentially incorporated into TAG by fish tissues (Hazel, 1979) and consequently, if fatty acids stored in leucocyte lipid as TAG are important in the immune response (TAG contributes up to 25 % of the total leucocyte lipid), analysis of the lipid composition of this fraction may have proved this. While the (n-6) PUFA level in the PC and PE fractions of leucocyte lipids were 3 times higher than found in the corresponding fractions in erythrocyte lipid, the leucocytes may have contained an even higher (n-6) PUFA content in their TAG.

The overall resistance of fish to disease is dependent on the nutritional status of the fish. Further research is necessary to identify nutrients capable of enhancing disease resistance and also determine the levels necessary for this enhancement. Dietary manipulation of the immune response by (n-3) and (n-6) PUFAs, and SFAs may improve pathological conditions in immunocompromised fish, but it is still necessary to clarify if, and at what levels, vitamin E and other antioxidants can control peroxide production in cells, and to establish the most efficient (n-3):(n-6) dietary fatty acid ratio for fish, to maintain the optimal fatty acid compositions for leucocyte function, be it lymphocytes or macrophages. A balance must be struck between optimal fish production and maintaining fish health, and a diet which could be specifically tailored to provide both maximum growth and active enhancement of disease resistance would be of great benefit to aquaculture.

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APPENDIX 1 BUFFERS

Complement inactivation of foetal calf serum:-

FCS, thoroughly thawed, was placed in a water bath at 56°C for 30 minutes, mixing periodically. It was aliquoted and stored at -20°C.

ELISA:-

Coating buffer

carbonate-bicarbonate solution

Na_2CO_3	1.59 g	Dissolved in 1 l distilled water
$NaHCO_3$	2.93 g	Adjusted to pH 9.6 and made freshly

Wash buffer (x10) (low salt)

Trisma base	24.2 g	Dissolved in 1 l distilled water
NaCl	222.2 g	pH adjusted to 7.3 with conc. HCl
Merthiolate	1 g	
Tween 20	5 ml	

Wash buffer (x10) (high salt)

Trisma base	24.2 g	Dissolved in 1 l distilled water
NaCl	292.2 g	pH adjusted to 7.7 with conc. HCl
Merthiolate	1 g	
Tween 20	10 ml	

Substrate buffer

sodium acetate/ citric acid buffer

Citric acid	21.0 g	pH adjusted to 5.4 with 1 M NaOH
Sodium acetate	8.2 g	

5 µl of H_2O_2 was mixed with 15 ml substrate buffer.

Substrate

3'3'5'5'-Tetramethylbenidine dihydrochloride (TMB) (42 mM) was added to 1:2 acetic acid: distilled water. 150 µl of this solution was added to 15 mls substrate buffer.

Stop reagent

2M H_2SO_4 in distilled water

Binding Buffer for Fatty acids:-

NaCl	0.116 M	pH 7.4
KCl	0.0049 M	
$MgSO_4$	0.0012 M	
$NaPO_4$	0.016 M	

Electrophoresis:- (nanopure water used throughout)

Sample buffer

Tris base	0.15 g	
2-Mercaptoethanol	1.0 g	
Sodium Dodecyl Sulfate (SDS)	0.4 g	Samples were diluted 1:4 in sample
Bromophenol blue	0.02 g	buffer
Glycerol	2.0 ml	

Electrode buffer

Glycine	14.4 g	Dissolved in 1 l of nanopure water
Tris base	3.30 g	pH 8.1 - 8.4 which must not be
Methanol	200.0 ml	adjusted.
SDS	1.0 g	

Separating gel

3 M Tris HCL pH 8.8	1.25 ml	Recipe for two mini-gels
30 % acrylamide and		
0.8 % bisacrylamide	[a]4.0 ml	[a]de-aerated for 10 min under vacuum
10 % SDS	0.1 ml	
Ammonium persulphate	[b]50 µl	[b]made prior to use at 0.1 gml^{-1}
N,N,N',N'-Tetromethyl-ethylenediamine (TEMED)	5 µl	
Nanopure water	4.6 ml	

Stacking gel

3 M Tris HCL pH 6.8	0.4 ml	Recipe for two mini-gels
30 % acrylamide and		
0.8 % bisacrylamide	[a]1.25 ml	[a]de-aerated for 10 min under vacuum
10 % SDS	0.1 ml	
Ammonium persulphate	[b]80 µl	[b]made prior to use at 0.1 gml^{-1}
N,N,N',N'-Tetromethyl-ethylenediamine (TEMED)	8 µl	
Nanopure water	8.2 ml	

Phosphate Buffered Saline (0.02 M)

$NaH_2PO_4.2H_2O$	0.911 g	Dissolved in 1 l distilled

$Na_2HPO_4.12H_2O$	4.799 g	water (nanopure water for tissue culture) pH 7.2

Purification of antibodies by DEAE 52-matrix chromatography:-

DEAE-matrix was washed with 0.5 N HCL, then with 0.5 N NaOH through a glass sintered filter. The matrix was then washed with 20 volumes 10 mM Tris buffer (pH 8.5) before transferring to a column. Serum, ascites fluid or purified antibody which had been dialysed against 3 x 2l changes 10 mM Tris buffer (pH 8.5) was passed down the column with 10 volumes of 10 mM Tris buffer (pH 8.5). Antibody was eluted with x 3 volumes of 50 mM NaCl in 10 mM Tris buffer followed by x 3 volumes of 100 mM NaCl in 10 mM Tris buffer.

Acid treatment of sodium sulphate percipitated antisera:-

A solution of PBS/saline (pH 2.1) was added to sodium sulphate percipitated antisera to give a concentration of 3.6 mg ml^{-1} protein. The solution was stirred slowly for 30 minutes, before adjust the pH to 7.0 by adding solid Tris base.

Saline:- (0.85 %)

NaCl (pH 7.4-7.6)	8.5 g	dissolved in 1 l distilled water

Western Blot:-

(1) Transblot buffer

Glycine	14.4 g	Dissolved in 1 l nanopure water
Tris Base	3.03 g	

(2) Tris Buffered Saline (TBS)

Tris base	6.07 g	Dissolved in 1 l nanopure water.
Disodium EDTA	0.41 g	pH 8.0
NaCl	8.7 g	

(3) Tween-Tris Buffered Saline (TBS)

Tris base	6.07 g	Dissolves in 1 l nanopure water.
Disodium EDTA	0.41 g	pH 8.0
NaCl	8.7 g	
Polyoxyethylene Sorbitan Monolaurate	1.0 ml	

(4) Stock Substrate Solution

4-chloro-nanopure	0.15 g	Made freshly
methanol	50.0 ml	

APPENDIX 2 SUPPLIERS OF MATERIALS

Aldrich Chemical Co., Gillingham, England
celite

Amersham International, Amersham, England

^{3}H-thymidine
harvest mats (Skatron Inc., V.A., USA)
[1-^{14}C] fatty acids
Soluene-350 (Canberra-Packard Instruments, Berks, U.K.),
Ecoscint Scintillation fluid
Ecoscint (Canberra-Packard Instruments, Berks, U.K.)

Animals
Atlantic salmon:- Buckieburn fish farm (Stirling Salmon Ltd., Stirling
rainbow trout:- Swanswater fish farm (Stirling)
African catfish:- Institute of Aquaculture, University of Stirling
Rabbits:- University of Stirling animal house.

Biorad Labs. Ltd., Watford, Hertfordshire
Dye concentrate reagent
Low weight molecular markers
4-chloro-naphthol solution
nitrocellulose paper

BDH Poole, England
$CaH_4(PO_4)_2H_2O$
K_2HPO_4
$ZnSO_4.7H_2O$
KI

Chance Propper Ltd, England
circular cover slips (13 mm diameter)

E. Merck, Darmstadt, Germany
HPTLC 10 x 10 cm glass plates (silica gel 60)

Ewos Ltd., Westfield, West Lothian, U.K.
LT-fishmeal
salmon and trout diets

Fisons Loughbourgh, Leics., England.
$CaCO_3$
$NaH_2PO_4.2H_2$
$FeSO_4.7H_2O$

Flow Ltd., Rickmansworth, England
phenol red-free hanks buffered saline salts
lymphocyte separation medium
minimum essential medium modified with Earles salts (EMEM), without sodium bicarbonate
10 HBSS
Leibovitz L-15 medium

Fosol, Seven Seas, Hull, U.K.
Sunflower oil

Fish oil

Gibco Ltd., Paisley, Scotland
foetal calf serum
tryptic soya broth
tryptic soya agar

ICN Biomedicals Inc., High Wycombe, Great Britian.
α-Cellulose

Konica
X-ray film
LX 24 developer
FX 40 fixer

Lancer, Div. of Sherwood Medical, Athy, Eire
haematocrit capillary tubes

Marvel, Cadburys
casein solution

Millipore Corp., Bedford
0.22 µm and 1.2 µm filters

Northumbria Biologicals Ltd. (NBL), England.
96 well flat bottomed microtitre plate (Costar, NBL).
96 well round bottom microtitre plates
6 well tissue culture plate
Serum-free medium (SF-1)
ELISA plates (high protein binding, NBL)
glutamine (Northumbria Biologicals Ltd. (NBL), England.)
potassium benzyl-penicillin/streptomycin sulphate (pen/strep) (NBL),

Passelli WA4, Tunnel Avebe Starches, Gillingham U.K.
Starch

Pierce Ltd., Cambridge
storage vials

Rathburn Chemicals Ltd., Walkerburn, Scotland
acetone
chloroform
cristaseal
diethyl ether
ethanol
glacial acetic acid
hexane
methanol
methyl acetate
propan-2-ol
toluene

Scottish antibody production unit (SAPU), Carluke, Scotland
Sheep red blood cells

anti-rabbit IgG-HRP
anti-mouse IgG-HRP
anti-rabbit IgG-FITC
Anti-mouse IgG-FITC

Sigma Chemical Co. Poole, Dorset
chromogen/substrate
α-tocopherol acetate
11-propylene glycol
2-mercaptoethanol
2,7-dichlorofluorescein
3(4.5-di-methylthiazoyl-2-yl)2.5 diphenyltetrazolium bromide
agarose
ascorbic acid
benzocaine (ethyl-*p*-aminobenzoate)
biotin
bovine serum albumin
butylated hydroxyanisole
butylated hydroxytoluene
calcium pantothenate
chloramine T
cholecalciferol
choline chloride
citric acid
con A
LPS
phytohaemagglutinin
copper acetate
$CoSO_4.7H_2O$
crystal violet stain
$CuSO_4.5H_2O$
cyanocobalamin
dextrin
DMSO
ethylene glycol
fatty acids [18:1(n-9); 18:2(n-6); 18:3(n-3); 20:4(n-6); 20:5(n-3) and 22:6(n-3)]
ferricytochrome C
fluorodinitrobenzene
folic acid
Freunds incomplete adjuvant
Freunds complete adjuvant
gelatin
Giemsa stain
glutaraldehyde
H_3PO_4
hen egg white lysozyme
heparin (ammonium salt)
hepes (4-[2-hydroxyethyl-1-piperazine-ethanesulfonic acid])
horse radish peroxidase
KCl
KOH
malachite green
May-Grunwald stain
menadoine
$MgCO_3$

Micrococcus lysodeikticus
$MnSO_4.4H_2O$
myo-inositol
n-propyl gallate
NaCl
NaH_2PO4
Na_2HPO_4
NaOH
nicotinic acid
nitroblue tetrazolium
Percoll
phenol red
phorbol myristate acetate
poly-l-lysine
prednisolone
pyridoxine hydrochloride
retinyl acetate
riboflavin
SDS
APS
TEMED
sodium sulphate
sodium bicarbonate
Sodium caseinate
Sodium borohydride
superoxide dismutase
thiamine hydrochloride
trichloroacetic acid
trypan blue
trypsin
Tween 20

Whatman, England
No.1 filter papers
DE 52