# REPLACEMENT OF DIETARY FISH OIL WITH VEGETABLE OILS: EFFECTS ON FISH HEALTH

A thesis presented for the degree of Doctor of Philosophy to the University of Stirling

By

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# Declaration

Declaration

I hereby declare that this thesis has been composed by myself and the results presented are a product of my own work, except where otherwise stated.

No parts of this thesis have been submitted in any previous application for a degree.

# Acknowledgements

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# Abstract

The work presented in this thesis examined the effects of dietary fish oil replacement on fish innate and adaptive immune function, disease resistance, tissue histopathology and fatty acid composition of lipids in peripheral blood leukocytes.

Dietary trials with Atlantic salmon (*Salmo salar*), Sea bass (*Dicentrarchus labrax*), Atlantic cod (*Gadus morhua*) and Arctic char (*Salvelinus alpinus*) were conducted in which fish oil was replaced by rapeseed oil, linseed oil, olive oil, palm oil, echium oil or a mixture of these oils. A significant reduction in respiratory burst activity was most pronounced in salmon and sea bass fed high levels of rapeseed oil-containing diets. In addition, rapeseed and olive oil inclusion in the diets of salmon and sea bass significantly reduced the head kidney macrophage phagocytic capacity to engulf yeast particles. A reduction in prostaglandin $E_2$ levels was found to be related to a reduction in macrophage respiratory burst activity in salmon fed linseed oil diets and sea bass fed a dietary blend of linseed, palm and rapeseed oils. Changes in macrophage function may be a contributing factor causing a reduction in serum lysozyme activity observed in some trials. No significant differences were detected in cumulative mortality of Atlantic salmon fed an equal blend of linseed and rapeseed oils challenged with *Aeromonas salmonicida*. However, resistance to *Vibrio anguillarium* was significantly impaired in Atlantic salmon fed a blended oil diet containing linseed, rapeseed and palm oil. The major histological difference of fish fed vegetable oil diets was the accumulation of lipid droplets in their livers. Dietary fatty acid composition significantly affected the fatty acid composition of peripheral blood leukocytes. Generally, fish fed vegetable oil diets had increased levels of oleic acid, linoleic acid and α-linolenic acid and decreased levels of eicosapentaenoic acid, docosahexaenoic acid and a lower n-3/n-6 ratio than fish fed a FO diet.

In conclusion, the results from these studies suggest that farmed fish species can be cultured on diets containing vegetable oils as the added oil source. However, feeding high levels of some vegetable oils may significantly alter some immune responses in the fish, especially head kidney macrophage function, disease resistance and, in addition, may cause an increase in tissue histopathology.

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## Abbreviations

The following abbreviations were used throughout the text.

| | |
|---|---|
| 14:0 | lauric acid |
| 16:0 | palmitic acid |
| 18:0 | stearic acid |
| 18:1 (n-9) | oleic acid |
| 18:2 (n-6) | linoleic acid (LA) |
| 18:3 (n-3) | α-linolenic acid (ALA) |
| 18:3 (n-6) | γ-linolenic acid (GLA) |
| 18:4 (n-3) | stearidonic acid (SDA) |
| 20:4 (n-6) | arachidonic acid (ARA) |
| 20:5 (n-3) | eicosapentaenoic acid (EPA) |
| 22:6 (n-3) | docosahexaenoic acid (DHA) |
| 2:1 | 2 parts chloroform : 1 part methanol |
| 2-ME | 2-mercaptoethanol |
| ARA | arachidonic acid |
| ACP | alternative pathway |
| ADP | adenosine 5'-diphosphate |
| ALA | α-linolenic acid |
| ANOVA | analysis of variance |
| ATP | adenosine 5'-triphosphate |
| BHT | butylated hydroxytoluene |
| BKD | bacterial kidney disease |

| | |
|---|---|
| BSA | bovine serum albumin |
| CCP | classical pathway |
| CD2 | Cluster of differentiation 2 |
| cfu | colony forming unit |
| CMI | cell mediated immunity |
| CoA | Coenzyme A |
| Con A | concanavalin A |
| CPM | counts per minute |
| CRP | C-reactive protein |
| DAG | diacylglycerol |
| Δ | delta |
| DHA | docosahexaenoic acid |
| DMSO | dimethyl sulphoxide |
| DPA | docosapentaenoic acid |
| EFA | essential fatty acid |
| EIA | enzyme immunoassay |
| ELISA | enzyme linked immunosorbent assay |
| EPA | eicosapentaenoic acid |
| $F_{ab}$ | part of antibody molecule which contains the antigen-combining site |
| FAME | fatty acid methyl esters |
| FAO | Food and Agriculture Organisation |
| FBS | foetal bovine serum |

| | |
|---|---|
| $F_c$ | part of the antibody molecule responsible for binding to antibody |
| FCR | food conversion rate |
| FCS | foetal calf serum |
| FFA | free fatty acids |
| FO | fish oil |
| g | gram |
| GALT | gut-associated lymphoid tissue |
| GIT | gastrointestinal tract |
| GLA | γ-linolenic acid |
| GLC | gas liquid chromatography |
| h | hour |
| H&E | haematoxylin and eosin |
| $H^+$ | hydrogen ion |
| HBSS | Hank's balanced salt solution |
| HDL | high-density lipoprotein |
| HEPE | hydroxy-5, 8, 10, 14, 17-eicosapentaenoic acid |
| HEPES | 4-[2-hydroxyehtyl-1-piperazine-ethabesulfonic acid] |
| HETE | hydroxy-5, 8, 10, 14, 17-eicosatetraenoic acid |
| HI | humoral immunity |
| HPTLC | high performance thin-layer chromatography |
| HRP | horse radish peroxidase |
| HSWB | high salt wash buffer |

| | |
|---|---|
| HUFA | highly unsaturated fatty acids |
| ICAM-1 | intercellular adhesion molecule 1 |
| IFN | interferon |
| IFN-γ | interferon-gamma |
| Ig | immunoglobulin |
| IgG | immunoglobulin G |
| IL-1(-2, -3, -4 ...) | interleukin – 1 (-2, -3, -4...) |
| i.p | intraperitoneal |
| kg | kilogram |
| KOH | potassium hydroxide |
| L-15 | Leibowitz L-15 medium |
| LA | linoleic acid |
| LDL | low-density lipoprotein |
| LFA-1 | lymphocyte function-associated molecule 1 |
| LNA | linolenic acid |
| LO | linseed oil |
| LPS | lipopolysaccharide |
| LSWB | low salt wash buffer |
| LT | leukotriene |
| $LTB_4$ | leukotriene $B_4$ |
| $LTB_5$ | leukotriene $B_5$ |
| LX | lipoxin |
| M | Molar |

| | |
|---|---|
| MAF | macrophage activating factor |
| mag. | magnification |
| MeOH | methanol |
| mg | milligram |
| MHC | Major Histocompatibility Complex |
| MIF | macrophage inhibition factor |
| min | minute |
| ml | millilitre |
| mM | millimolar |
| MTT | 3(4,5-di-methylthiazoyl-2-yl)2.5 diphenyltetrazolium bromide |
| NaCl | sodium chloride |
| $NAD^+$ | nicotinamide adenine dinucleotide phosphate |
| NADPH | nicotinamide adenine dinucleotide phosphate (reduced) |
| NBT | nitroblue tetrazolium |
| NCC | non-specific cytotoxic cells |
| ng | nanogram |
| OD | optical density |
| OO | olive oil |
| °C | degrees Celcius |
| NK | natural killer cells |
| PBS | phosphate buffered saline |
| PC | phosphatidylcholine |

| | |
|---|---|
| PCV | packed cell volume |
| PE | phosphatidylethanolamine |
| Pen/strep | potassium benzyl-penicillin / streptomycin sulphate |
| PG | prostaglandin (e.g. PGA, PGD, $PGE_2$, $PGF_{1\alpha}$, $PGF_{2\alpha}$). |
| $PGI_2$ | prostacyclin |
| Pi | phosphate |
| PI | phosphatidylinositol |
| PIT tags | passive integrated transponders |
| PLA | phospholipase A |
| PMA | phorbol myristate acetate |
| PMN | polymorphonuclear cells |
| PO | palm oil |
| PS | phosphatidylserine |
| PUFA | polyunsaturated fatty acids |
| PWM | pokeweed mitogen |
| RBC | red blood cell |
| RIA | radioimmunoassay |
| RO | rapeseed oil |
| RPS | relative percentage survival |
| S.I. | stimulation index |
| S1/2 | salmon advanced to go to sea within 6 months |
| SD | standard deviation |
| SE | standard error |

| | |
|---|---|
| SDS | sodium dodecyl sulphate |
| SGR | specific growth rate |
| SOD | superoxide dismutase |
| SPB | sodium phosphate buffer |
| SRBC | sheep red blood cells |
| SW | seawater |
| TAG | triacylglycerol |
| TBS | Tris buffered saline |
| TCA | tricarboxylic acid cycle |
| TCR | T-cell receptor |
| TLC | thin layer chromatography |
| TNF-α | tumour necrosis factor-alpha |
| TSA | tryptone soya agar |
| TSB | tryptone soya broth |
| TX | thromboxane |
| $TXB_2$ | thromboxane $B_2$ |
| U | Unit |
| µg | microgram |
| µl | microlitre |
| µM | micromolar |
| UV | ultraviolet |
| VLDL | very low density lipoproteins |
| VO | vegetable oil(s) |

## Abbreviations

| | |
|---|---|
| WBC | white blood cell |

Chapter 1 – Introduction

# Chapter - 1 General Introduction

## 1.1 General Introduction

While production of fish meal and oil from feed-grade fisheries is static, or even in decline, the demand for fish meal and fish oil (FO), a basic constituent of commercial fish feed, is predicted to increase steadily resulting in elevated raw material prices. In the same way that alternatives to fish meal are being investigated, the potential exists to replace FO with vegetable oil (VO) in many fish species. In addition to its effects on growth, any changes to immune response, cellular function and tissue histology as a consequence of FO replacement in the diets of fish require investigation.

Many authors have addressed the issue of fish meal replacement in aqua-feeds, but the study of alternative lipid sources to date is relatively limited, especially with regard to fish health. Several studies have shown that fish such as Atlantic salmon (*Salmo salar*), Brook char (*Salvelinus fontinalis*) and juvenile turbot (*Scophthalmus maximus*) can be fed many different types of dietary fats and oils, with no apparent adverse effects (Polvi & Ackman, 1992; Bell *et al.*, 1993a; Bell *et al.*, 1993b; Guillou *et al.*, 1995; Dosanjh *et al.*, 1998). It is also documented that different dietary fats affect the fatty acid composition of the fish, as found in other animals and man. However, additional information is needed concerning the effects of dietary fats on the health and immune status of fish. It is well established that many freshwater fish, including salmonids, can metabolise $C_{18}$ polyunsaturated fatty acids (PUFA), abundant in many VO, to their long-chain highly unsaturated $C_{20}$ and $C_{22}$ products (Sargent *et al.*, 1995).

Currently, the culture of Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) relies heavily on marine FO, to supply essential fatty acids (EFA) and energy for the whole production cycle. The feed grade fisheries that supply these oils are currently diminishing and with global aquaculture production expanding the need to investigate alternatives to FO is clear. The global production of FO, based on fisheries landings, is static and it is predicted that by 2010 the fish feed industry will require more than 80 % of the total world production of FO (Barlow, 2000). In addition, natural phenomena, notably El Nino, in 1997/1998 caused a severe reduction in the South American feed grade fisheries creating shortages and price increases in feed raw materials. This is illustrated by the change in FO prices from 400 USD / Mt in 1996 to 700 USD / Mt in 1998. Although the supplies are now restored and the price of fish meal and oil are at their lowest level, it is still important to seek alternative oil sources so that should future supplies of fish meal and FO can be reduced, the aquaculture industry would have a secure and sustainable future.

The role of fish in human nutrition has attracted increasing public interest in recent years, as the expansion of aquaculture has substantially increased the availability of fish as a food source. Particular attention has been focused on the levels of lipid in the flesh of the fish, as consumers are continuously concerned about the amount and type of fat in their diet. Some of the currently cultured fish species, such as salmonids, contain a considerable amount of flesh lipid, while others, such as cod (*Gadus morhua*), are generally lean. Fish lipids, however, have been shown to have a favourable fatty acid composition, due to their high levels of PUFA. PUFA, especially those of the (n-3) series, which are abundant in fish lipids, have proven beneficial for human health, as they are essential for normal physiological functions (Carroll, 1986; Sargent, 1996), and are thought to be helpful in the

prevention of cardiovascular disease (Lampila, 1987), cancer and other chronic diseases (Carroll, 1986). The beneficial effects of the inclusion of FO in the diets of humans, farmed animals and poultry are well documented (Pike, 1999; Lee & Arm, 1988). Potential benefits include reduced risk or severity of cardiovascular disease, inflammatory disease, arthritis and asthma (Lee & Arm, 1988). These benefits are known to be the result of the long chain omega-3 (n-3) PUFA (Pike, 1999) in relation to the amount of omega-6 (n-6) PUFA present in the diet. The restricted availability and the high cost of FO have directed the interest of many researchers to identify alternative lipid sources to be used in aquatic feeds. Consequently, the high levels of lipid in fish have a high PUFA content, particularly (n-3) fatty acids, rich in eicosapentaenoic acid (EPA, 20:5(n-3)) and docosahexaenoic acid (DHA, 22:6(n-3)). Increased consumption of oily fish including, herring, sardines, mackerel and salmon can provide the necessary (n-3) PUFA in human diets. At the present time, salmon produced by aquaculture, using feeds containing only marine FO rich in (n-3) PUFA, is a valuable component of the human diet (Bell *et al.*, 1998a; Aursand *et al.*, 2000; Schmidt *et al.*, 2001). Therefore, substitution of FO with VO should be carried out in such a way that it ensures the product quality of the cultured salmon, and maintains the healthy image of seafood as part of the human diet.

## 1.2 Lipid composition of fish

### 1.2.1 *Lipids*

Lipids are a group of fat-soluble compounds occurring in the tissues of plants and animals and consist broadly of fats, phospholipids, waxes, sphingomyelins and sterols (Ackman,

1980; Eckey, 1954). Dietary lipids are important as a source of energy and EFA for fish, which are needed for normal growth and development (Watanabe, 1982). Dietary lipids serve a variety of important physiological and metabolic functions in fish and also assist in the absorption of fat-soluble vitamins (Castell, 1979; Watanabe, 1982; Sargent *et al.*, 1989). Fish cannot synthesise either linoleic acid (LA, 18:2(n-6) or α-linolenic acid (ALA, 18:3(n-3) *de novo*. Hence, one or both of these fatty acids must be supplied preformed in the diet (Owen *et al.*, 1975; Watanabe, 1982). In general, freshwater fish require dietary LA or ALA, or both; (Castell *et al.*, 1972a,b; Owen *et al.*, 1975; Takeuchi & Watanabe, 1977a; Kinsella *et al.*, 1977) whereas marine fish require dietary EPA and/or DHA (Owen *et al.*, 1972; Yone & Fujii, 1975a,b; Yamada *et al.*, 1980; Watanabe, 1982).

### 1.2.2 *Functions and effects of dietary lipids*

Relative to proteins and carbohydrates, the digestion and absorption of lipids is more complex. In salmonids, the proximal intestine and associated caeca are the major sites for absorption of lipids (Ostos Garriso *et al.*, 1993). Mono and diacylglycerols resulting from hydrolysis of triacylglycerols (TAG) and phospholipids are passively absorbed. The absorption of lipids has been studied in several teleost fish including rainbow trout (Lutton, 1983). Further studies are needed to elucidate the mechanisms of uptake for fatty acids by fish intestine and their relative importance.

The other product of TAG hydrolysis are free fatty acids which are re-esterified in the intestinal mucosal cells with glycerol, monoacylglycerols, and lysophospholipids to form TAG and phospholipids, respectively (Sire *et al*, 1981). These lipids are then transported to

the liver in large lipoprotein complexes, mainly chylomicron particles (Sire *et al.*, 1981) and very low-density lipoproteins (VLDL), via either the blood or lymphatic system (Bergot, 1981a). Some lipid can be stored in the intestinal epithelial cells as lipid droplets (Bergot, 1981a), but the majority is transported to the liver. Lipid is subsequently transported from the liver to the extrahepatic tissues in the form of the plasma lipoprotein complexes, VLDL, and low-density lipoprotein (LDL) (Fremont & Leger, 1981). High-density lipoprotein (HDL) is the main serum lipoprotein found in rainbow trout followed by LDL and then VLDL (Perrier *et al*, 1979). The structures, apoproteins, and lipid class compositions of fish plasma lipoproteins are similar to their mammalian counterparts (Fremont & Leger, 1981). Following digestion and transportation, some of the fatty acids once absorbed by cells and tissue, are metabolised as energy sources. The biosynthesis of fatty acids in fish, resembles that of mammals. The process outlined by Gurr and Harwood (1991), as shown in Figure 1.1, occurs mainly in the liver and to a lesser extent in the adipose tissue (Henderson *et al.*, 1985).

## 1.3 Polyunsaturated fatty acids (PUFA) and their role in fish.

The PUFA can be divided into two main groups omega-6 (n-6) and omega-3 (n-3), which have different physiological functions and effects. Humans and animals cannot synthesise PUFA from basic carbon sources and so require them through their diet. The main (n-6) PUFA are LA and its metabolites arachidonic acid (ARA, 20:4(n-6)) and γ-linolenic acid (GLA, 18:3(n-6)), occurring particularly in VO such as corn, sunflower, soyabean and

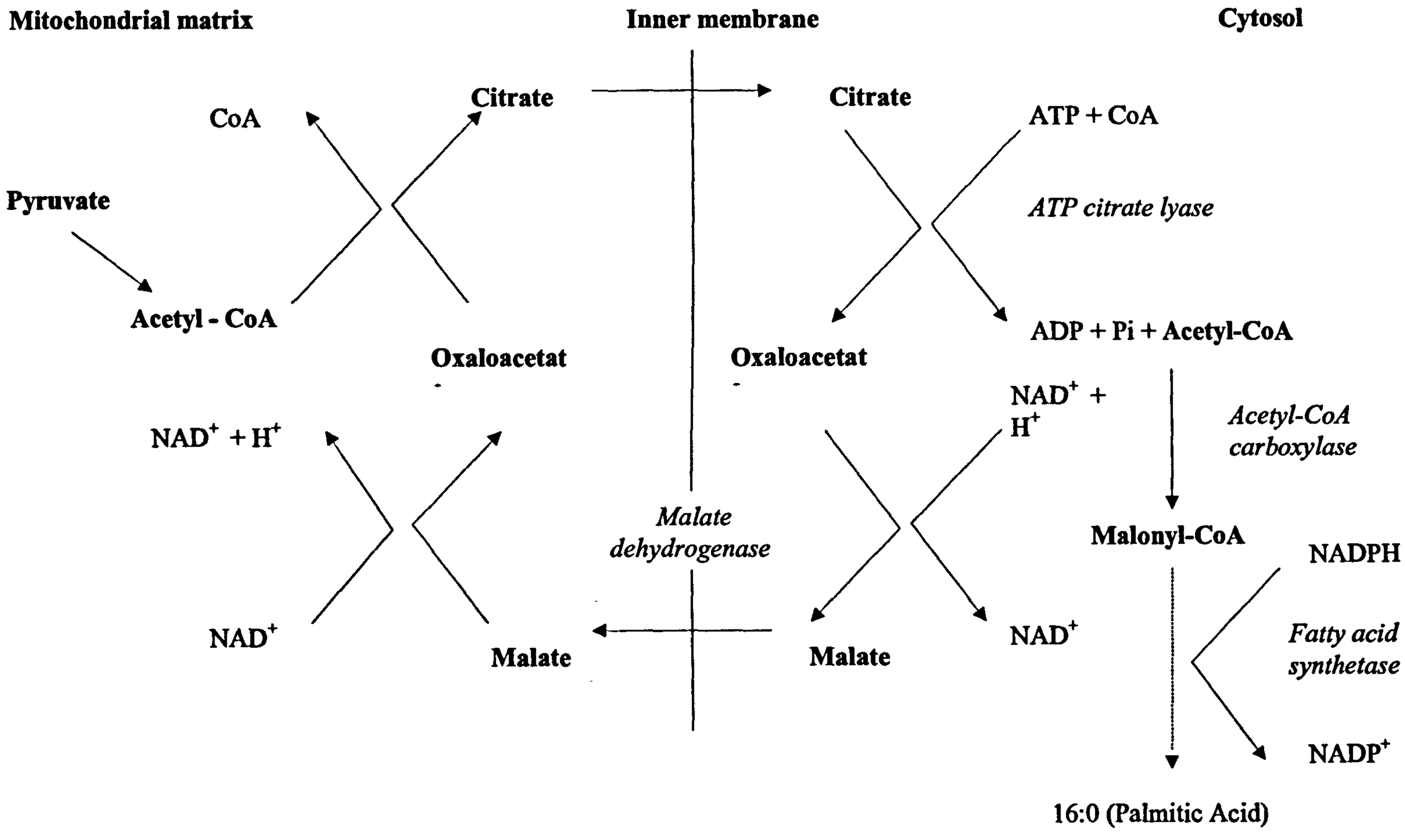
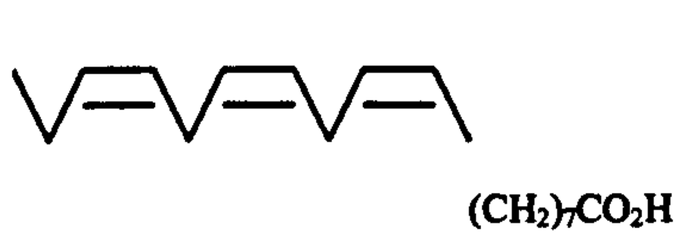



Figure 1.1 The biosynthesis of fatty acids in vertebrates.

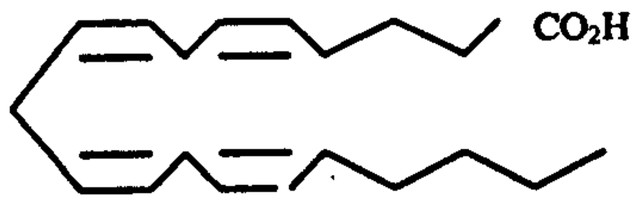
safflower oils (Bernardini, 1985). The main (n-3) PUFA is ALA acid found in soyabean, RO and LO, and its metabolites EPA and DHA, which are found predominantly in fish (Bernardini, 1985). The general structure of fatty acids is presented in Figure 1.2. The fatty acids, LA and ALA are transformed into longer-chain PUFA and their derivatives by enzymes called desaturases and elongases (Owen *et al.*, 1975; Castell, 1979; Ackman, 1980; Sargent *et al.*, 1989). Fish, like all other animals, do not possess the desaturases necessary for the formation of LA and ALA from oleic acid. Consequently, all (n-3) and (n-6) PUFA in fish lipids ultimately originate from (n-6) and (n-3) PUFA formed in plants (Sargent *et al.*, 1989). These same enzymes metabolise both (n-6) and (n-3) fatty acid groups and if the dietary intake of one is too great, metabolism of the other group can be impaired and this can lead to an imbalance in the production of prostaglandins (PG), leukotrienes (LT) and thromboxanes (TB) (Hwang, 1989; Johnston & Marshall, 1984). These hormone-like compounds are involved in important physiological processes including inflammatory reactions and modulation of the immune system. (Sargent *et al.*, 1989; Hwang, 1989; Johnston & Marshall, 1984). Consumption of (n-3) PUFA results in decreased cellular membrane ARA (20:4(n-6)) levels, and increased levels of EPA and DHA. EPA and DHA competitively inhibit the conversion of ARA to PG. Thus, a replacement of membrane ARA by (n-3) PUFA is accompanied by a decreased capacity of inflammatory cells to produce eicosanoids from ARA, such as $PGE_2$ and the 4-series LT (Hwang, 1989). The pathway of PUFA conversion to eicosanoids is shown in Figure 1.3.

Linoleic acid n-6 (LA)

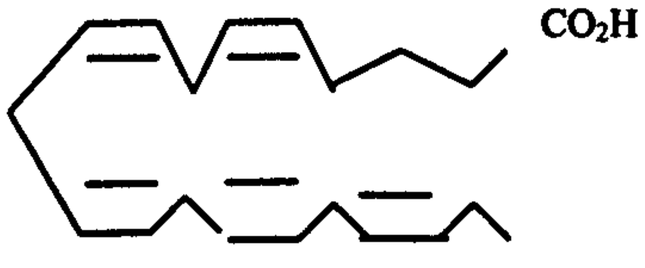
γ-Linolenic acid (GLA)



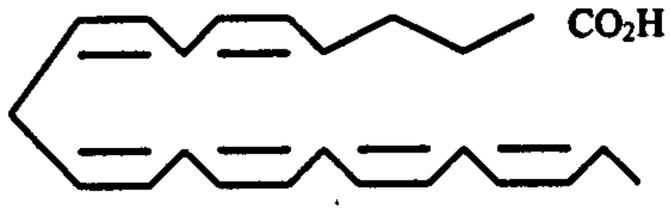
α-Linolenic acid (ALA)



Arachidonic acid (ARA)



Eicosapentaenoic acid (EPA)



Docosahexaenoic acid (DHA)

Figure 1.2 The structure of the principle fatty acids.

(Taken from the International Fishmeal and Oil Manufacturers Association - IFOMA No. 28 May, 1999).

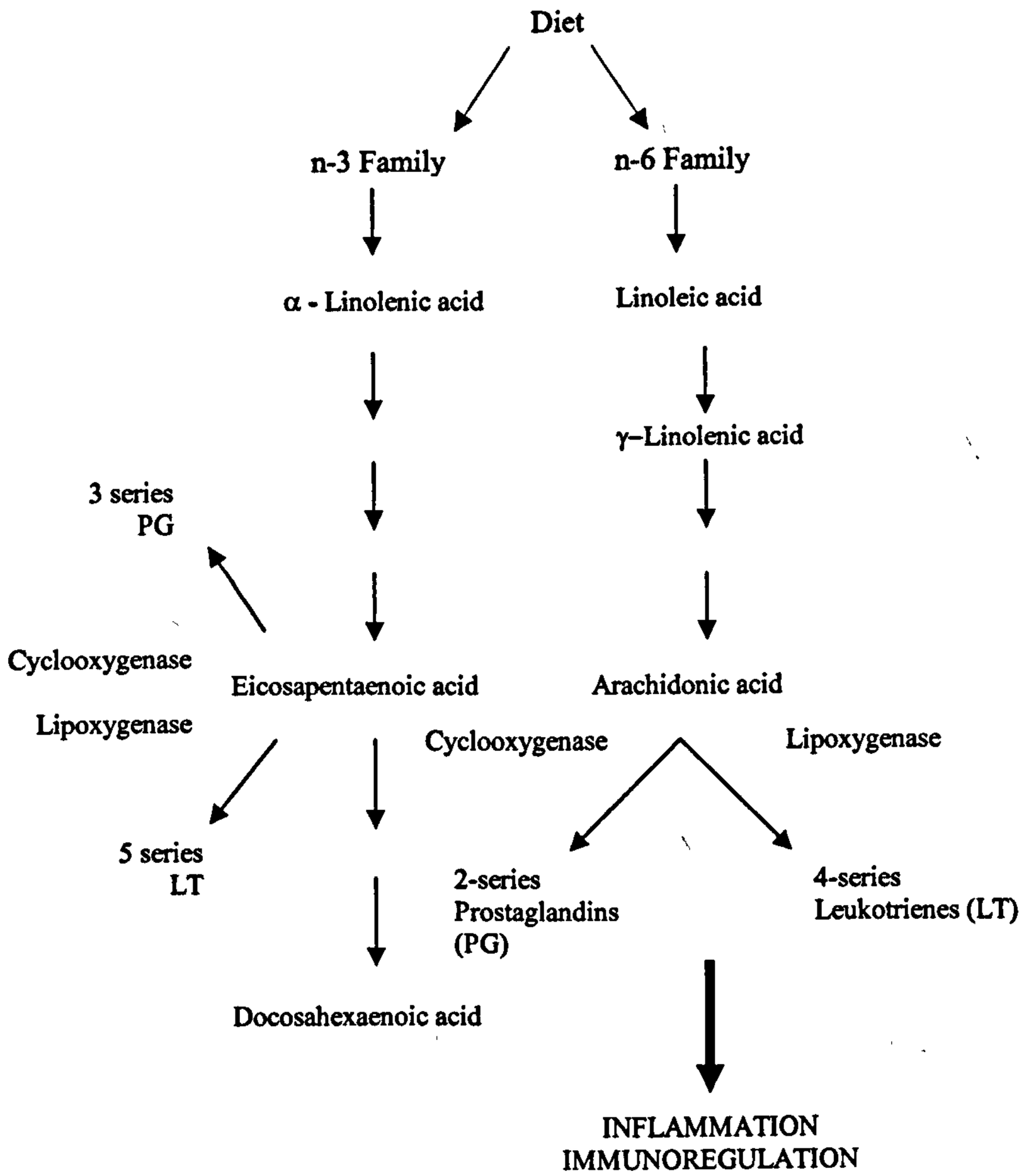



Figure 1.3 Metabolism of polyunsaturated fatty acids in animals.

(Adapted from Trends in Immunology Today, "Fat chance of immunomodulation" Vol.19 No.6 1998).

The pathways of elongation and desaturation of the EFA to form longer chain PUFA are shown in Figure 1.4. Garg *et al.* (1988, 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 LO (high in ALA) were combined with beef tallow, the replacement of (n-6) by (n-3) fatty acids was reduced considerably when diets containing LO were combined with safflower oil (high in LA). It has been suggested that the ALA content of lipid in the diets of rats has to be greater than 15 % to inhibit the conversion of LA to ARA (Kramer, 1980).

From Figure 1.4, the dietary EFA, ALA, is converted to its biologically active forms, EPA, and DHA, by a pathway whereby ALA is converted by a delta (Δ) 6 fatty acid desaturase to 18:4(n-3), which is chain elongated to 20:4(n-3) and then converted by a Δ5 fatty acid desaturase to EPA, which is further chain elongated to 22:5(n-3). It was believed until recently, that 22:5(n-3) was further converted to DHA by a Δ4 desaturase enzyme. However there was no substantial experimental evidence to suggest that Δ4 desaturase activity actually exists. As a result of studies carried out by Voss *et al.*, (1991, 1992) on rat liver microsomes, the authors discovered that 22:5(n-3) was efficiently chain-elongated to 24:5(n-3) and, further, desaturated to 24:6(n-3) as shown in Figure 1.4.

Voss and colleagues reported that the primary metabolic fate for 24:6(n-3) is chain shortening via limited β-oxidation to DHA. It is worth noting though, that this pathway is complicated by the movement of fatty acid intermediates between organelles in the cell as the partial degradation by β-oxidation appears to occur in the peroxisomes (Sprecher *et*

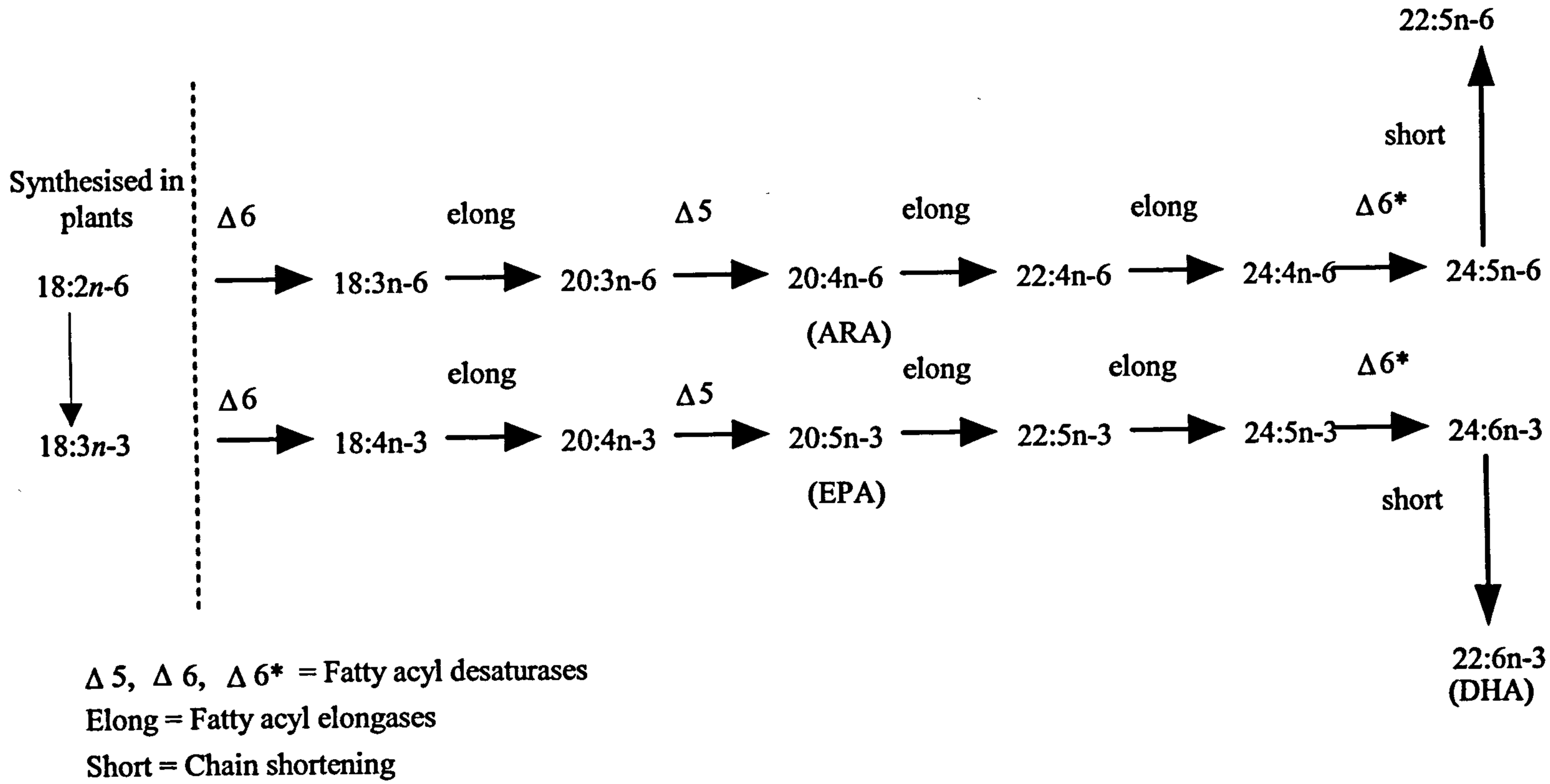



Figure 1.4 Metabolism of Essential Fatty Acids showing fatty acid desaturase and elongation activity (Sprecher pathway)

*al.*, 1995; Sprecher & Chen, 1999). Thus it is believed that the $C_{24}$ fatty acids move from the microsomes to the peroxisomes to be partially degraded to $C_{22}$ fatty acids.

Similarly, LA is desaturated by Δ6 and chain elongated to 20:3(n-6), and then converted by a Δ5 fatty acid desaturase to ARA which in turn can be elongated and then desaturated by Δ6 desaturase to generate 22:5(n-6). The same fatty acid desaturases and fatty acid elongases act on (n-6) and (n-3) intermediates, thus, there is competition between the two PUFA series (Owen *et al.*, 1975; Kinsella *et al.*, 1990; Buzzi *et al.*, 1996). A noteworthy feature of the pathway given in Figure 1.4 is that a Δ6 desaturation occurs twice, and there is continuing debate as to whether only one Δ6 enzyme exists or whether chain length specific enzymes exist (Voss *et al.*, 1992). There is good evidence now that the same Δ6 can desaturate both $C_{18}$ and $C_{24}$ substrates.

It has been established that rainbow trout readily converts ALA to DHA through the enzymatic reactions involving Δ5 and Δ6 desaturase activity (Owen *et al.*, 1975). Turbot were first believed to have an active Δ6 but deficient in Δ5 activity (Bell *et al.*, 1985). However, recent data on turbot cells are more consistent with a deficiency in the $C_{18}$-$C_{20}$ elongase multi-enzyme complex (Ghioni *et al.*, 1999) rather than a deficiency in Δ5 desaturase.

In general, therefore, freshwater fish convert ALA to DHA and also LA to ARA (Kinsella *et al.*, 1977), and marine fish are not capable of these conversions (Castell, 1979). However marine fish have little or no need to convert dietary ALA to EPA in the wild as they ingest plentiful quantities of DHA and EPA through their natural diet. Freshwater fish consume

substantial amounts of both ALA and LA and substantially less DHA in their natural diets and so it is normal for them to readily convert ALA to DHA and LA to ARA (Owen *et al.*, 1975; Kinsella *et al.*, 1977; Castell, 1979).

There are two main physiological functions of PUFA, firstly as precursors of eicosanoids and secondly, as essential constituents of biomembranes (Johnston & Marshall., 1984; Hwang, 1989; Kinsella *et al.*, 1990). In order to function correctly biomembranes must have the correct composition of lipid classes and fatty acids. Any alteration in the lipid structure and composition could profoundly influence cellular metabolism. Eicosanoids, which include PGs, affect many aspects of health both positively and, in some cases, negatively. Eicosanoids are formed from ARA, EPA, DHA and GLA, and the synthesis of various eicosanoids from ARA is illustrated in Figure 1.5. ARA is the major precursor of the eicosanoids in fish as well as in mammals. However, there is evidence that, in fish and other animals, production of eicosanoids from ARA is competitively inhibited by EPA, and also 20:3(n-6), with the eicosanoids from these latter two fatty acids being less active or having different activities from those produced from ARA. For example, feeding juvenile Atlantic salmon diets rich in sunflower oil with high levels of LA increased the levels of both LA and ARA in cell phospholipids. In addition it also increased levels of eicosanoids produced from ARA i.e. leukotriene $B_4$ ($LTB_4$) and thromboxane $B_2$ ($TXB_2$), and decreased eicosanoids produced from 20:5(n-3) i.e. $LTB_5$ and 12-hydroxeicosapentaenoic acid (Bell *et al.*, 1993a,b).

The amounts and types of eicosanoids synthesised following stimulation vary with tissue and cells (Goldyne & Stobo, 1981; Kinsella *et al.*, 1990). Eicosanoids have high biological

activity, are usually short-lived, and are produced by cells in very low concentrations to act in their immediate environment. Prostanoids are involved in haemostasis and thrombosis, renal function, vascular regulation, blood pressure and reproductive processes (Goodwin & Ceuppens, 1983). LT exhibit a number of potent effects, including bronchoconstriction, erythema and the activation of white blood cells and are, therefore, important mediators of inflammatory and hypersensitivity reactions (Johnston & Marshall, 1984; Hwang 1989; Kinsella *et al.*, 1990).

The first step in eicosanoid biosynthesis is the release of the PUFA from the cell membrane phospholipids, a reaction catalysed by phospholipase $A_2$ (Murota, 1982; Kinsella *et al.*, 1990). Depending on cell type, this free fatty acid is further metabolised by either cyclooxygenase or a lipoxygenase enzyme (Lands *et al.*, 1977; Johnston & Marshall, 1984; Hwang, 1989). The action of cyclooxygenase results in the formation of unstable cyclic endoperoxidases (Meade & Mertin, 1978). In the case of ARA, these cyclic endoperoxidases are the precursors of (i) prostaglandins $D_2$, $E_2$ or $F_2\alpha$ through isomeration; of (ii) prostacyclin ($PGI_2$) by the action of prostacyclin synthase; and of (iii) thromboxane $A_2$ ($TXA_2$) formed by thromboxane synthase (Figure 1.5).

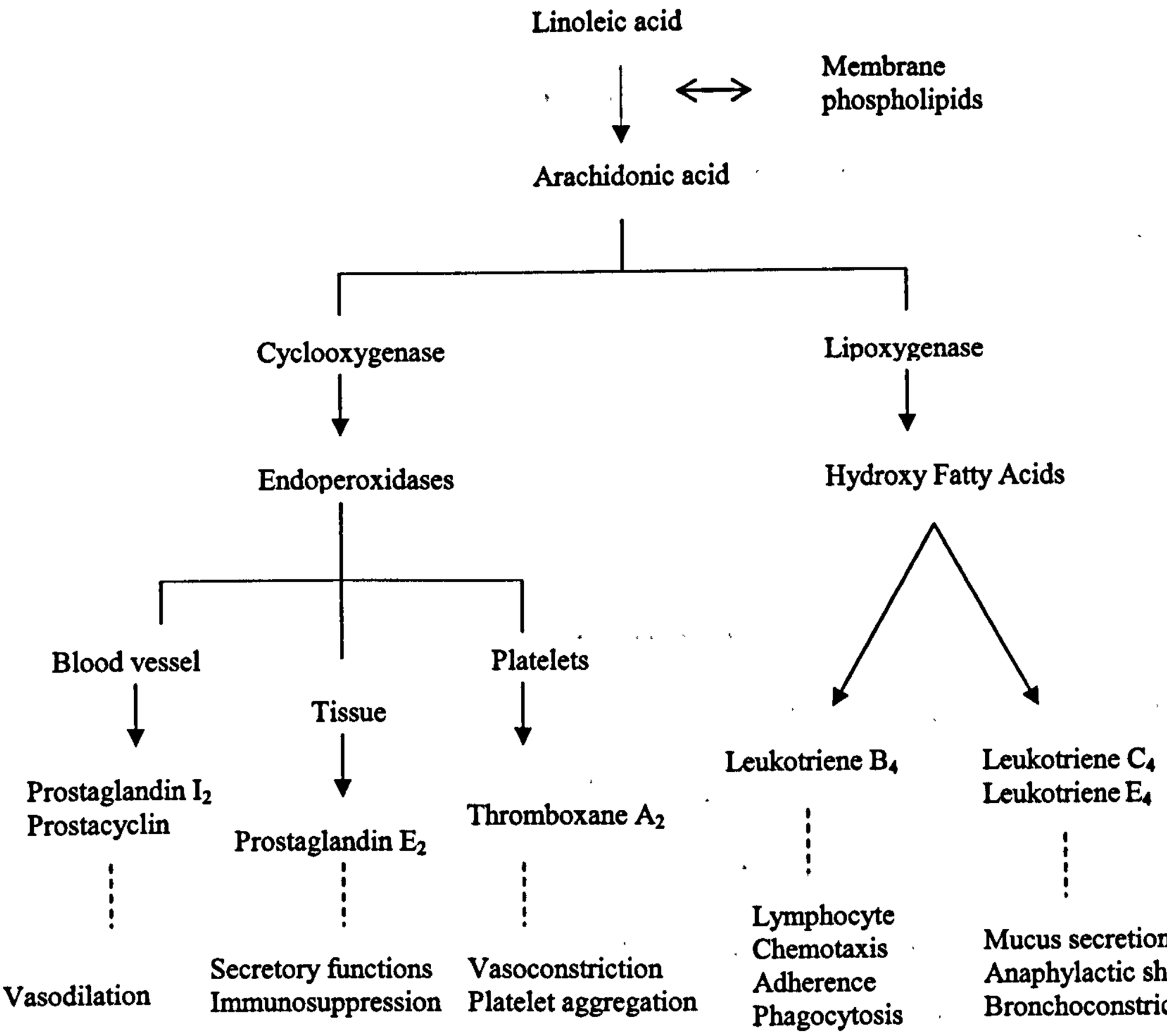



Figure 1.5 Synthesis of eicosanoids from ARA (20:4 n-6).

Castell *et al.*, (1972b) first documented the EFA deficiency signs in fish, and observed reduced growth, poor feed efficiency, increased mortality, and elevated muscle water content in rainbow trout fed diets deficient in (n-3) fatty acids. Indications related to lipid function included altered permeability of membranes, fatty degeneration of livers, decreased haemoglobin levels, and decreased red blood cell volume.

Kiron *et al.*, (1995) investigated the impact of EFA nutritional status on different mechanisms involved in the function of the immune system of rainbow trout. The authors found that the bactericidal activity of macrophage cells from rainbow trout *in vitro* was reduced when the diet was deficient in EFA. The macrophage activity was superior in all groups receiving EFA (LA, ALA and (n-3) HUFA) compared with the deficient group receiving palmitic acid. Antibody production was also reduced in rainbow trout receiving EFA deficient diets. In addition, Blazer *et al.*, (1989) studied the effect of lipids on disease resistance in channel catfish (*Ictalurus punctatus*) and suggested that dietary lipids may influence the levels of circulating antibody in fish.

It is now well documented that manipulating dietary EFA can modulate the amounts and types of eicosanoids synthesised in tissues (Hwang, 1989). Availability of the direct precursor acid is an important limiting factor in regulating the biosynthesis of eicosanoids in animal tissue. The amounts and types of precursor acids depend on the composition of fatty acids in tissue phospholipids, which, in turn, is influenced by the composition of dietary fatty acids. Accordingly, manipulating the composition of dietary fatty acids can also modify biological responses that are sensitive to eicosanoids.

## 1.4 Fish feed production

The production of feeds for aquaculture has so far been dependent on the use of fish meal and FO. The use of fish meal and FO in fish diets essentially involves the conversion of low value wild caught fish into higher value cultured animals. Fish meal and FO are not only excellent sources of highly digestible protein and energy, but they also provide essential amino acids, fatty acids, phospholipids, cholesterol, liposoluble vitamins, minerals and trace elements. Besides ecological and ethical opposition to the use of fish meal and FO as a feed ingredient, there is growing economic concern about the uncertain market availability and cost of fish meal and FO. The demand for fish meal and FO, as major ingredients in aqua-feeds, has steadily grown with the aquaculture expansion in the last decades. Future demands projected by Barlow (2000), who assumed moderate reduction of fish meal and FO inclusions, seem to predict a continuously high pressure on the fish meal and FO market and even a shortage in years with low fish meal and FO production. A more optimistic prediction was made by Tacon and Forster (2001), who assumed a drastic reduction in fish meal and FO inclusion in the coming decades, due to consumer pressure and market forces, resulting in acceptable supply/demand ratios for fish meal and FO.

There is a wide range of raw materials that make up a food mixture to meet the nutritional requirements of the fish for energy, amino acids, fatty acids, carbohydrates, vitamins and minerals. A major factor to consider in feed formulation is the total energy and protein/energy ratio of the final product. The protein content must be calculated according to the amino acid balance desired and the lipids included to satisfy the best fatty acid profile for the species concerned and the energy level required (Behnke *et al.*, 1994).

## 1.5 Fish oils

Lipid in feeds have a number of important functions, for example; energy source; intracellular energy reserve; transport form for metabolic energy; source of EFA to achieve normal growth and development; source of phospholipids (Sargent *et al.*, 1989; Watanabe, 1982). FO is the generic name given to preparations of oil extracted from the body tissues or livers of fish (e.g. herring, sardines, mackerel, cod). It has excellent proportions of the fatty acids required by most fish species. FO is rich in the long chain n-3 PUFA such as EPA and DHA. Typically these fatty acids comprise 15-30 % of the fatty acids present in FO with absolute amounts and ratios depending on the species used and the season (Calder, 2001a). The fatty acids, EPA and DHA derived from marine FO, often termed highly unsaturated fatty acids (HUFA), are nearly always the major dietary PUFA used in the production of farmed fish. FO are very different from VO and animal fats since they have a wider range of chain lengths (12-24 carbons), low levels of odd-numbered and branched chain fatty acids and higher degrees of unsaturation. For example, soyabean and rapeseed oils (RO) each contain about 10 different fatty acids in their TAG structure, compared with up to 40 different fatty acids that could be present in FO ranging from 12:0 to 24:1 (Tucker & Pigott, 1993). FO uniquely contain significant quantities of EPA and DHA. Some common fatty acids found in marine FO are shown in Table 1.1.

Table 1.1 Polyunsaturated fatty acid commonly found in FO.

| Fatty Acid | Menhaden | Sardine | Tuna | Herring | Mackerel | Salmon |
|---|---|---|---|---|---|---|
| 18:4 n-3 | 2.1 | 4.7 | 0.8 | 3.0 | 1.3 | 1.4 |
| 20:3 n-3 | 0.2 | - | - | - | - | 2.0 |
| 20:4 n-3 | 1.7 | 0.5 | 2.0 | 0.7 | 4.0 | 2.0 |
| 20:5 n-3 | 16.7 | 7.4 | 6.9 | 9.0 | 11.5 | 3.2 |
| 22:5 n-3 | 1.9 | 0.6 | 2.2 | 0.6 | 1.8 | 5.1 |
| 22:6 n-3 | 7.0 | 5.2 | 19.7 | 4.0 | 15.1 | 5.2 |

(William E. M. Lands, 1986c, Fish and Human Health)

## 1.6 Vegetable oils

Almost all plants contain fats or oils, mainly in their seeds (e.g. linseed, soybean). Five main fatty acids are found in most VO, namely, myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1(n-9)) acids and LA, with some allowance for ALA (Eckey, 1954). They are straight-chain homologous series of saturated and unsaturated carboxylic acids of even number chain lengths from $C_8$ to $C_{24}$ (Bockish, 1993). VO are largely TAG that are liquid at room temperature, but differ greatly in fatty acid composition (Table 1.2). They can be classified as short-chain ($C_{6-8}$), medium-chain ($C_{10-12}$), and long-chain ($C_{14-18}$) and may be saturated or have one, two or three double bonds of the *cis* configuration (Bockish, 1993). VO generally have a relatively high concentration of PUFA with the exceptions being coconut and palm oils, which are primarily saturated, and olive and RO, which have relatively less PUFA and more monounsaturated fatty acids. The most abundant PUFA is LA, with linseed, RO and soya oils having appreciable concentrations of ALA (Bockish, 1993), whereas ARA, DHA and EPA are all absent in plant oils (Volkman *et al.*, 1989).

The fatty acid composition of lipids in different oils and fats is illustrated in Table 1.2 (Pike, 1999).

The world's annual production of plant oils is a hundred times higher than that of FO (Bimbo, 1990), with the major producers being the United States, China, Brazil, India, Russia, Argentina and Canada. The current solution to the problem of high fish meal and FO prices is partial replacement by vegetable protein and oil sources. These alternative ingredients exhibit numerous disadvantages like low nutrient content, anti-nutritional factors, imbalanced amino acid and fatty acid profiles. However there are also numerous advantages to their use, for example, there would be a more consistent supply of raw materials, lower cost, the lack of a 'fishy' taste may be a bonus to some consumers and with regard to replacing the marine FO with VO, there would be reduced levels of dioxins and polychlorinated biphenyls (PCB).

Potential changes in product quality resulting from VO inclusion in fish feeds should be minimised if the health benefits of fish in the human diet is to be maintained. To achieve these goals, any oil replacing FO should provide sufficient energy in the form of saturated and monounsaturated fatty acids, and other easily catabolised fatty acids, to maintain high growth and development and optimal health.

RO is a potential candidate for FO substitution as it has both moderate levels of LA and ALA, and is rich in oleic acid. In addition, the ratio of ALA/LA in RO of 1:2 is regarded as

Table 1.2 Fatty acid composition of lipids in oil and fats (as g / 100g fatty acids).

| Lipids in oil/fats | Total n-6 | $C_{18}$ n-3 | $C_{20\geq}$ n-3 | Total n-3 | n-6 : n-3 |
|---|---|---|---|---|---|
| Menhaden | 3.9 | 0.9 | 23 | 23.9 | 0.16 |
| Herring | 3.3 | 0.6 | 16.2 | 16.8 | 0.20 |
| Animal (pig) | 11 | 0.6 | 0 | 0.6 | 18.30 |
| Soyabean | 54.4 | 7.1 | 0 | 7.1 | 7.67 |
| Maize | 50.4 | 0.9 | 0 | 0.9 | 56 |
| Linseed | 14.5 | 56 | 0 | 56 | 0.26 |
| Rapeseed | 29.5 | 10 | 0 | 10 | 2.95 |
| Cotton seed | 27.5 | 0 | 0 | 0 | >100 |
| Sunflower | 65 | 0.1 | 0 | 0.1 | >100 |

beneficial to human health and should not be detrimental for fish health (Ackman *et al.*, 1990). A number of earlier studies have utilised RO in feed formulations for salmonids and no changes in growth rates and feed conversion were observed (Tocher *et al.*, 2000; Dosanjh *et al.*, 1988; Guillou *et al.*, 1995; Polvi & Ackman, 1992). Linseed oil (LO) is also a potential candidate for FO replacement being rich in ALA, the substrate for synthesis of n-3 PUFA, and also contains significant levels of LA in a ratio of 3-4:1. LO has also been used in a number of studies, either alone or blended with RO, with no apparent detrimental effects on fish growth (Bell *et al.*, 1997; Tocher *et al.*, 2000; Sowizral *et al.*, 1990; Yang & Dick, 1994a). However, some of these studies used purified, or semi-purified diets with relatively low dietary lipid levels and were conducted for relatively short periods of time, compared with the trials carried out in this thesis.

## 1.7 The Immune system in fish

The ontogeny of the fish immune response is a vast subject but has been comprehensively reviewed by Tatner (1996). The immune system of fish is broadly categorised into specific or acquired immunity and non-specific or innate immunity. However, both systems use cellular and humoral mechanisms to provide protection against infection. In comparison, fish depend more heavily on non-specific defence mechanisms than mammals (Landolt, 1989). The specific immune system is induced by an initial interaction between an antigen and lymphocytes resulting in an initial production of antibodies against the antigen and the development of a memory response. The memory response enables a rapid secondary response upon re-exposure to an antigen. Long-term recognition of specific antigens may

be dependent on the ability of fish to retain antigens for extended periods of time. For instance, Atlantic salmon retain *Aeromonas* lipopolysaccharide (LPS) for up to sixteen weeks in lymphoid tissues (Press *et al.*, 1996). However, this capability apparently does not enhance immune responses to later exposure. The control mechanisms of specific immune responses, as in mammals, seem to involve T-helper and suppressor cells (Ruglys, 1985).

The non-specific defence system in fish is often the first line of defence against pathogens and consists of a multitude of cells, proteins and peptides in tissues and body fluids (Robertsen, 1999). Non-specific immunity includes host defences such as physical barriers for example mucus; enzymes e.g. lysozyme; phagocytic cells and blood proteins (Ellis, 1988).

Two populations of lymphocytes mediate specific immunity. One population is responsible for antibody (immunoglobulin, Ig) secretion and express Ig on their surface. The second is responsible for direct cell killing and regulation of immune responses via a range of secreted cytokines (Secombes, 1994a) and are characterised by the presence of a different type of antigen receptor, the T-cell receptor (TCR). These separate populations of lymphocytes are equivalent to T- and B-cells (Clem *et al.*, 1991). Early research found that teleosts produce predominantly one antibody of a single class, IgM (Ellis, 1982; Wilson & Warr, 1992) which appears primarily as a tetramer. However it is now known that fish have structural variations amongst the immunoglobulins that are produced (Killie *et al.*, 1991). Fish immunoglobulins have a similar structure to the mammalian types i.e. composed of heavy and light polypeptide chains with a variable antigen binding site (Fab) and a constant carboxyl-terminal effector region (Fc). It is generally considered though, that fish antibodies are of lower affinity and diversity than those found in birds and

mammals, however they do display good memory and specificity (Warr, 1997). Interactions between cells of the immune system are mediated not only by cell-cell contact but also through the release of soluble factors. Cytokines play a regulatory role within the immune system, and their range is usually limited to cells in the immediate vicinity of the cytokine producing cell. Such cytokines are IL-2, Interferon-gamma (IFN-γ)/Macrophage activating factor (MAF), chemotactic factor and macrophage inhibition factor (MIF) (Secombes, 1991).

The peripheral blood in teleosts shows both mature and immature cells at various transitional stages of development. Erythrocyte numbers vary with species but usually are in the range of $1.05 - 3.0 \times 10^6$ mm$^3$. These cells are ovoid in shape and 13-16μm long and 7-10μm broad and possess a centrally located nucleus. A variety of leukocyte types are involved in non-specific cellular defences of fish, and include monocytes/macrophages, granulocytes, and non-specific cytotoxic cells (NCC) the latter being equivalent to mammalian NK cells (Evans *et al.*, 1992). Monocytes form about 0.1 % of the circulating leucocyte population and are between 9-25 μm in diameter and contain small granules in their cytoplasm with a large nucleus. Macrophages and granulocytes are mobile phagocytic cells found in the secondary lymphoid tissues and in the blood. These cell types are particularly important in inflammation. Changes in macrophage activity are often observed by changes in their phagocytic and respiratory burst activity. Although macrophages are often classed as components of the non-specific immune response, they are not independent of the specific immune response. Activated macrophages are known to act as antigen presenting cells (APC) triggering lymphocyte activation (Secombes & Fletcher; 1992). The development of inflammatory reactions is controlled by a number of mediators, including

cytokines, eicosanoids, complement factors and other vasoactive compounds released by phagocytes. Lymphocytes are separated into large (10-15 μm diameter) and small (7-10 μm diameter) categories, which may represent different functional states. In fish, lymphocytes constitute 70-90 % of the total number of leucocytes and are round or oval in shape with a nucleus occupying virtually the whole cell with the cytoplasm often showing a pseudopodia-like projection on the cell surface. Neutrophils are morphologically similar to those in mammals and are commonly found at sites of inflammation. The nucleus is often kidney shaped although mature cells can have around a five lobed nuclei and cell size can vary from 4 to 13 μm in diameter.

Phagocytosis is the process whereby cells, internalise, kill and digest invading microorganisms. Phagocytes are attracted to a site of infection by both pathogen and host chemo-attractants e.g. complement components and eicosanoids (Secombes *et al.*, 1992). On arrival the phagocytes attach themselves to the microorganisms via their non-specific cell surface receptors, ingest the pathogen by the formation of a phagosome and finally, attempt to kill the invading microorganism.

Humoral molecules present in the blood and mucus of fish also have a major role in the non-specific defence system. These substances are predominantly proteins or glycoproteins and include lysozyme, complement, anti-proteases, transferrin and C-reactive protein (CRP). These substances are able to directly lyse bacteria, or fungi to inhibit bacterial or viral replication, to act as opsonins to increase uptake of bacteria by phagocytes and to neutralise bacterial proteases.

There are various immune parameters that may possibly be used as markers for disease resistance, lysozyme is one. In fish, lysozyme plays an important role in the host defence mechanisms against infectious diseases. It is distributed mainly in tissues rich in leucocytes, such as the head kidney, at sites where the risk of bacterial invasion is high, such as the skin, the gills, and the alimentary tract, and the eggs. (Fletcher & White, 1973; Murray & Fletcher, 1976; Siwicki & Studnicka, 1987; Grinde, 1989; Mock & Peters, 1990; Yousif *et al.*, 1991). 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 & 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. Lysozyme occurs mainly in neutrophils and monocytes of fish with smaller amounts detectable in macrophages (Ingram, 1980). Its activity has been detected in fish with levels varying between species (Mock & Peters, 1990); for example, rainbow trout has a twenty times greater activity of the enzyme than Atlantic salmon. In addition, lysozyme concentrations increase during infection and injection of foreign material (Fletcher & White, 1973; Siwicki & Studnicka, 1987). Although lysozyme has a direct antibacterial and antiviral role it can also enhance phagocytosis by acting as an opsonin, or by directly activating macrophages and leukocytes (Jolles & Jolles, 1984).

The complement system is one of the major effector pathways of the process of inflammation. It consists of a group of about twenty serum proteins that include several acute phase proteins. Fish have a serum protein system comprising of a large number of complement components (Sakai, 1992). Bony fish possess a complement system similar to mammals, consisting of two pathways, the classical and the alternative pathway. A number

of microorganisms spontaneously activate the complement system, via the "alternative pathway" (ACP), which is an innate, non-specific reaction. This results in the complement molecules covering the entire microorganism, leading to its uptake by phagocytes. The complement system can also be activated by antibodies bound to the pathogen surface, ("classical pathway" (CCP)), when it constitutes a specific, adaptive response. To date the existence of both pathways have been shown in salmonid fishes (Nonaka, *et al.*, 1981a,b). Complement activation is a cascade reaction with each component sequentially acting on others. The consequences of the complement activation are (i) opsonisation, (ii) activation of leukocytes, and (iii) lysis of target cells. The complement pathway may have an anti-viral function also. Sakai (1992) demonstrated that complement is capable of inactivating pathogenic fish viruses.

The major lymphoid tissues of the fish are the thymus, kidney and spleen (Rowley *et al.*, 1988). The thymus in most teleosts is a paired lymphoid organ located near the gill cavity. The thymus produces large numbers of lymphocytes and is thought to be the main source of immunocompetent T-cells (Chilmonczyk, 1992). The kidney is usually the most important lymphoid tissue and is subdivided into the head kidney (pronephros) and the middle kidney (mesonephros). This organ includes lymphocytes in all stages of development, suggesting that it has a role as both a primary lymphoid tissue and a secondary one, and it is the main producer of antibodies in fish (Ellis, 1982). Both the spleen and the gut-associated lymphoid tissue (GALT) constitute the main peripheral lymphoid organs of the fish. The spleen is a large, blood-filtering organ, which is efficient in trapping and processing antigens. However, the lymphoid tissue of a teleost is poorly developed (Zapata & Cooper, 1990). The GALT consists of lymphoid tissue that contain mainly lymphocytes,

macrophages, and plasma cells as well as different types of granulocytes. In all teleosts, diffuse accumulations of lymphoid tissue appear along the gut (Doggett & Harris, 1991). In addition, macrophages appear in the intestinal epithelium as well as in the lamina propria and are involved in scavenging and/or antigen presentation.

## 1.8 Tissue Histology

A diagrammatical illustration of the anatomy of a teleost fish showing the major organs present is shown in Figure 1.6. The intestine of most teleost species can be divided into two regions, often called the proximal (anterior) and the distal (posterior) regions. The walls of the intestine do not include a submucosa or muscularis mucosa, however, it does include stratum compactum and stratum granulosum in the mucosa. It also needs to be recognised that unlike other vertebrates, the intestines of fish can include accessory structures that increase absorptive surface area, for example pyloric caecae, which are extensions of the proximal intestine. Among fish there is phylogenetic variation in the organisation of the mucosa, with the structure ranging from folds to villi (Kanou, 1984). Microvilli dimensions are responsive to diet composition and environmental conditions, with changes probably mediated by hormonal signals (Nonnotte *et al.*, 1995).

The intestinal epithelium of fish includes four basic cell types, and all originate from stem cells located at the base of each villous fold. Three of the cell types migrate up the folds; these include the enterocytes, mucous secreting goblet cells, and enterochromaffin cells and are considered to have endocrine functions.

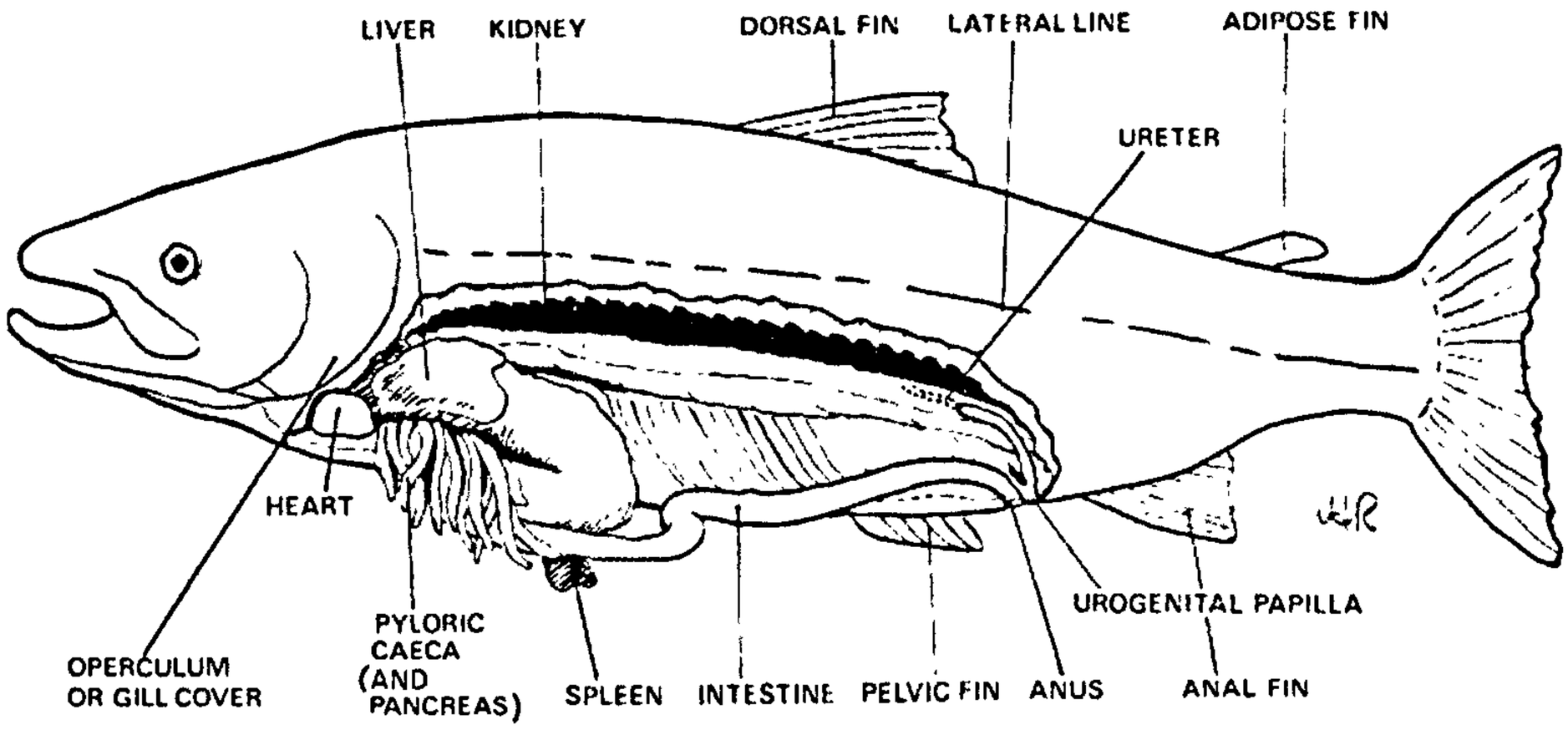



Figure 1.6 The anatomy and physiology of teleosts adapted from the Handbook of Trout and Salmon Diseases R.J.Roberts and C.J. Shepard, 1997.

The fourth type, Paneth cells, migrate deeper into the tissue where they are thought to be involved in enteric immune functions. The relative proportions and functions of the different cell types vary between species and regions of the intestine and this has been related to feeding habits (Murray *et al.*, 1996). Intestinal structure can respond rapidly and reversibly to changes in dietary intake. The intestines of fish fed commercial formulated feeds are known to be less diverse, which is thought to reflect the more consistent composition compared to natural diets (Deplano *et al.*, 1991a,b).

The metabolic activities of the liver are essential for providing fuel to the brain, muscle and other peripheral organs. Most compounds absorbed by the intestine pass through the liver,

which enables it to regulate the level of many metabolites in the blood. As previously mentioned the liver also plays a central role in the regulation of lipid metabolism, where fatty acids are synthesised, esterified, and then secreted in the blood in the form of plasma lipoprotein complexes. The teleost liver is a relatively large organ, as it is in mammals and is supplied with venous blood by the hepatic portal system. The histology of fish liver differs from mammalian in that the hepatocytes are not arranged into lobules or cords as they are in mammals. Fish hepatocytes are polygonal and have a distinctive centrally located nucleus.

The circulatory system of fish is anatomically simple, compared with that of higher vertebrates. The blood circulates from the heart to the gill arches, where it becomes oxygenated by diffusion through the epithelium of the gill lamellae. It is then distributed via the dorsal aorta to the arteries and peripheral capillaries and returned to the heart through the venous system. The walls of the heart are made of three layers; an inner endocardium, a medial endocardium and a peripheral pericardium. The endocardium consists of an endothelium and a subendocardium of loose connective tissue that attaches the endocardium to the myocardium. The pericardium consists of two layers; the visceral epicardium, a single layer of mesothelial cells and underlying connective tissue that encloses the heart. In addition, in the ventricle there is a distinct outer compact layer of muscle and an inner spongy layer with numerous trabeculae.

From the available literature there does not seem to be a vast amount of information on any pathological changes in fish tissues caused by the substitution of FO with VO. Cowey *et al.*, (1976a) reported pathological changes comprising thickening and distortion of fat cell

membranes, increased vascularisation and some loss of cellular integrity in the lateral muscle wedge and the liver of turbot fed hydrogenated coconut oil and to some degree corn oil. Studies carried out by Bell *et al.*, (1993a) reported that fish fed a sunflower oil diet developed a marked cardiac histopathology and increased heart phopholipase A activity. This pathology was evident in a less severe form in fish given a control FO diet, but was virtually absent in fish fed LO. The authors concluded that feeding diets high in 18:2 (n-6) can result in undesirable pathophysiological conditions that may, in part, be due to alterations in fatty acid composition and metabolism.

In a more recent study Olsen *et al.*, (1999) reported major ultrastructural differences in the enterocytes of Arctic char (*Salvelinus alpinus*) fed LO or soybean lecithin. In fish fed the LO diet there were substantial accumulations of lipid droplets in the enterocytes from the pyloric caecae and midgut but not the hindgut. These accumulations were not observed in fish fed the soybean lecithin diet. In some cases, deposition of fat in the enterocytes was so great that it resulted in epithelial damage with lipid droplets and cell debris appearing in the intestinal lumen. It was suggested that these changes were likely to be pathological and may lead to intestinal malfunction and represent a major infection route to pathogenic bacteria.

## 1.9 Nutrition and Fish Health

Overall diet and dietary components affect the immune system of fish as well as mammals. *In vivo*, the fatty acid composition of the cells of the immune system can also be altered by diet (Meade & Mertin, 1978). Nutrient deficiencies, imbalances or excess may impair the

proliferation and differentiation of both lymphoid and non-lymphoid stem cells; the capacity of immune cells to recognise foreign stimuli, alter the proliferative responses of the various cells, impair antigen presentation, or reduce phagocytic and cytolytic capacity of cells. Nutrition may also indirectly affect the immune response by altering metabolic and endocrine parameters that modulate immune functions (Kinsella *et al.* 1990).

In mammals, Ferber & Resch, (1977) showed *in vitro* that when a lymphocyte is activated there is a marked turnover in membrane fatty acids with increased incorporation of PUFA. Incorporation of PUFA into lymphocyte membranes enhances the cytotoxic capacity of restimulated memory cells, whereas saturated fatty acids lead to decreased cytotoxic functions (Bialick *et al.*, 1984). In addition, dietary (n-3) PUFA markedly affect lymphocyte functions (Bly *et al*, 1990). Feeding laboratory animals' diets rich in FO resulted in decreased mitogen-stimulated lymphocyte proliferation, decreased natural killer (NK) cell activity and decreased production of interleukin-2 (IL-2) by lymphocytes (Calder, 1998a,b). Evidence of a decreased expression of the IL-2 receptor on activated lymphocytes and a reduced level of expression of the adhesion molecules intercellular adhesion molecule 1 (ICAM-1), CD2 and lymphocyte function-associated molecule 1 (LFA-1) on the surface of lymphocytes has been found. Feeding FO also resulted in decreased production in IL-1, IL-6 and tumour necrosis factor-α (TNF-α) by inflammatory macrophages, decreased expression of major histocompatibility complex (MHC) class II molecules and adhesion molecules on the surface of macrophages, decreased expression of MHC class II, ICAM-1, CD2 and LFA-1 on dendritic cells, and decreased ability of dendritic cells to present antigen (Hughes & Pinder 2000; De Pablo & De Cienfuegos, 2000).

The relatively new field of nutritional immunology in fish is the study of the interactions between nutrients and the immune system. Changes in the fatty acid composition of the lipids in the diet of fish have been shown to affect the non-specific defence mechanisms, (Sheldon & Blazer, 1991; Obach *et al.*, 1993; Waagbϕ *et al.*, 1993a,b, 1995) the specific immunity (Erdal *et al.*, 1991; Waagbϕ *et al.*, 1993a,b, 1995; Fracalossi & Lovell, 1994; Thompson *et al.*, 1996) and the resistance to infectious diseases (Salte *et al.*, 1988; Erdal *et al.*, 1991; Waagbϕ *et al.*, 1993a; Fracalossi & Lovell, 1994; Thompson *et al.*, 1996). The majority of these studies were carried out with diets containing lipid levels below 25 % and at present commercial diets for Atlantic salmon can contain lipid levels of 30 % and above.

There is little information on the effect of plant oils on serological parameters of fish and on their non -specific immune system. Most studies have assessed the suitability of various dietary lipid sources for finfish species in terms of growth, feed utilization and flesh quality. By contrast, relatively little attention has been directed toward the possible adverse effects of novel lipid sources of varying fatty acid composition on the immune response and disease resistance of fish. The latter effects could potentially occur, especially if tissue levels of eicosanoid precursors such as ARA and EPA are altered.

Researchers are beginning to realise that fish feeds producing the fastest growth may not provide for the best disease resistance (Blazer, 1992; Sealey & Gatlin, 1999). Early fish nutrition studies were based strictly on growth and feed conversion. Attention is now focused on the interactions of nutrients, physiological effects, disease susceptibility and overall health. In addition, the nutritional state is known to influence immune functions

(Landolt, 1989; Blazer, 1992) with dietary protein of critical importance. The overall resistance of fish to disease is therefore dependent on the nutritional status of the fish.

The majority of research into the effects of dietary fatty acids on the immune response of finfish has primarily concentrated on teleost fish that are commercially important for aquaculture. Therefore, most of the studies have been carried out using salmonids. A balanced diet has long been recognised as a necessity in preserving health and maintaining the animal's ability to resist diseases (Lall & Oliver, 1993). Nutrients may alter immune responses by acting on immune cells directly or indirectly through metabolic, neurological, or endocrine pathways (Reddy & Frey, 1992). A proper balance of macro- and micronutrients, including amino acids, PUFA, vitamins and trace elements, is essential for the development of immune system beginning from the larval stages. Adequate nutrition is required for cells of the immune system to divide and synthesise effector molecules. The diet supplies the immune system with the amino acids, PUFA, enzyme co-factors and energy necessary to support lymphocyte proliferation and the synthesis of effector (e.g. Ig's, lysozyme and complement) and communication molecules (e.g. cytokines and eicosanoids).

It is known that high ratios of (n-3)/(n-6) PUFA found in FO tend to reduce mammalian T-cell immune activity e.g. transplant rejection and, rheumatoid arthritis whilst high ARA levels enhance inflammatory responses, for example, to bacterial infections. Many studies have shown that in humans and laboratory animals, oils rich in (n-3) PUFA such as FO are more suppressive than LO, which, in turn is more suppressive than oils rich in (n-6) such as sunflower oil (Calder, 1998b). This suppression is manifest through reduced macrophage

and T-cell functions such as lymphocyte proliferation and the expression of T-cell derived cytokines and the changes appear to reflect reductions in (n-3) content.

Whilst suppression of cellular responses by the long chain PUFA, EPA, DHA, has been demonstrated in humans and animals, specific immune responses in the form of antibody levels are increased in birds (Pike, 1999). This also appears to be the case in fish. When Atlantic salmon were fed high and low ratios of (n-3)/(n-6) PUFA (Thompson *et al.*, 1996), it was shown that there were no differences in any of the non-specific immune parameters measured. Rather than immunosuppression being a consequence of a high (n-3)/(n-6) ratio, the groups of fish fed diets with low (n-3)/(n-6) ratio were shown to be less resistant to infection with *Aeromonas salmonicida* and *Vibrio angullarum.*

In an internal EWOS communication (Burrells & Buttle, personal communication) Atlantic salmon were fed diets coated at a level of 16 % of the diet with FO or VO (LO or RO or sunflower oil) chosen for their difference in (n-3)/(n-6) ratios. These diets were fed for six weeks after which a normal FO diet was given for a further 6 weeks. After feeding with the trial diets the fatty acid profiles in the flesh reflected those of the diets. The mean weights of fish fed LO and sunflower oil diets were lower than the controls but this was shown to be statistically insignificant. A significant reduction in growth was observed in the group fed the RO diet. The capacity of B-cells was significantly reduced in fish fed all three VO diets whilst the macrophage activity was significantly lowered in fish fed the sunflower oil diet. When the fish were returned to the FO diet, there appeared to be no significant difference in this non-specific macrophage activity. The most significant finding was the effect of the VO on tissue pathology, particularly that of the intestines. All three VO produced changes

to the histopathological appearance of the intestinal mucosa particularly in the distal portion of the gut. The most severe abnormalities were seen after feeding the diet for six weeks in the fish given the RO diet. A surprising outcome of the study was that after cessation of alternative oils and return to normal FO diet for 6 weeks, evidence of pathological disruption in the intestines was still evident, with the LO group showing the least resolution.

Parker and Hendricks (personal communication) observed abnormalities in the structure of the heart, liver, kidney and gills in coho salmon (*Oncorhynchus kisutch*) after feeding diets containing high erucic acid levels from RO. Alternatively, Dosanjh *et al.*, (1984) reported no induced pathology in coho salmon fed RO diets for eighty-four days. The supplemental lipid comprised about 56 % of the dietary lipid content, and 15 % of the dry matter. They reported that the dietary treatment had little influence on the coho salmon's growth. Furthermore, mean haematocrit, blood haemoglobin levels, and differential white blood cells counts were normal in all the groups tested. High levels of dietary (n-3) PUFA have also been proposed to increase disease resistance in cultured Atlantic salmon by increasing the strength of the blood cell membrane in fish suffering from cold-water vibriosis (Salte *et al.* 1988).

Thomassen & Rϕsjϕ., (1989) substituted FO with soybean oil or low- or high-erucic acid RO for up to 68 % of the lipid normally contained in the feed for Atlantic salmon. No statistically significant differences in growth of Atlantic salmon grown in seawater were observed. By comparison, in rats and other warm-blooded animals marked inhibition of growth was reported when fed RO (Beare-Rogers, 1977). These authors reported an

increase in the lipid level in the hearts of salmon or rats when fed RO possibly affecting heart performance after longer feeding periods.

Dietary lipids strongly influence the fatty acid composition of membrane phospholipids. The fatty acid composition of membranes, determine physical properties such as fluidity and permeability. Hence, the activity of membrane associated enzymes, membrane receptors, and the binding of mitogens, antigens and soluble mediators can all be affected by fatty acid composition (Blazer, 1992). The roles of different fatty acids must therefore be related to the function of the membrane phospholipids, perhaps by conserving a correct membrane fluidity for cellular function. Bly *et al.*, 1988 noted that lymphocytes would need a dynamic membrane for rapid signalling and active transport to be involved in the immune response. Johnston (1988) has shown that dietary fat type and amount can affect PG synthesis, mitogenic responses of T-cells, the humoral immune response and macrophage phagocytosis in homeotherms. In previously reported studies, Sheldon & Blazer, (1991) investigated the effects of either menhaden oil, soybean oil or beef tallow on channel catfish macrophage function and humoral immune response and found that diet was beneficial to this activity. The humoral immune response and macrophage function in both non-immunised fish and fish immunised by intraperitoneal injection of a formalin-killed *Edwardsiella ictaluri* vaccine, were assessed. The menhaden oil group showed significantly increased killing of engulfed bacteria when compared to macrophages from either of the other groups. The fish grown on the menhaden oil diets had significantly higher antibody titres than the other groups, in addition, to a higher killing index of the macrophages (Sheldon & Blazer, 1991).

Two studies carried out by Waagbϕ *et al.*, (1993a,b) indicated that dietary lipids, varying in the contents of (n-3) PUFA and vitamin E, affect the non-specific immunity and disease resistance of Atlantic salmon. In the first experiment, Atlantic salmon were fed diets containing soybean oil, capelin oil or sardine oil for 12 months and non specific immune factors were measured. In the second experiment, Atlantic salmon smolts, fed the same experimental diets from the onset of first–feeding, were challenged with *V. salmonicida* at 7 °C and 13 °C to evaluate the effects of the diets on disease resistance. The fatty acid compositions of spleen and erythrocyte phospholipids reflected the fatty acids in the diets but the contents of (n-3) PUFA were not directly related to the content in the diet. Water temperature also affects the fatty acid composition of membrane phospholipids. The change in phospholipid fatty acid composition, caused by the diets, may inflict temperature-like effects on immune functions. According to Bly and Clem (1992) the temperature sensitivity is expressed early in cell activation, probably at receptor level or trans-membrane signalling. The spleen contains haematopoietic stem cells, and a variety of immune cells, and the dietary lipids may affect the composition and activity of these differently. This was shown by differences in the phospholipid fatty acid composition of spleen and erythrocytes. The erythrocyte phospholipids differed from spleen phospholipids mainly by higher proportions of (n-3) PUFA and lower levels of saturated fatty acids.

Macrophages from fish fed sardine oil showed reduced bacterial killing activity at 12°C incubation compared to capelin oil groups (Waagbϕ, 1993a). Reduced activities were also found in phagocytosis when macrophages from sardine oil fed fish were compared to those fed with soyabean oil, although these were not significant. In contrast to these findings Sheldon and Blazer (1991) demonstrated that the bactericidal activity of macrophages in

channel catfish was positively correlated to the dietary level of (n-3) PUFA at two temperatures and post-vaccination. Waagbϕ *et al.*, (1993a) found no effects with regard to dietary treatment on IL-1 production from isolated macrophages. However, other findings have suggested that immunosuppression can be caused by dietary lipids through reduced cytokine production (Meydani, 1990). Sardine oil fed fish supplemented with vitamin E showed the best survival at low water temperature, while fish fed a capelin oil diet with vitamin E was the preferable dietary lipid source at higher water temperatures. Differences in survival after bacterial infections are probably related to cell membrane structure. The structure may be affected by dietary fatty acid composition, vitamin E and water temperature (Waagbϕ *et al.*, 1993a).

The influence of dietary lipids on trout erythrocytes is reflected in 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, *V. salmonicida*. Applying this to a clinical situation, Salte *et al.* (1988) investigated the effects of high levels of dietary (n-3) PUFA (20:5 (n-3) and 22:6 (n-3)) on the physical properties of erythrocyte membranes from salmon fed at low water temperatures. Results showed reduced membrane fragility and decreased mortalities due to Hitra disease (cold water vibriosis) in the high (n-3) PUFA group. In juvenile Atlantic salmon, Erdal *et al.*, (1991) found that increased dietary (n-3) PUFA 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) PUFA, possibly due to oxidative stress as described above.

A balance must be achieved between optimal fish production and maintaining fish health, and a diet that could be specially tailored, with regard to fatty acid composition, to provide both maximum growth and active enhancement of disease resistance would be of great benefit to the aquaculture industry as a whole.

## 1.10 Project Aims

The aim of this research programme was to examine possible pathological and immunological effects caused by replacement of FO by VO in feeds for Atlantic salmon (*S. salar*), Sea bass (*Dicentrarchus labrax*), Atlantic cod (*G. morhua)* and Arctic char (*Salvelinus alpinus*). A number of different approaches have been used. Firstly, it is intended to establish if any immunosuppression or enhancement results from different fatty acid incorporation *in vivo* by measuring various immunological parameters and eicosanoid levels. Secondly, the effects of VO on the fatty acid composition of lipids of immunocompetent cells and, disease resistance of the fish was investigated. Finally, histological examination of specific tissues to determine any pathological consequences of dietary inclusion of alternative oils was carried out.

Chapter -2 General Materials and Methods

## 2.1 Introduction

The methodology outlined in this chapter consists of a general description of the techniques used throughout the study. Buffer formulations are given in Appendix 1 and suppliers of materials in Appendix 2. Materials and methods specific to individual experiments will be included in the relevant chapters. All dissecting equipment and glassware used for immunological and bacteriological experiments were sterilized prior to use using an Astell Swiftlock 6000 autoclave. All buffers were prepared with double distilled water obtained from a Purite select Analyst HP system.

## 2.2 Fish Husbandry

The experimental dietary trials carried out in this thesis were conducted at a number of different fish culture facilities throughout Scotland, Spain and Norway. The sites used in Scotland were mostly operated by Marine Harvest (Scotland) Ltd., situated at Loch Duich, Lochailort, Kinlochmoidart, Invergarry or the Scottish Executive Environment and Rural Affairs Department (S.E.E.R.A.D) Fish Cultivation Unit, Aultbea, Wester Ross which are approximately a 3-4 h drive away from the Institute of Aquaculture, where most of the analyses was carried out. However, the analyses from the dietary trials in Chapters 1 and 2 were carried out at EWOS Technology Centre, Livingston, Scotland, before it was relocated to Dirdal, Norway. All further analyses from the dietary trials presented in Chapters 3-7 were conducted at the Institute of Aquaculture, University of Stirling, Scotland. The trial sites used in Spain were the Departamento de Biologia, Facultad de Ciencias del Mar y Ambientales, Universidad de Cadiz and at the facilities of Acuinova,

Andalucia in San Fernando, Cadiz. The trial sites used in Norway were the research station at Gildeskal, Inndyr, near Bodo and Nutreco ARC's research station in Stavanger, Norway. Ultimately problems were encountered due to the distance of the trial sites from the laboratory facilities. The sampling regime used took this into account as discussed in Section 2.2.1. All experiments were conducted in accordance with British Home Office guidelines regarding research on experimental animals.

### *2.2.1 Experimental animals*

The fish species examined in this thesis are commercially important species to the European aquaculture industry. The anadromous species, Atlantic salmon (*Salmo salar*), and Arctic char (*Salvelinus alpinus*), and the marine species, sea bass (*Dicentrarchus labrax*) and Atlantic cod (*Gadus morhua*) were studied. All fish were given time to acclimate to experimental conditions prior to commencing experimentation. It is important to consider that when fish experience stress, not only is their immune response suppressed, but also a variety of metabolic processes, including lipid metabolism may be affected (Barton & Iwama, 1991). Stress has been implicated in modulating the immune system of fish, making them more susceptible to pathogens (Ellis, 1981), by affecting both the specific and nonspecific immune system of the animal (Schreck, 1996). The fish studied throughout the course of this study inevitably experienced stressful procedures, such as handling, anaesthesia and transport, however care was taken to minimise the stress levels experienced by the fish, which can have detrimental effects on the animals physiological and immune responses.

### *2.2.2 Tank system*

The various tank systems used, the number and size of fish, and the water temperatures used will be discussed in the appropriate chapters. Tank cleanliness was an important factor in disease prevention. Tank walls and pipes were cleaned regularly according to the procedures in place at the relevant trial site facilities. From time to time fish need periodical treatment with malachite green and in more recent trials with the safer alternative Pyceze®, however the technical staff on each trial site administered the treatment.

### *2.2.3 Feeds*

The dietary compositions of the feeds used throughout this study are shown in the relevant chapters. The feeding schedules of all experimental fish complied with the recommended daily feeding guide produced by fish feed companies' (EWOS, Biomar, Nutreco) and took into consideration the size of the fish, with respect to pellet size and diet composition. Water temperature and fish weight dictated the amounts of diet fed daily. The experimental diets used throughout the studies differed in their oil composition and were formulated to satisfy the nutritional requirements of the species involved (US National Research Council, 1993). In most of the dietary trials, fish were fed by automatic feeders unless otherwise stated.

### *2.2.4 Marking of fish*

When it was necessary to identify individual fish, a Pan jet inoculator was used to mark fish. The pan jet injects a fine jet of aqueous Alcian blue dye (1% w/v), subcutaneously, and was applied to the underside of the fish. Combinations of mark positions were applied and these were identifiable for up to 5 months after injection. The position of the pan jet marks are indicated by the stars shown in Figure 2.1 and various combinations of these were used to identify the dietary history of the fish.

### *2.2.5 Vaccination of fish*

Anaesthetised fish were vaccinated by intraperitoneal (i.p.) injection along the central line of the body between the pelvic and pectoral fins administering 0.1 ml of the Alphaject 1200 furunculosis vaccine (Chapter 4 and Chapter 6). The vaccination of fish in Chapters 4 and 6 was carried out at Marine Harvest sites using a commercial vaccination syringe.

## 2.3 Sampling of experimental fish

In assessing how dietary change influences the health of fish, any alterations in immune function resulting from changes in cellular fatty acid composition arising from VO incorporation, *in vivo*, were investigated. The effects of dietary change on the immune function and tissue histology of the different fish species by partially replacing FO with VO was examined. Fish were fed the experimental regimes for different lengths of time prior

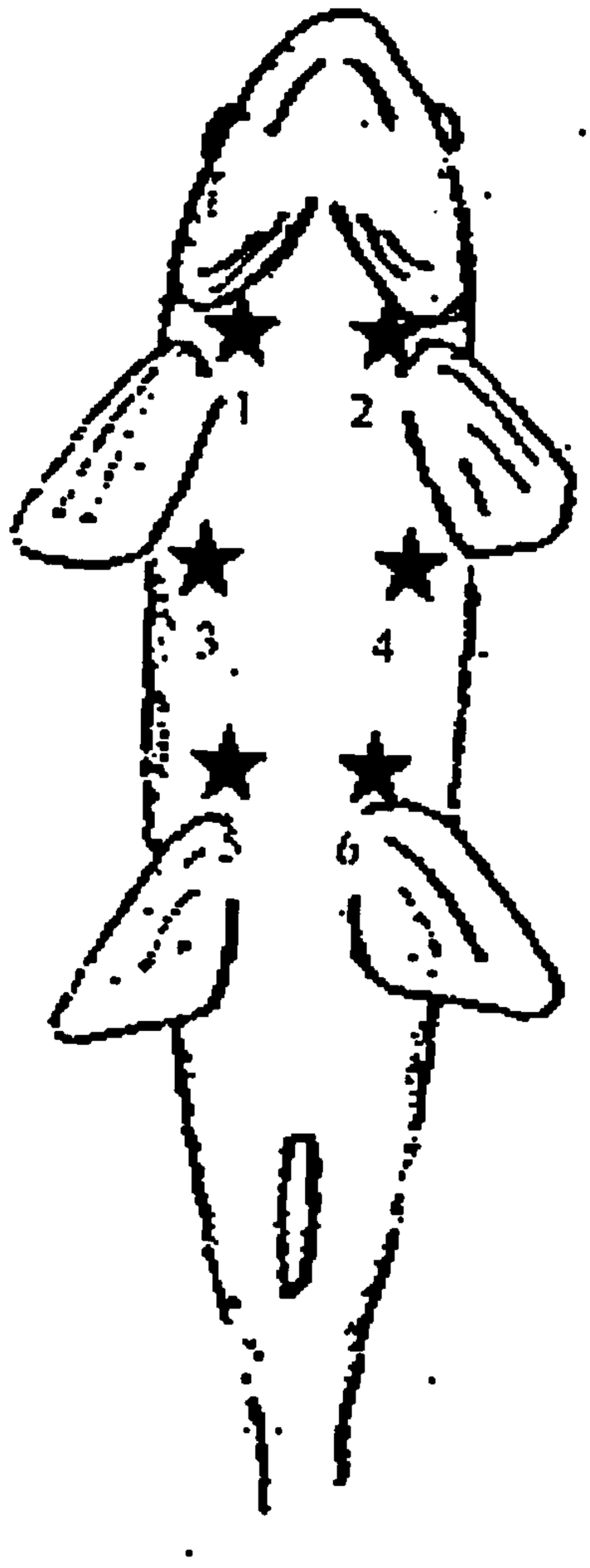



Figure 2.1 Positioning of pan jet markings

to sampling and this will be discussed in the relevant chapters. In order to evaluate the effect of VO on fish health different immunological parameters were determined, including both humoral immunity (serum lysozyme activity) and cellular immunity (phagocytic index and respiratory burst activity of head kidney macrophages). Haematocrit and the total number of circulating erythrocyte and leukocyte counts were determined from heparinised blood samples. In addition, the effect of dietary VO on the fatty acid composition of peripheral blood leukocytes (PBL) was also studied in some of the dietary trials. Finally samples of heart, liver and intestine were removed from individual fish for processing and histological examination.

### *2.3.1 Anaesthesia of fish*

Ethyl p-aminobenzoate or MS-222, tricaine methanesulphonate were used throughout the study at a stock concentration of 40 g $l^{-1}$ in ethanol, which was generally, diluted 1:1000. For anaesthesia, 40 mg $l^{-1}$ of the anaesthetic in holding water was appropriate, and for fatal anaesthesia 100 mg $l^{-1}$ of anaesthetic was adequate. An anaesthetic bath was prepared by slowly adding the stock solution to water with thorough stirring to prevent the drug coming out of solution.

### *2.3.2 Isolation of blood*

Blood samples were taken to assess various serological, haematological and biochemical parameters. Blood is the most convenient tissue for sampling leukocytes. Wherever possible, blood was used as the source of cells throughout the study unless stated

otherwise. However, additional immune cells were obtained from the head kidney of experimental fish.

Blood samples were normally taken from anaesthetised fish, or from fish freshly sacrificed immediately following culling. Once anaesthetised, the fish were killed with a sharp blow to the head using a blunt instrument and placed on its side. The vacutainer needle, attached to the plastic connector was put into the correct position and pressure applied to the needle until the skin was punctured and the vertebrae felt. The vacutainer was pierced and the position of the needle adjusted to slightly below the vertebrae until the caudal vein was punctured and blood appeared in the tube. Blood was sampled from a region approximately 3 scales below the lateral line, diagonally to the left of the adipose fin. If plasma or whole blood was required, heparin was added to the vacutainer prior to collection (100 µl), to prevent clotting. A final concentration of 10 units of heparin / ml of blood was added to a 10 ml vacutainer depending on the size of fish to be bled and the size of the vacutainer. The various sizes of vacutainers and needles used throughout this study are shown in Table 2.1.

Table 2.1 Guidelines for choosing appropriate needle and vacutainer size for sampling blood.

| Size of fish (g) | Vacutainer size (ml) | Needle size (G) |
|---|---|---|
| >200 | 10 | 20 |
| 100-200 | 5 | 21 / 22 |
| <100 | 2.5 | 22 |

### 2.3.2.1 Leukocyte counting

Considerable importance is based on white blood cell counts, as stressed, and diseased fish can give different numbers to normal base line levels. After sampling blood, a 1 in 100 dilution of the sample was prepared in phosphate buffered saline (PBS). The suspension was thoroughly mixed and the counting chamber filled. The white blood cells were counted using a 4 mm objective and x 10 eyepiece. The final cell count was expressed as the number of cells / $mm^3$, using the following calculation:

$$N \times 5 \times 10 \times 1000 \times 100 = \text{number of white blood cells / } mm^3$$

Where N = number of cells counted in 5 large squares

5 = multiplication factor to give the number of cells in one $mm^2$

10 = multiplication factor to take into account the depth of the chamber

1000 = multiplication factor to give result in $mm^3$

100 = the dilution factor

### 2.3.2.2 Erythrocyte counts

Red blood cell counts can give an indication of the health status of the fish depending on whether the levels are above or below normal counts. A 1 in 1000 dilution of blood in PBS, was mixed and counted. The calculation to express the results as the number of red blood cells / $mm^3$ is as above, however the dilution factor is 1000.

#### 2.3.2.3 Determination of the packed cell volume (PCV) of erythrocytes (Haematocrit)

Simple haematocrit readings give levels or percentages of erythrocytes in the blood, which is a valuable guide in diagnosing certain blood disorders and gives an indication of whether the fish is stressed or not. Blood samples, transported on ice, were shaken gently to ensure thorough mixing. A heparinised microcapillary tube was inserted into the blood sample and filled by capillary action. One end of the tube was sealed with Cristaseal and the tube was placed in a Hawksley micro-haematocrit centrifuge for 4 min at 9,300 x g. Haematocrit levels were determined using a micro-haematocrit reader and results were expressed as the percentage packed red cell volume in relation to the whole blood volume.

### *2.3.3 Isolation of serum and plasma*

If serum was required, the blood was left undiluted, without heparin and allowed to clot overnight a 4 °C then centrifuged at 900 x g for 10 min and the serum collected. If plasma was required then the blood was centrifuged at 900 x g for 10 min as soon as possible following collection. If any delay existed between sampling and centrifugation, the blood was held at 2-6 °C. For most of the haematological assays the blood was stored between 2-6 °C for no longer than a 24 h period (due to the distance of the trial sites from the laboratory at the Institute of Aquaculture).

### *2.3.4 Isolation of head kidney*

The removal of head kidney (pronephros) from fish renders a supply of macrophages available for immunological assays. Macrophages are the main phagocytic cells in fish and their role as effectors of the immune system and the inflammatory response is clearly suggested. During this procedure, surfaces, equipment and containers were all clean but not necessarily sterile at the farm trial sites. Immediately after bleeding the fish, it was placed on a dissecting board, ventral side up and the mucus covering the skin scraped off. The skin was disinfected using 70 % ethanol and the ventral side of the fish opened, cutting from the anus to the oesophagus using sterile scissors and scalpels, taking care not to puncture the intestines. Once the cavity was exposed, the intestine was cut at the anus and pulled gently away from the abdominal walls taking care not to puncture the bile sac. The swim bladder was then pierced and pulled away from the kidney. Once the kidney was exposed (Figure 2.2), the membrane was cut using a sterile blade and pulled away using fine sterile forceps. All the edges of the head kidney were cut and then scraped together and removed into a sterile bijou bottle containing the appropriate culture medium required for the subsequent analysis.

### *2.3.5 Sampling tissues for histology*

Microscopic examination of the structure of tissues requires histological processing of tissues and staining of subsequent tissue sections. Collected tissues were "fixed" immediately in 10 % buffered formalin to preserve their structural integrity. The intestine

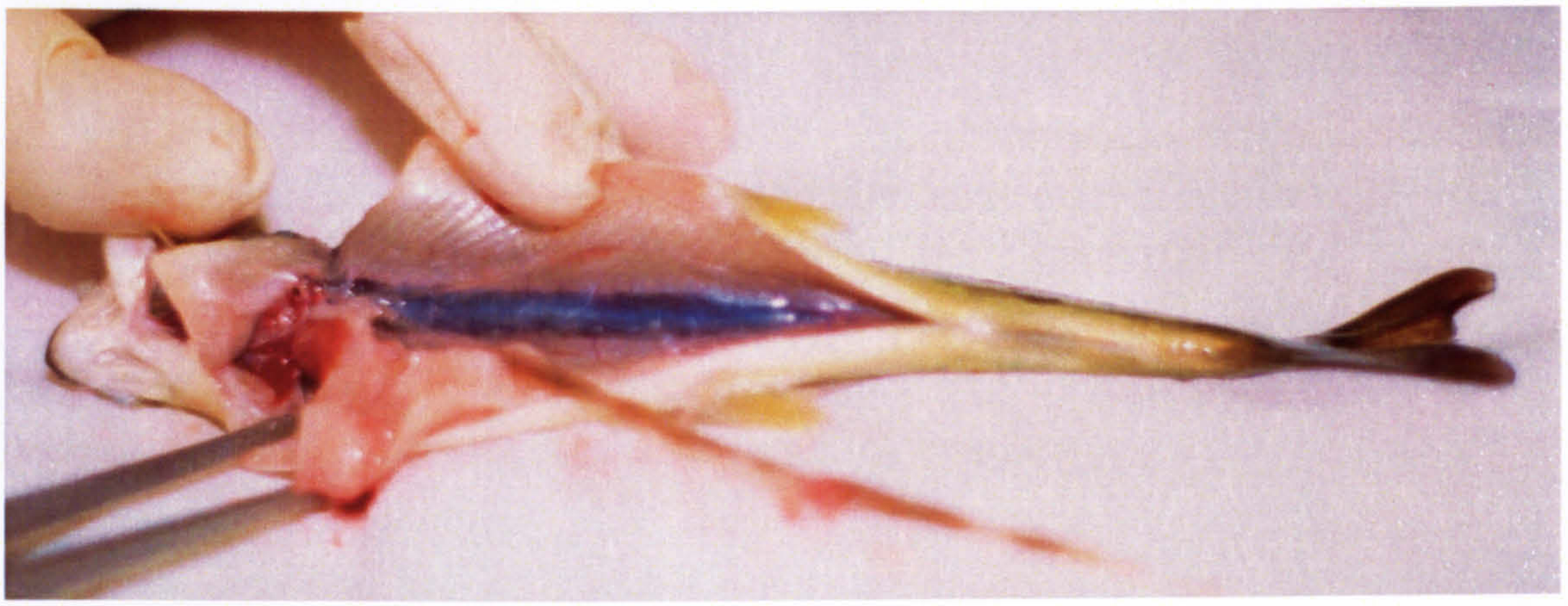


Figure 2.2 Sampling head kidney (pronephros)

was cut at the anus, pulled away from the abdominal walls and finally the alimentary tract was cut free and laid out on a clean surface. With a scalpel, approximately 1 cm sections were excised from each of the proximal, mid and distal segments of the intestine (Figure 2.3). The excised sections of intestine were then placed into separate Petri dishes containing a few ml of PBS so that individual sections were not mixed. The sections were cut open and rinsed in the PBS to remove most of the faeces. Finally the washed tissues were placed into specimen jars three-quarters filled with fixative (10% buffered formalin). These were then stored at 20 °C during transport and prior to processing. In most cases the whole heart including the bulbus artenosus were removed for histological processing whereas only a section of the whole liver was removed for histology unless the fish were small then the whole liver was removed.

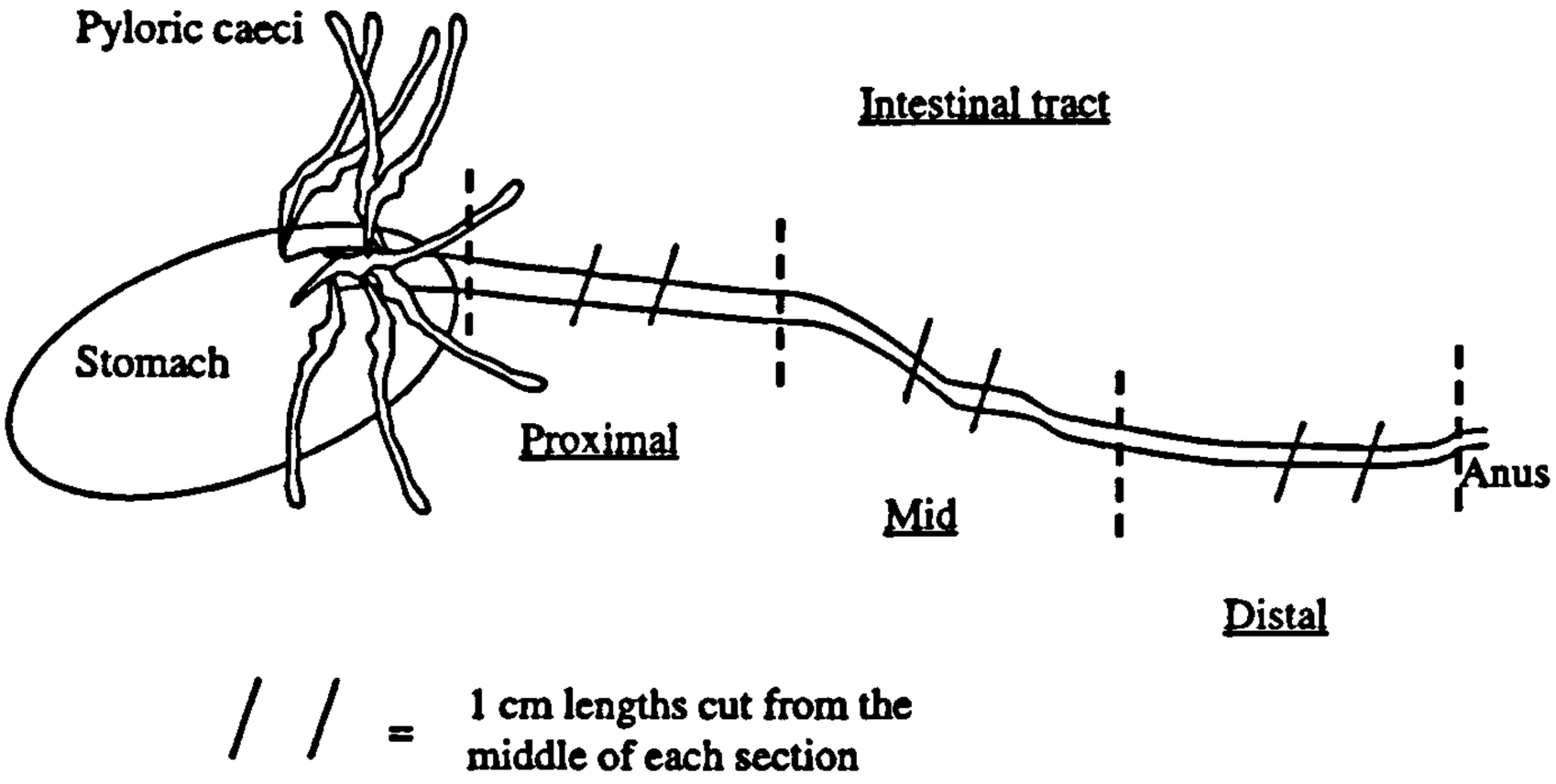



Figure 2.3 Positioning of excised sections from the intestine of fish.

### *2.3.6 Separation of peripheral blood leukocytes (PBLs)*

All manipulations excluding centrifugation, microscope work and incubation were carried out aseptically in a Laminar Air-Flow cabinet. Whole heparinised blood was centrifuged in a bench centrifuge at 600 x g for 10 min, after which the "buffy coat" at the surface of the red cell pellet was collected using a sterile glass Pasteur pipette and placed into 5 ml of Leibowitz L-15 Medium containing 0.1 % foetal calf serum (FCS)(0.1% FCS L-15). Care was taken not to aspirate too many red blood cells (RBC) from the buffy coat. Using a 5 ml pipette the suspension was carefully layered onto 5 ml 51 % Percoll (1.080 g $l^{-1}$) in a 10 ml conical centrifuge tube taking care to avoid intermixing of the fractions. The tube was then centrifuged at 900 x g for 35-45 min. The band of cells (lymphocyte-enriched) at the

interface of the medium and the 51 % Percoll, was collected using a sterile glass Pasteur pipette, and placed into 5 ml 0.1 % FCS L-15 in a plastic universal bottle. The band of cells at this interface were composed predominately of lymphocytes, granulocytes and thrombocytes, while the erythrocytes were pelleted at the bottom of the tube. If the RBC contamination was greater than 2 % of the cells, the cells were replaced onto the Percoll and centrifuged again. The cells were washed twice in 0.1 % L-15, at 600 x g for 10 min, and the pellet resuspended in a volume of L-15 medium containing 5 % FCS (5 % FCS L-15) considered appropriate to the size of the cell pellet (e.g., 1 ml for most pellets). The concentration of live cells in the suspension was estimated using trypan blue exclusion.

### *2.3.7 Preparation and culture of head kidney macrophages*

Macrophages derived from the head kidney of salmonids were used in a variety of assays to determine macrophage function, which reflects the level of the host's ability to resist infection. The macrophages were isolated from the head kidney dissected in Section 2.3.4 and cultured for a period of time prior to assaying.

#### 2.3.7.1 Isolation of head kidney macrophages under laboratory conditions

Head kidney macrophages were prepared as described by Braun-Nesje *et al.*, (1981) and modified by Secombes, (1990). The procedure below was followed when head kidneys were removed from fish at the EWOS Technology Centre or the Institute of Aquaculture, University of Stirling. All manipulations were carried out aseptically in a sterile environment. Using sterile forceps, a sterile sieve was placed into a sterile Petri dish and

0.1 % FCS L-15 medium containing the head kidney was poured into the sieve. The tissue was disrupted by gently rubbing it through the nylon gauze (ĩ μm) with sterile forceps, after which, the resulting suspension was transferred to a sterile siliconised bijou and allowed to stand for 2-3 min to allow large aggregates to settle. The cell suspensions were placed onto previously prepared 51 % / 34 % Percoll gradients (1.070 g $l^{-1}$). Percoll (2.5 ml of 51 %) was placed into a sterile 10 ml conical centrifuge tube and 2.5 ml of a 34 % Percoll gradient was gently layered on top avoiding intermixing of the solutions. The cell suspension was placed onto the 34 % fraction and centrifuged at 900 x g for 35-45 min at 4 °C. The band at the interface of the 34 % / 51 % fractions was enriched with macrophages and was collected into 5 ml of attachment medium and washed twice by centrifugation at 600 x g for 10 min. The resulting cell pellet was resuspended in a volume of attachment medium considered appropriate to the size of pellet (in most cases this was 1 ml). The cell concentration was adjusted to 2 x $10^7$ viable cells $ml^{-1}$ in 0.1 % FCS L-15 medium. Concentrations of FCS greater than 0.1 % impedes the adherence of macrophages to plastic (Secombes, 1990). Macrophage cell suspensions were used in a variety of immunological assays described in Section 2.4.

### 2.3.7.2 Isolation of head kidney macrophages at fish farm trial sites

This procedure was followed when head kidneys were sampled from fish at trial sites, due to the length of time involved in transporting the tissue to EWOS Technology Centre or the Institute of Aquaculture. The same procedure outlined in Section 2.3.7.1 was followed, however instead of preparing a density gradient, 200 μl of the cell suspension were transferred to 4 replicate wells of a sterile 96-well flat-bottomed microplate. The plate was

then covered and placed in an insulated container to avoid rapid or extreme variations in temperature from ambient. The plates were then transported back to the laboratory, and on arrival the macrophage cultures were washed twice and 5 % FCS L-15 added as in the laboratory method described in Section 2.3.7.1. These cells were used in the NBT respiratory burst assay and when measuring macrophage phagocytosis activity.

## 2.4 Immunological analyses

All procedures were carried out in a laminar airflow cabinet using sterile materials. Many reports of fish cell culture can be found in the literature, and reported culture systems vary widely depending on the fish species and the tissue being cultured (Faulmann *et al.*, 1983; Tocher *et al.*, 1988). Differences between the optimal culture conditions of salmon head kidney macrophages and cod head kidney macrophages exist and details are outlined in Chapter 7 Section 7.2.1.3.

### *2.4.1 Macrophage Function*

### *2.4.2 Nitroblue Tetrazolium Reduction Assay (NBT assay)*

Cell suspensions (100 µl well$^{-1}$) were plated into 4 replicate wells of a 96 well flat-bottomed microtitre plate and incubated in a cooled incubator at 18 °C for 2-3 h. Non-adhering cells were removed washing twice with 0.1 % FCS L-15 medium. Fresh 5 % FCS L-15 medium was added to the macrophage monolayer and the plates incubated for 24 h prior to any assay procedure (e.g. nitroblue tetrazolium assay (NBT)).

This procedure was applied to macrophage cultures following removal of the head kidney from fish fed the experimental diets as described in Section 2.3.4. Precipitation of the formazan (3,5-Diphenyl-1-(m-tolyl) formazan) can be visualized and measured spectrophotometrically. The reagent containing the NBT (1 mg $ml^{-1}$, in tablet form) and phorbol 12-myristate 13-acetate (PMA, 1 µg $ml^{-1}$) as the cell stimulant was freshly made up at the time of the assay and any excess was discarded. From 3 of the 4 culture wells for each sample, 10 ml of L-15 medium was pipetted into a plastic universal bottle. One NBT tablet (10 mg) was dissolved in the medium using a magnetic stirring bar followed by the addition of 10 µl of PMA stock. Using a 50 –250 µl multichannel pipettor set to 200 µl the cells were washed twice to remove unattached cells. A freshly prepared solution of the NBT/PMA reagent (100 µl) was added to each well and placed in an incubator at 18 – 20 °C for 45 min. Following incubation the supernatant was aspirated off using a multi-channel pipettor and 100 µl methanol added to the wells. A further 100 µl of methanol was added to the wells and the cells fixed for 5 min. The methanol was then aspirated off and the plate allowed to air dry. The formazan was dissolved by adding 120 µl 2M KOH and 140 µl dimethyl sulphoxide (DMSO) to each well and mixed by pipetting up and down. Finally the optical density (OD) at 620 nm was determined for each well using an ELISA plate reader (Dynatech MR5000). From the fourth culture well the number of macrophage nuclei $well^{-1}$ was found as described in Section 2.4.2.1 and the results were expressed as "Macrophage activity" for $10^5$ cells ml.

#### 2.4.2.1 Macrophage nuclei counts

The medium containing macrophage cultures in the microplate wells was pipetted up and down 2-3 times before being discarded, thus removing unattached cells. Fresh L-15 medium was aspirated and the cells washed again. Macrophage lysis buffer (50 µl) (100 ml distilled water, 0.0021 g citric acid, 1 ml Tween, 0.05 g crystal violet) was added to each well and pipetted up and down 5-6 times to ensure the release of the nuclei from the attached macrophages. The counting chamber was charged and using a magnification of > 600 x (eyepiece magnification = 12.5 x with 50 x magnification objective) the number of macrophage nuclei were counted. From this the number of nuclei per 50 µl of lysis buffer and consequently per microplate well can be determined by dividing the count / ml by 20.

### *2.4.3 Phagocytosis by head kidney macrophages*

Phagocytic activity of macrophages was evaluated using a microscopic counting technique. Head kidney macrophages were isolated as described in Section 2.3.4. Microscope slides were washed with absolute ethanol, allowed to air dry then two circles were drawn on the slide with a wax (PAP) pen. Macrophage suspension (100 µl ) was added to each circle and slides were incubated in a humid Petri dish at room temperature (21°C) for 1 h. The slides were then washed 5 times with PBS to remove non-adherent cells. To one of the circles 100 µl of yeast suspension (*Saccharomyces cerevisiae*) (5 mg $ml^{-1}$ in L-15) was added and to the other circle 100 µl of L-15 medium (control) was added and incubated again at 21°C for 1 h. Non-adherent cells were washed off with PBS, fixed with 70 % methanol and allowed to air dry. Slides were then immediately stained with a RapiDiff

staining system, dried and mounted. Cells were examined under oil immersion (x 100) and the number of yeast particles engulfed in 100 macrophages was determined.

### *2.4.4 Lymphocyte Stimulation Assay*

Due to transport of the samples from trial sites to the laboratory, if a delay of up to 24 h occurred between the blood sampling and separation of the lymphocytes; the blood was stored in a cooled insulated box or kept in the laboratory refrigerator at 2-6 °C. The isolation of peripheral blood leukocytes, as described in Section 2.3.6 was followed. The cell concentration was adjusted to 1 x $10^6$ $ml^{-1}$ in 5 % FCS L-15. L-15 medium containing 5 % FCS (20 µl) was dispensed into 3 wells of a 96-well sterile flat-bottomed microplate and 20 µl of the desired stimulant dispensed into appropriate triplicate wells. The prepared lymphocyte suspension (200 µl , 2 x $10^5$ cells $well^{-1}$) was added into all of the wells containing medium or Concavalin A (ConA) (25µg $ml^{-1}$, previously determined optimal concentration (unpublished)) or Lipopolysaccharide (LPS) (100µg $ml^{-1}$, previously determined optimal concentration (unpublished)) as the stimulant. The microplates were then incubated in a cooled incubator at 18-20 °C for 3 days. Radiolabelled $^3$H-thymidine was diluted to 0.74 Bq $ml^{-1}$ in L-15 medium and 50 µl added to each well and the plate incubated for approximately 18 h. The cells were harvested with a semi-automatic multiple harvester transferred onto glass fibre filters and washed in distilled water. The radioactivity content of the filters was counted in a liquid scintillation counter. Results were expressed as 'Mean counts per minute' (CPM) of the stimulated cultures compared with control cultures or as 'Stimulation Indices' (SI) (Erdal & Reitan, 1992).

SI = Mean CPM stimulated cultures/Mean CPM non-stimulated control cultures

All procedures using radioactivity were carried out at Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik, Scotland.

### 2.4.4.1 Cell viability counts

Trypan blue (180 µl, 0.4 % (w/v) aqueous solution) was dispensed into a sterile plastic bijou and the cell suspension was inverted 2-3 times to ensure an even suspension, after which 20µl of cell suspension was added. This resulted in an initial dilution of the cells of 1/10. Measurements of cell counts were made using an improved Neubauer's counting chamber (haemocytometer) as shown in Figure 2.4.

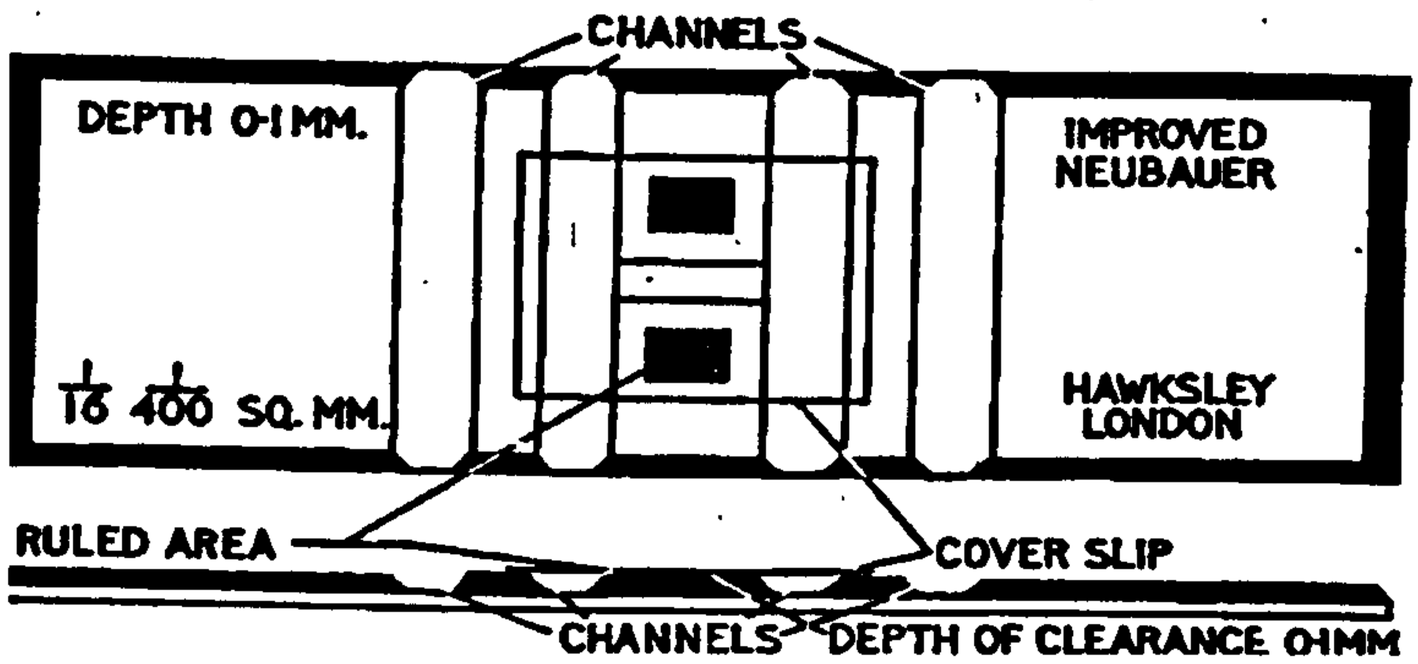



Figure 2.4 Illustration of an Improved Neubauer haemocytometer used for counting cells.

The 1/10 cell suspension in trypan blue was thoroughly mixed and 20 µl aspirated into the counting chamber (chamber depth = 0.1 mm). The charged counting chamber was then placed under a Leica inverted microscope (magnification of > 300, eyepiece magnification = 12.5 x with the 25 x magnification objective) and the number of viable cells contained within the middle square mm of the ruled area were counted (Figure 2.5). If the suspension contained a heavy load of cells, the 4 corner squares along with the central square (5 x $1/25^{th}$ sq.mm.) were counted then the result multiplied by 5.

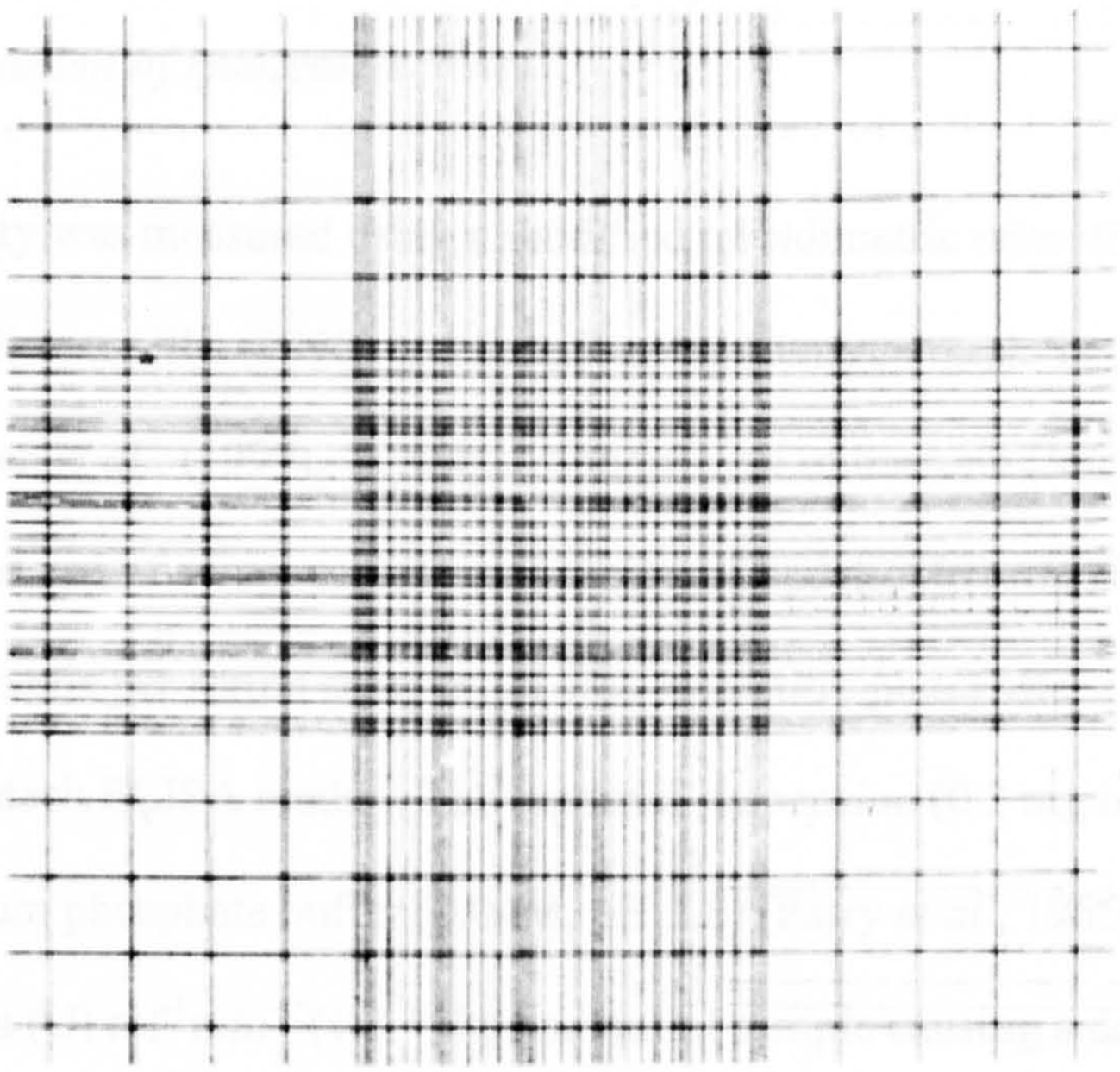


Figure 2.5 Ruled area of the haemocytometer.

The number of viable cells within the 1 sq. mm area (n) was noted as the number of cells / $mm^2$. Then the concentration of cells /ml of the original suspension was calculated by multiplying "n" by $10^5$. Which is derived from the following:

Multiplying n by 10 ($10^1$): original dilution in trypan blue

Multiplying 10 ($10^1$) to achieve the number of cells / $mm^3$ (the depth of the chamber is 0.1mm).

Finally multiply by 1000 ($10^3$) to convert $mm^3$ to $cm^3$ (ml).

### *2.4.5 Determination of lysozyme activity*

Lysozyme activity was measured using a modified turbidimetric microtitre plate technique according to Ellis (1990) and described by Rungruangsak-Torrissen *et al.*, (1999) and Lygren *et al.*, (1999). A suspension of 1□µl of bacteria (*Micrococcus lysodeikticus*) and 1 µl of serum sample was measured spectrophotometrically at 540 nm in five replicate wells per serum sample (96 well microtitre plate) after 1 and 5 min at 25 °C, using a Dynatech ELISA reader. The bacterial suspension (0.2 mg $ml^{-1}$) was prepared in sodium phosphate buffer (0.04M, pH 5.8) (Parry *et al.*, 1965). The results are given as units (U) $ml^{-1}min^{-1}$ (1U = the amount of sample causing a decrease in absorbance of $0.001min^{-1}$).

## 2.5 Enzyme Linked Immunosorbent assay (ELISA) to measure antibodies in vaccinated fish

### *2.5.1 Antigenic Preparation*

*Aeromonas salmonicida* (courtesy of the Bacteriology laboratory, Institute of Aquaculture) was maintained on tryptic soy agar (TSA) plates at 22 °C by sub-culturing the plates when required. Bacterial suspensions were prepared for use as antigen in ELISA by seeding the bacteria into tryptic soy broth (TSB) and grown at 22 °C for 24 h. The bacterial suspension was centrifuged at 2000 x g (Mistral 3000 centrifuge) for 20 min to collect the bacterial pellet and further resuspended in 50 ml of sterile PBS and then centrifuged again to wash bacteria. The resultant pellet was resuspended in a few ml of PBS and the OD read at 610 nm. The concentration of bacteria was adjusted to $1 \times 10^8$ colony forming units (cfu) $ml^{-1}$ in sterile PBS using the standard curve proposed in Section 2.5.2. The bacteria were killed by heating in a water bath for 60 min at 60 °C.

### *2.5.2 Preparation of standard curve*

*A. salmonicida* colonies were placed into 30 ml of TSB broth and incubated overnight at 22 °C. The following day the OD of the undiluted broth culture (100 % sample reading) was read at 610 nm using a Cecil CE 2041 2000 Series spectrophotometer, blanking against PBS. Serial dilutions of the 100 % bacterial culture were made in PBS from $10^{-1}$ to $10^{-7}$ after which each dilution was plated out individually (6 x 20 µl spots on the TSA plate). Plates were allowed to dry then incubated overnight at 22 °C. From the 100%

bacterial suspension, 80%, 60%, 40% and 20% suspensions were prepared in sterile PBS and their respective OD recorded. Serial dilutions ($10^{-1}$ to $10^{-7}$) of each bacterial culture concentration were prepared, plated out and incubated overnight at 22 °C. The number of cfu were determined for each concentration of bacterial suspension prepared. The colonies were counted on the first plate in which it was possible to count 10-50 colonies easily per drop (usually around $10^{-5}$ -$10^{-4}$). The total number of counted colonies was then multiplied by 50 to convert to the number of cfu $ml^{-1}$ (e.g. the average number of colonies x 50 x dilution (e.g. 45 x 50 x $10^5$) = 2.25 x $10^8$ cfu $ml^{-1}$). The OD of each % sample was plotted against cfu $ml^{-1}$ (viable bacteria) to obtain a standard curve (Appendix 3). The regression equation and $R^2$ of the curve was determined and used to calculate the number of bacteria $ml^{-1}$ from the OD from which the appropriate dilution required could be calculated.

### *2.5.3 ELISA*

ELISA plates were coated using 50 µl per well of coating buffer with 0.05 % (w/v) poly-L-lysine (Appendix 1) and incubated for 1h at 21 °C. Plates were washed twice with low salt wash buffer (LSW, Appendix 1) and 100 µl per well of bacterial suspension (Section 2.5.1) added to the plate and incubated overnight at 4°C. Gluteraldehyde in PBS (50 µl of 0.05% (v/v)) was added to the wells containing the bacteria for 20 min. Plates were washed again three times with LSW buffer and post-coated with 200 µl per well of a 1% (w/v) bovine serum albumin (BSA) in PBS (Appendix 1 - antibody buffer), used to block non-specific binding by incubating for 2 h at 21 °C. After incubation, plates were washed a further three times with LSW buffer and 2-fold dilutions of serum made from 1/4 to 1/4096 in PBS

across the ELISA plate. PBS was added as a negative control and fish serum known to be positive to *A. salmonicida* was obtained from the Bacteriology laboratory at the University of Stirling and diluted 1/40 prior to use. Plates were incubated overnight at 4 °C.

The following day, the plates were washed with a high salt wash solution (HSW) (Appendix 1), incubating for 5 min on the last wash. Anti-Atlantic salmon IgM monoclonal antibody (100 µl) (Appendix 1) was added to all the wells and incubated for 1 h at 21 °C. After washing with HSW buffer five times, 100 µl of sheep anti-mouse IgG horse radish peroxidase (HRP) conjugate diluted 1/1000 in conjugate buffer (Appendix 1) was added to all the wells and incubated at 21°C for 1 h. Unbound conjugate was washed off with HSW buffer and 100 µl chromogen/substrate (Appendix 1) was added to each well and incubated for 10 min at 21°C. The reaction was stopped with 50 µl per well of 2M $H_2SO_4$ and the plates read spectrophotometrically at 450 nm on a Dynex MRXII ELISA plate reader. A positive result was recorded when the OD was more than 3 times that of the background PBS wells. Specific antibody titres were expressed as a Log of the first positive dilution for each sample (Antibody titre (-Log 2+1)).

## 2.6 Eicosanoid Measurements ($PGE_2$ Enzyme immunoassay)

The $PGE_2$ assay was performed using a commercially available enzyme immunoassay (EIA) kit. The assay is based on the competition between $PGE_2$ and a $PGE_2$-acetylcholinesterase (AchE) conjugate ($PGE_2$ tracer) for a limited amount of $PGE_2$ monoclonal antibody. Since the concentration of the $PGE_2$ tracer is held constant while the

concentration of $PGE_2$ varies, the amount of $PGE_2$ tracer that is able to bind to the $PGE_2$ monoclonal antibody is inversely proportional to the concentration of $PGE_2$ in the well. The antibody - $PGE_2$ complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's reagent (which contains the substrate for AchE) is added to the wells. The product of this enzymatic reaction has a distinct yellow colour and absorbs strongly at 412 nm. The intensity of this colour, determined spectrophotometrically, is proportional to the amount of $PGE_2$ tracer bound to the well, which is inversely proportional to the amount of free $PGE_2$ present in the well during the incubation; or

$$\text{Absorbance} \propto [\text{Bound } PGE_2 \text{ tracer}] \propto 1/[PGE_2]$$

Plasma samples previously collected from heparinised bloods were thawed prior to use. Two hundred µl of plasma were required per sample, which was centrifuged in an eppendorf centrifuge to remove any particulate debris before use. The supernatant was then loaded onto a C18 reverse-phase 'Sep-Pak' mini-column that had been previously washed with 5 ml methanol followed by 10 ml distilled water. The sample was then washed with 10 ml distilled water followed by 5 ml 15% ethanol (v/v), followed by 5 ml hexane: chloroform 65:35 (v/v). The eicosanoids were then eluted into stoppered test tubes with 10 ml ethyl acetate. Washing with 10 ml methanol then regenerated the Sep-Pak and 10 ml distilled water prior to loading the next sample. The extracts were then dried under nitrogen, re-dissolved in 0.1 ml methanol and stored in a small glass vial at –20 °C before analyzing by immunoassay.

Ultra Pure water was used to prepare all EIA reagents and buffers. The contents of one vial of EIA buffer concentrate was diluted with 90 ml of Ultra Pure water, rinsing the vial to ensure collection of all the salts that may have precipitated. Wash Buffer concentrate was diluted with Ultra Pure water and Tween 20 to a give a final volume of 500 ml (498.75 ml Ultra Pure water, 1.25 ml concentrate and 0.25 ml Tween 20). The $PGE_2$ standard was prepared by diluting 100 µl of the standard with 900 µl of Ultra Pure water (bulk standard solution 10 $ngml^{-1}$). To prepare the standard for use in the EIA, eight test tubes were numbered (1-8) and 360 µl of EIA buffer was transferred to tube 1 and 200 µl to tubes 2-8. The bulk standard (40 µl, 10 $ngml^{-1}$) was transferred to tube 1 and mixed thoroughly. The standard was then serially diluted by removing 200 µl from tube 1 and placing it in tube 2, mixed thoroughly and repeated this process for tubes 3-8. The $PGE_2$ AchE tracer (100 dtn) was prepared by reconstituting it with 6 ml of EIA buffer and stored at 4 °C. $PGE_2$ monoclonal antibody (100 dtn) was reconstituted with 6 ml of EIA buffer and stored at 4 °C.

EIA buffer (100 µl) was added to non-specific binding (NSB) wells and 50 µl of EIA buffer added to maximum binding ($B_o$) wells. To appropriate wells in the microplate, 50 µl of each diluted standard was added. As each sample should be assayed at two dilutions 5 µl and 50 µl were added to the sample wells. $PGE_2$ AchE tracer (50 µl) was added to each well except the total activity (TA) and the blank wells. $PGE_2$ monoclonal antibody (50 µl) was added to each well except the total activity, non-specific binding, and the blank wells. The plate was then covered with plastic film and incubated for 18 h at 21 °C.

One vial of Ellman's Reagent (100 dtn) was reconstituted with 20 ml of Ultra Pure water and protected from the light until use. The microplate wells were emptied and washed five times with wash buffer. Ellman's reagent (200 µl) was added to each well and 5 µl of tracer added to the total activity wells. The plate was then covered with a plastic film, placed in the dark and put on an orbital shaker to develop for around 45 min. The chromophore produced by this enzyme reaction is yellow and was read at a wavelength of 405 nm. A standard graph of % B / $B_o$ (% bound/maximum bound) versus log $PGE_2$ concentration was compiled.

## 2.7 Histology

### *2.7.1 Tissue sampling, preservation and processing*

Fish organ samples (heart, liver and gut) were removed according to the method outlined in Section 2.3.5 and placed in 10 % neutral buffered formalin to prevent autolysis and to safeguard the tissues against the damaging effects of processing. The tissues were cut to the correct size and placed into coded cassettes, the cassettes were immediately placed into water until processing. Processing was carried out in an automatic tissue processor. The tissues were dehydrated in a graded series of alcohols followed by treatment with chloroform to remove the dehydrating agent and lastly impregnated in wax to remove the clearing agent. Tissue processing was in most cases carried out at the Institute of Aquaculture however in some cases where the sample numbers were large tissues were then sent to BS&S Scotland (Edinburgh, Scotland) for processing and haematoxylin and eosin staining. The processing schedule was as

follows: 50 % methylated spirit (1 h), 80 % methylated spirit (2 h), 100 % methylated spirit (2 h), 100 % methylated spirit (2 h), 100 % methylated spirit (2 h), 100 % ethanol (2 h), 100 % ethanol (2 h), chloroform (1 h), chloroform (1 h), molten wax (2 h), molten wax (2 h), molten wax (2 h), molten wax (2 h).

After overnight processing, the cassettes were placed into a molten wax bath of the histoembedder. The organs were then embedded in paraffin wax in suitable moulds and immediately placed onto a cool plate. Once solidified the blocks were removed from the mould and stored at room temperature until sectioning. Blocks were trimmed and sectioning was carried out on a Leica 2035 BIOCUT microtome. Blocks were sectioned at 5 µm, and the best section slipped onto a glass slide and left to dry on a drying rack at 40 °C. When all the sections had been taken the slides were dried in a drying oven at 60 °C overnight. Slides were stained with haematoxylin and eosin (H&E) and mounted with pertex.

### *2.7.2 Haematoxylin and eosin staining*

The slides were dewaxed using xylene for 5min followed by the staining procedure outlined below: absolute alcohol (2 min); methylated spirit (1.5 min); wash in tap water; haematoxylin (5 min); wash in tap water; acid alcohol (3 quick dips); wash in tap water; Scot's tap water (1 min); eosin (5 min); wash in tap water; methylated spirit (30 sec); absolute alcohol (2 min); absolute alcohol (1.5 min); xylene (5 min); xylene (until coverslipped).

### *2.7.3 Pathological Assessment*

All histopathology assessments were examined and assessed "blind" to eliminate bias in interpretation by the fish veterinary surgeon Dr Pete Southgate from the Fish Vet Group in Inverness. Stained sections of heart were assessed for signs of endocarditis (level of inflammation affecting the internal endocardial epithelium of the heart chambers), pericarditis (level of inflammation affecting the pericardial sac) and pathology (principally the presence of any areas of necrosis of the cardiac muscle). Liver sections were evaluated on four characteristics; fat content (the level of fat deposition within each hepatocyte, represented by the presence of fat vacuoles within the cells), inflammation (the presence of inflammatory cells within the tissue), the degree of peri-vascular cuffing (pvc) (the presence of an accumulation of inflammatory or extra haematopoietic cells surrounding blood vessels within the tissue and forming a 'cuff' as this is often thought to be linked to poor performance) and finally the presence of necrosis (the level of any cell death within the organ, sometimes represented by the death of individual cells (single cell necrosis, scn) or by more extensive cell death (focal or more generalised). Intestinal sections were evaluated for four characteristics; mucus (prominence and level of mucus cells within the mucosa); vacuoles (prominence and level of absorptive vacuoles within the mucosa); cells (level of inflammatory cell infiltration into the lamina propria) and slough (the degree of sloughing of the mucosa into the lumen of the gut – this is sometimes associated with necrosis of the mucosa i.e. true ante-mortem pathology, but is a frequent finding due to some post mortem deterioration of the mucosa or processing artefact where the sectioning causes the delicate mucosa to slough into the lumen). Often there is more sloughing when there is a higher level of vacuolation presumably because the vacuoles make the mucosal

cells more prone to processing damage. All parameters mentioned were scored from 0 to 5, with 0 being the lowest score or the least amount of pathology seen and 5, the highest score, or greatest amount of pathological change. An Olympus BH-2 microscope was used in the assessment of the histology sections. Histopathology photographs were taken of various fish tissues using a Leitz Orthloux microscope and camera. The total magnification was calculated according to Appendix 4.

## 2.8 Lipid Analysis

All solvents used throughout this procedure were of HPLC grade and were obtained from Fisher Scientific, Loughborough,UK. All solvents contained 100mg $l^{-1}$ (0.01 %) of butylated hydroxytoluene (BHT) as an antioxidant.

### *2.8.1 Separation of peripheral blood leukocytes for lipid determination*

Peripheral blood leukocytes were isolated from blood from four fish per dietary treatment using the lymphocyte separation medium, Histopaque® and density gradient centrifugation. One ml of blood was diluted with 4 ml of L-15 medium and 3 ml of the diluted blood was layered onto 4 ml of Histopaque® and centrifuged at 400 x g for 45 min. The leukocytes located around the interface of the separation medium were collected and stored in 1ml of chloroform:methanol (2:1 v/v) at –20 °C until required for lipid extraction. If erythrocyte contamination was considered to be excessive then the gradient centrifugation was repeated.

### *2.8.2 Extraction of total lipid from fish peripheral blood leukocytes(PBL)*

Total lipid was extracted from fish PBL by a modification of the method of Folch *et al.*, (1957). Chloroform: methanol (4 ml, 2:1) was added to each leukocyte suspension, vortexed and put on ice for 30 min. KCl (1.3 ml, 0.88 %) was added and the solutions were mixed on a vortex mixer. After separation by centrifugation at 1000 x g for 2 min, the chloroform layer was collected and evaporated under a stream of oxygen-free nitrogen (N-Evap 112). The lipids were dried by vacuum desiccation before the weight of lipid was determined gravimetrically. The extracts were redissolved in 500 µl of chloroform: methanol (2:1) and stored at –20 °C. Fatty acid methyl esters (FAME) were prepared by acid-catalysed transesterification of total lipid according to the method of Christie, (1982). Toluene (1 ml) was added to the lipid sample if it contained total lipid, triacylglycerol, or partial acylglycerols and 2 ml of a 1 % sulphuric acid in methanol methylating reagent was added prior to the samples being flushed with nitrogen and incubated for 16 h at 50 °C in a hot block. $KHCO_3$ (4 ml), isohexane:diethyl ether + BHT (1:1 v/v) (1 ml) and isohexane: diethyl ether (1:1 v/v) (4 ml) was added to each sample tube and mixed using a vortex mixer. After centrifugation at 1000 x g for 2 min, the upper organic layer was transferred to another test tube and a further 5ml of isohexane:diethyl ether (1:1 v/v) was added to the original tube, shaken and centrifuged as before. The upper organic layer was again taken and combined with the previous one. The solvent was evaporated under oxygen-free nitrogen and the sample redissolved in 100 µl of isohexane. The methyl esters were purified by thin-layer chromatography (TLC) with lipid loading not exceeding 1.5 $mgcm^{-1}$. The TLC plates (20 x 20 cm Silica gel 60) were developed in

isohexane:diethyl ether:acetic acid (90:10:1 (v/v)). The plates were sprayed with 1 % (w/v) iodine in chloroform to visualize the fatty acid methyl esters (FAME). The FAME bands were scraped from the plates and eluted with 5 ml of isohexane:diethyl ether 1:1 (v/v) and 1 ml of isohexane:diethyl ether + BHT 1:1 (v/v). Finally the silica solvent mixture was filtered using a Buchner funnel and the FAME were separated and quantified by gas-liquid chromatography (Carlo Erba GLC8000 Vega series 2) using a 30 m x 0.32 mm i.d. fused silica wall coated capillary column (CP Wax 52CB) with an on-column injection system and flame ionization detection (FID). The injection temperature was 50 °C and the FID temperature was set to 250 °C. Hydrogen was used as the carrier gas and temperature programming was from 50 to 150 °C at 40 °C $min^{-1}$ and then to 225 °C at 2 °C $min^{-1}$. Fatty acids were separated in terms of carbon number with the most saturated eluted first followed by the monounsaturated and then the di-unsaturated etc. in the same series. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman, 1980; Bell *et al.*, 1983). Data were collected and processed using the Chromcard software for Windows (version 1.19) computer package.

## 2.9 Statistical analysis

Significance of difference ($p<0.05$) between dietary treatments was determined by either one-way analysis of variance, ANOVA or Student's 't' test, depending on the number of test dietary groups. Differences between means were determined by Newman-Keuls post test. ANOVA and regression analysis was performed using a GraphPad Prism (version 3.0) statistical package. Significant differences in values are indicated by a different superscript letter. Details of additional analysis can be found with the relevant chapters.

Chapter - 3 - Replacement of dietary fish oil with rapeseed and linseed oils in Atlantic salmon (*Salmo salar* L.): Effects on fish health

## 3.1 Introduction

### *3.1.1 Wild Atlantic salmon*

Atlantic salmon (*S. salar*) are an anadromous species and in the wild their life cycle starts in fresh water with the hatching of alevins, which emerge from gravel after 3-4 weeks ready to feed as fry. The fry then develop into the next stage known as "Parr" and can remain in fresh water for 2-3 years. Parr then become smolts in the spring of their second, third or fourth year of life and migrate to the sea. The transformation from parr to smolt involves a number of morphological, physiological and behavioural changes which pre-adapt young salmon for life in the sea while they are still in fresh water (Hoar, 1976). Folmar and Dickhoff (1980) reported "the onset and synchronisation of smoltification and migration appear to be regulated by environmental factors; primarily increasing day length and water temperature". On returning to fresh water, the salmon stops feeding. Eggs are laid in gravel in late autumn and after spawning the fish are known as kelts and many die at this stage.

### *3.1.2 Farmed Atlantic salmon*

The life cycle of farmed Atlantic salmon is somewhat different from that of its wild counterpart. The initial production stages of fertilisation, hatching and fry production are mainly conducted indoors. Fry develop in tanks indoors until, on reaching the parr stage, the fish are transferred to larger tanks, usually located outside, or to cages in fresh water. (FAWC, 1996). All the final stages of salmon production take place in cages in sea water, where the smolts are transferred, and fed until they are harvested.

The proximate composition of farmed fish (moisture, lipid, protein and ash content) can be influenced by a variety of factors, which can be classified as endogenous and exogenous (Shearer, 1994). Endogenous factors are genetically controlled and are mainly associated with the life cycle, while factors such as diet and farming environment are considered exogenous (Love, 1970; Shearer, 1994). Among the endogenous factors, the life cycle stage, sex, age, size and genotype can be considered as the most important. The lipid content of farmed Atlantic salmon has been shown to be significantly higher than that of its wild counterpart (Ackman & Takeuchi, 1986; Farmer *et al.*, 1997; Bell *et al.*, 1998a).

As mentioned in Chapter 1, the culture of Atlantic salmon relies heavily on marine FO to supply EFA for the production cycle. The capture fisheries that supply these oils have levelled off, or even declined, in recent years, while aquaculture has shown dramatic growth (10 % / annum worldwide) since the mid 1980's (Pike & Barlow, 2002). Therefore with global aquaculture production expanding, the need to investigate alternatives to FO is clear.

Numerous studies have shown that salmonids in seawater can effectively utilise VO with no apparent adverse effects on growth, providing that the oils contain sufficient 18:3(n-3) to satisfy their EFA requirements (Polvi & Ackman, 1992; Bell *et al.*, 1993a, b; Guillou *et al.*, 1995; Dosanjh *et al.*, 1998). Studies have shown that RO and LO are possible alternatives to FO as sources of EFA in Atlantic salmon parr up to smoltification (Bell *et al.*, 1997; Tocher *et al.*, 2000). In the same way that alternatives to fish meal are being investigated (Gomes *et al.*, 1995; Kaushik *et al.*, 1995; Helland & Grisdale-Helland, 1998; Carter & Hauler, 2000) the potential exists to replace FO with VO in the diets of many

cold-water fish species. However, the effects that this replacement has on growth of the fish and on its immune response, cellular function and tissue histology need further investigation. The present study was designed to examine the possible immunological and pathological effects of alternative oil inclusion in feeds for Atlantic salmon. Firstly, it was proposed to establish if any immunosuppression or enhancement resulted from changes in cellular fatty acid composition arising from VO incorporation *in vivo*, by measuring a variety of immunological parameters in experimental fish. Secondly, tissue histology was evaluated to determine if pathological changes occurred as a result of the inclusion of VO in fish diets.

## 3.2 Materials and Methods

### *3.2.1 Dietary Groups*

The following study was designed to examine the effects of combinations of two dietary VO, LO and RO, on the immune response of Atlantic salmon post smolts in seawater and to see if any negative effects that occurred could be restored by the subsequent feeding of a FO-only finishing diet. Photoperiod manipulated smolts (initial weight 120 ± 10 g) were distributed into ten cages (5 x 5 m; 600 fish cage$^{-1}$) in Loch Duich, Lochalsh, Scotland (Figure 3.1) and fed one of ten diets, a control diet containing FO alone and nine diets containing different combinations or ratios of FO and/or RO and LO. Specifically, the ten diets produced were comprised of 100 % FO, 100 % LO or 100 % RO, FO/RO (2:1 and 1:2, w/w), FO/LO (2:1 and 1:2, w/w), RO/LO (2:1 and 1:2, w/w) or FO/RO/LO (1:1:1, w/w/w) forming a triangular experimental design as shown in Figure 3.2. Each square

Figure 3.1 Marine Harvest Fish Trials Unit (FTU), Loch Duich, Lochalsh, Scotland.

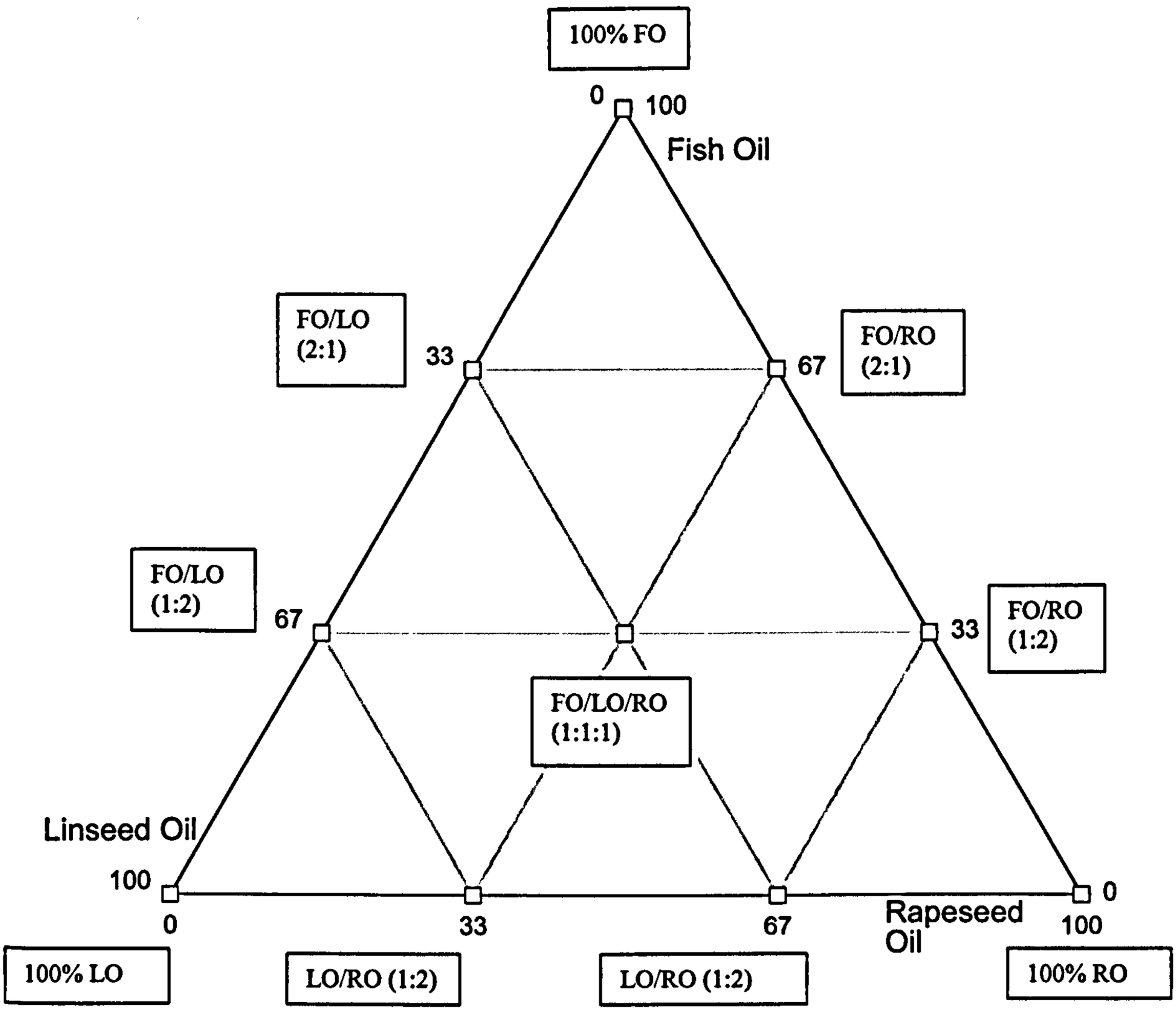



Figure 3.2 Triangular dietary experimental design.

Numbers refer to percentage oil added and ratios in parenthesis refer to the ratio of the oils added. FO = fish oil, LO = linseed oil, RO = rapeseed oil

represents a diet with the three corners representing the diets containing 100 % FO or RO or LO, with intermediary diets having graded amounts of the two oils on that side of the triangle. The diet in the centre represents the diet containing all three oils in equal proportion. The experimental diets were prepared by EWOS Technology Centre, Livingston, Scotland. Initially, fish were fed 3 mm pellets containing 47.0 % crude protein, 24.1 % crude lipid and 7.6 % moisture. Fish were later fed 6 mm pellets containing 41.8 % protein, 30.5 % lipid and 6.8 % moisture once they reached an average weight of 500 g. The dietary formulations and fatty acid compositions of each diet are shown in Tables 3.1 and 3.2 respectively. The 100 % FO diet was fed to triplicate pens of fish, whereas the experimental diets were fed to individual pens. Fish were fed for 50 weeks on the experimental trial diets, and then fish of 1.8 to 2.3 kg were transferred onto a FO only diet for a further 12 week "wash-out" phase. Fish were sampled twice, after feeding the experimental diets for 50 weeks, with a final sampling performed after 12 weeks feeding with a FO diet. Twelve fish from each dietary group were sampled at both sample points. The average temperature over the experimental period (February 1999-July 2000) was 10.8 ± 3.4 °C. After sampling at week 50, the remaining fish in pens fed the 100 % FO, RO and LO diets were redistributed into three pens, with each pen containing 100 fish from each treatment. The fish were marked by fin clipping to identify their previous dietary history.

All three pens were fed a diet containing 100 % FO, in a 9 mm pellet, for a further 12 "week wash out" period. The composition of this diet was essentially similar to the 6 mm 100 % FO diet. The experimental diets offered differed only in their oil composition and

Table 3.1. Formulations of the experimental diets

| Component g/100g | FO 100% | FO/LO (2:1) | FO/LO (1:2) | LO 100% | FO/RO (2:1) | FO/RO (1:2) | RO 100% | LO/RO (2:1) | LO/RO (1:2) | FO/LO/RO (1:1:1) |
|---|---|---|---|---|---|---|---|---|---|---|
| Fishmeal[1] | 53.8 | 53.8 | 53.8 | 53.8 | 53.8 | 53.8 | 53.8 | 53.8 | 53.8 | 53.8 |
| Soya (Hi Pro)[2] | 7.6 | 7.6 | 7.6 | 7.6 | 7.6 | 7.6 | 7.6 | 7.6 | 7.6 | 7.6 |
| Wheat[3] | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 |
| Fish oil[4] | 23.6 | 15.7 | 7.9 | 0 | 15.7 | 7.9 | 0 | 0 | 0 | 7.9 |
| Rapeseed oil[5] | 0 | 0 | 0 | 0 | 7.9 | 15.7 | 23.6 | 7.9 | 15.7 | 7.9 |
| Linseed oil[5] | 0 | 7.9 | 15.7 | 23.6 | 0 | 0 | 0 | 15.7 | 7.9 | 7.9 |
| Micronutrients[6] | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 1 |

[1]Norsemeal, London SW, England.

[2]Grosvenor grain, Perth, Scotland.

[3]Stewarts of Larbert, Larbert, Scotland.

[4]United fish Products, Aberdeen, Scotland.

[5]Meade-King Robinson & Co., Liverpool, England.

[6]Vitamins, minerals and

Astaxanthin (Roche Products, Heanor, Derbyshire, England to specification by Ewos Ltd., Bathgate, Scotland)

Astaxanthin, as Carophyll pink®, 50 $mgkg^{-1}$ diet; Vitamin A as retinyl acetate, 2.6 $mgkg^{-1}$; Vitamin D as cholecalciferol, 0.1$mgkg^{-1}$ and Vitamin E as DL-α-tocopherol acetate, 250 $mgkg^{-1}$. All other vitamins and minerals provided at levels in excess of nutritional requirements for salmonid fish (US National Research Council 1993).

FO = fish oil, LO = linseed oil, RO = rapeseed oil. Ratios in parenthesis represent ratio of oil added.

Table 3.2. Fatty acid compositions of the ten experimental diets used in the feeding trial (Values are weight % of total fatty acids).

| Fatty acid/diet | 100 % FO | 100 % LO | 100 % RO | LO:FO (1:2) | LO:FO (2:1) | RO:FO (1:2) | RO:FO (2:1) | LO:RO (1:2) | LO:RO (2:1) | LO:RO:FO (1:1:1) |
|---|---|---|---|---|---|---|---|---|---|---|
| 14:0 | 5.6 | 1.1 | 1.2 | 3.6 | 2.2 | 3.7 | 2.3 | 1.1 | 1.2 | 1.9 |
| 16:0 | 13.6 | 7.6 | 7.2 | 11.6 | 9.6 | 11.7 | 9.4 | 7.6 | 7.7 | 8.8 |
| 18:0 | 2.5 | 3.6 | 1.9 | 2.9 | 3.4 | 2.3 | 2 | 2.4 | 3 | 2.7 |
| **Total saturates[1]** | **24** | **13.2** | **11.4** | **20.2** | **16.7** | **19.9** | **15.5** | **10.9** | **13** | **14.9** |
| 16:1n-7 | 5.5 | 1 | 1.3 | 3.5 | 2.2 | 3.8 | 2.5 | 1.3 | 1.2 | 1.9 |
| 18:1n-9 | 15.1 | 16.6 | 48.3 | 16.1 | 16.4 | 26.7 | 37.5 | 36.9 | 25.9 | 28.8 |
| 20:1n-9 | 9.6 | 1.9 | 3 | 6 | 3.7 | 6.5 | 4.6 | 2.6 | 2.2 | 3.5 |
| 22:1n-11 | 13.2 | 2.3 | 2.7 | 8.2 | 4.8 | 8.3 | 5.4 | 2.7 | 2.6 | 4.3 |
| **Total monounsaturates[2]** | **44.3** | **22.1** | **55.6** | **34.6** | **27.6** | **46.2** | **50.6** | **45.5** | **32.3** | **39** |
| 18:2n-6 | 4.5 | 13.6 | 17.9 | 7.9 | 11.4 | 9.5 | 14 | 16.3 | 14.6 | 13.7 |
| 20:4n-6 | 0.6 | 0.2 | 0.2 | 0.4 | 0.3 | 0.5 | 0.3 | 0.2 | 0.2 | 0.2 |
| **Total n-6[3]** | **5.5** | **13.9** | **18.1** | **8.7** | **11.8** | **10.4** | **14.6** | **16.8** | **15** | **14.1** |
| 18:3n-3 | 1.7 | 45.4 | 8.9 | 18.6 | 33 | 4.7 | 6.6 | 20.2 | 32.9 | 22 |
| 18:4n-3 | 2.8 | 0.5 | 0.4 | 1.8 | 1 | 1.9 | 1.1 | 0.4 | 0.4 | 0.9 |
| 20:4n-3 | 0.8 | 0.1 | 0.1 | 0.6 | 0.3 | 0.6 | 0.4 | 0.1 | 0.2 | 0.2 |
| 20:5n-3 | 7.3 | 1.8 | 1.9 | 5.3 | 3.3 | 5.7 | 3.9 | 2.1 | 2.2 | 3.1 |
| 22:5n-3 | 1.2 | 0.3 | 0.3 | 1 | 0.6 | 1.1 | 0.6 | 0.4 | 0.4 | 0.5 |
| 22:6n-3 | 10.5 | 2.7 | 2.9 | 8.4 | 5.3 | 8.7 | 5.9 | 3.5 | 3.4 | 4.9 |
| **Total n-3** | **24.5** | **50.8** | **14.5** | **35.8** | **43.6** | **22.8** | **18.6** | **26.7** | **39.6** | **31.5** |
| **n-3/n-6** | **4.4** | **3.7** | **0.8** | **4.1** | **3.7** | **2.2** | **1.3** | **1.6** | **2.6** | **2.2** |

[1]Includes 10:0, 12:0, 17:0, 20:0 and 22:0; [2]Includes 14:1, 17:1, 20:1n-7, 22:1n-9 and 24:1; [3]Includes 18:3(n-6), 20:2(n-6), 20:3(n-6) and 22:5(n-6). FO = fish oil, LO= linseed oil, RO = rapeseed oil.

were formulated to satisfy the nutritional requirements of salmonid fish, including (n-3) EFA which are provided both by the inclusion of dietary fish meal and by ALA present in LO and RO (US National Research Council, 1993). The experiment was conducted in accordance with the British Home Office guidelines regarding research on experimental animals.

### *3.2.2 Measurement of cellular immune responses*

#### 3.3.3.1 Measurement of mitogen stimulation response

The capacity of T and B lymphocytes, derived from peripheral blood, to be stimulated by mitogens was determined using a lymphocyte stimulation assay as previously described in Section 2.4.2. At week 50 and after the 12 week "wash-out" period, heparinised blood and head kidneys were collected from 12 fish maintained on each of the following four diets: 100 % FO, 100 % LO, 100 % RO and the equal mix of the three oils (FO: LO: RO 1:1:1). Due to the time required for sampling and assay procedures, no other dietary groups were sampled for measurement of immune function apart from the four diets mentioned above.

#### 3.3.3.2 Macrophage function

As the trial was located some distance from the laboratory, it was not possible to isolate the cells on Percoll density cushions as described by Secombes (1990) so the methods outlined in Sections 2.3.3 and 2.3.5.2 were followed. Macrophage suspensions were prepared for measuring the respiratory burst activity by the reduction of NBT as outlined in Section 2.4.1.

### 3.3.3.3 Histopathology

Samples were collected at Week 50 to identify any effects of oil type on the histology of the heart, liver or intestine. Samples were also examined after the 12 week "wash-out" period to determine if any pathological effects observed had been attenuated by the resumption of the FO diet. Both at Week 50 and at the end of 12 weeks feeding with the FO finishing diet, samples of proximal, mid and distal intestine were collected from 6 fish from all ten dietary groups, in addition to the heart and liver for histopathological examination. Sections were fixed in 10 % buffered formalin at the time of dissection, embedded in paraffin wax and 5 μm sections were cut and stained with haematoxylin and eosin as outlined in Sections 2.8.1 and 2.8.2. Processed sections were examined and assessed "blind" to eliminate bias in interpretation (Section 2.8.3).

## *3.2.3 Statistical Analysis*

All the data are presented as means ± SEM (n = 12) unless otherwise stated. Significant differences between dietary treatments were determined by analysis of variance (ANOVA) using $p<0.05$ as a cut off for significance. Differences between means were determined by Newman-Keuls post test.

## 3.3 Results

### *3.3.1 Growth*

Presentation of growth data is not shown in this Chapter but is covered in Bell *et al.*, (2003a). Weights of fish sampled after 32 weeks did not differ ($p>0.05$). However, after feeding the experimental diets for 50 weeks, there were differences between dietary treatments ($p=0.0004$). Despite the weight differences, regression analysis carried out by Bell *et al.*, (2003a) showed no correlation between final weight and any dietary fatty acid, indicating that the dietary treatment was not responsible for differences in final weight. Bell *et al.*, (2003a) suggested that the differences in final weight may be explained by feeding methodology. There were no differences in final weight among the three replicate FO treatments. Fish were sampled again after a further 20 week feeding period with a 100 % FO diet (finishing diet), however no differences in final weights were observed.

### *3.3.2 Assessing Immune response*

#### 3.3.3.1 Mitogen stimulation

After feeding with diets containing 100 % LO or 100 % RO for 50 weeks, the T-cell responses assessed using Con A as the mitogen showed no significant differences (mean stimulation index (S.I.) = 0.698 and 0.778 respectively) compared to the 100 % FO fed fish (mean S.I. = 1.413) as shown in Figure 3.3. In fish fed the 100 % LO (mean S.I.= 3.0) and the FO:LO:RO (1:1:1) diet (mean S.I.= 3.2) (Figure 3.3), there were no significant differences found in the B-cell response assessed using LPS compared to the fish fed the

100 % FO diet. Feeding with 100 % RO had no statistical effect on B-cell responses (Figure 3.3).

After resumption of the FO diet at week 50, fish which had been fed the 100 % RO, 100 % LO and the FO:LO:RO mix (1:1:1) had no significant differences in T-cell response to Con A (Figure 3.3) compared to the control FO diet. No significant differences were found in the B-cell response to LPS either, although the group that had been fed the 100 % LO diet showed a lower mean stimulation index (S.I. = 0.84) than the other dietary groups (Figure 3.3).

### 3.3.3.2 Macrophage function

Fifty weeks after maintaining the salmon on the different diets the activity of macrophages isolated from the head kidney was significantly depressed in the 100 % RO group ($p=0.0006$) (mean macrophage activity = 22.5) compared to the macrophage activity measured in the other three dietary groups (Figure 3.4). No other differences between the treatments were significant.

After resumption of a FO only diet, both the LO (mean macrophage activity = 39.6) and RO (mean macrophage activity = 34.4) diets had significantly depressed macrophage activity ($p=0.0387$) compared to the control, while the macrophage activity of fish fed the equal mix diet was not affected by the dietary changes (Figure 3.4).

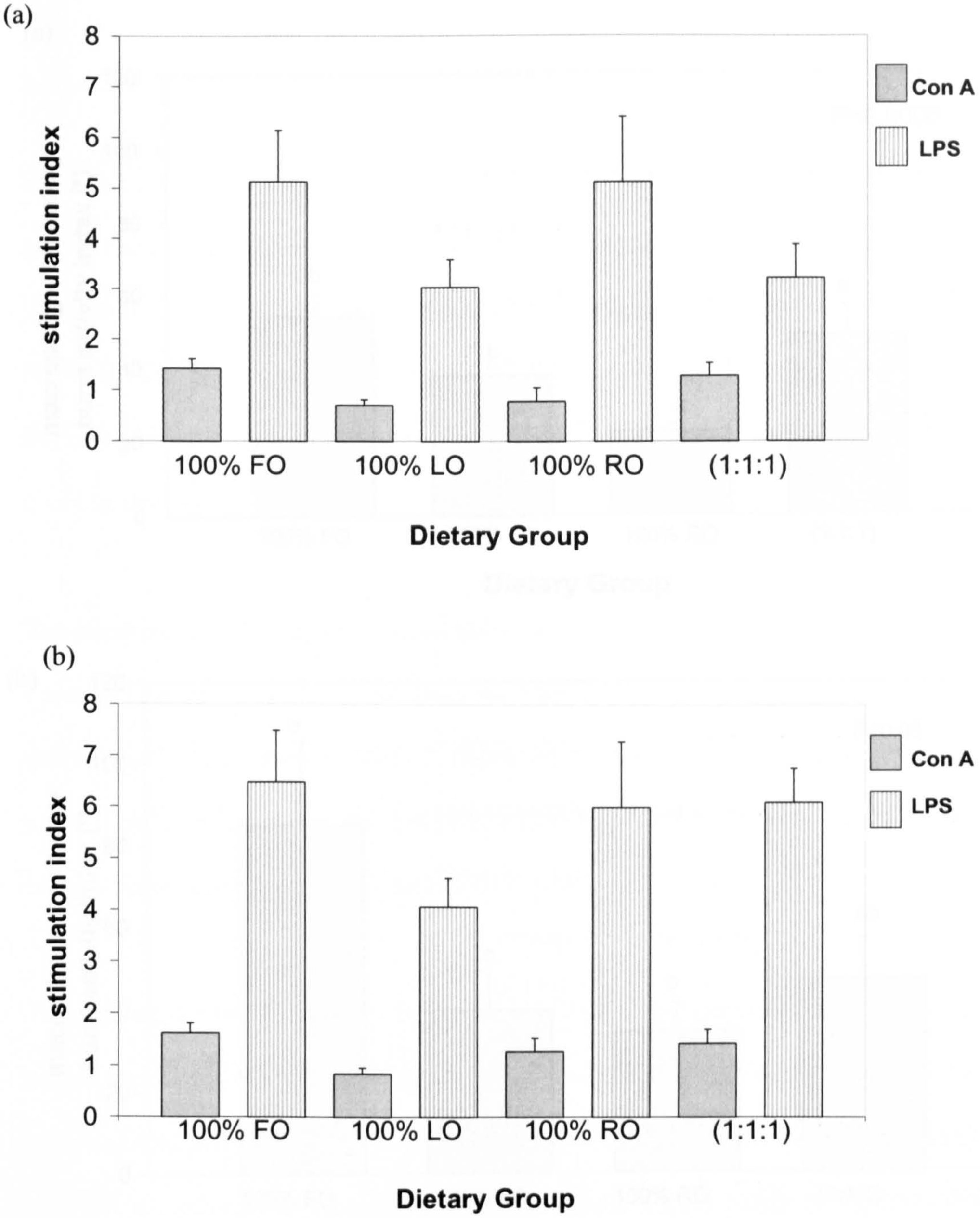



Figure 3.3 Effect of feeding the experimental diets on salmon (a) response of lymphocytes to ConA and LPS after 50 weeks feeding the diets (b) response of lymphocytes to ConA and LPS after feeding a FO diet for a 12 week 'finishing' period. FO = fish oil, LO = linseed oil, RO = rapeseed oil, ConA = Concanavalin A, LPS = lipopolysaccharide.

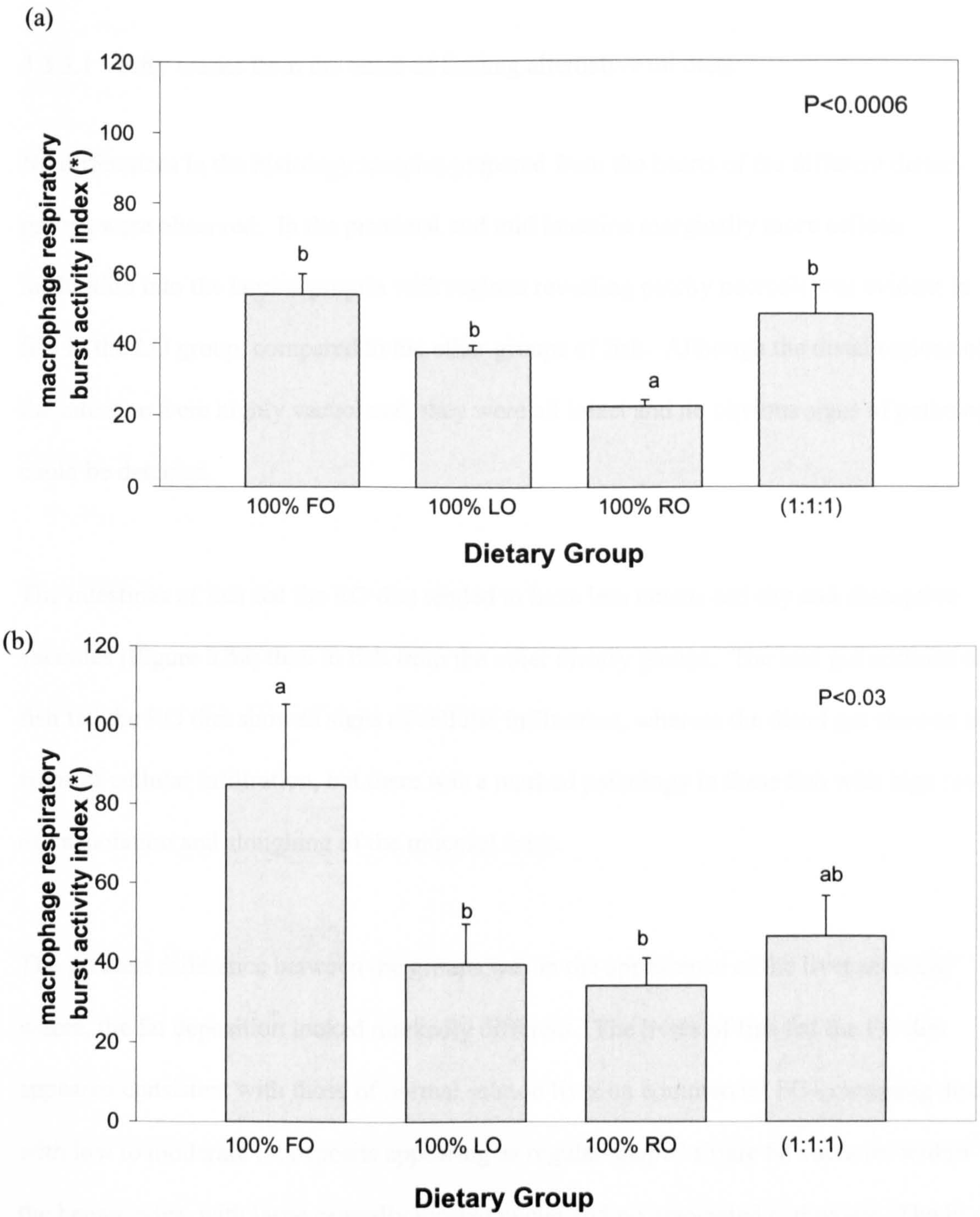



Figure 3.4 Effect of feeding the experimental diets on salmon head kidney macrophage respiratory burst activity (a) after feeding the experimental diets for 50 weeks (b) after feeding with a FO diet for a 12 week 'finishing' period. * = Absorbance per $10^5$ cells x 100. FO = fish oil, LO = linseed oil, RO = rapeseed oil.

### *3.3.3 Histopathology*

#### 3.3.3.1 Fifty weeks from the onset of feeding alternative oil diets

No differences in the histology samples prepared from the hearts of the different dietary groups were observed. In the proximal and mid intestine marginally more cellular infiltration into the lamina propria with regions revealing patchy necrosis was evident in fish in the LO group, compared to the other groups of fish. Although the distal regions of the intestine were highly vacuolated, they were all intact and no obvious signs of pathology could be detected.

The intestines of fish fed the RO diet tended to have less mucus activity and absorptive vacuoles (Figure 3.5a) than in fish from the other dietary groups. The mid gut sections of fish fed the RO diet showed signs of cellular infiltration, whereas the distal gut showed no signs of cellular infiltration, but there was a marked pathology in these fish with high levels of vacuolation and sloughing of the mucosal folds.

The greatest difference between the groups was in the appearance of the liver sections, where, the fat deposition looked markedly different. The livers of fish fed the FO diet appeared consistent with those of normal salmon liver on commercial FO-containing diets with low to moderate fat deposits appearing as regular-shaped single fat vacuoles within the hepatocytes, with large centrally located nuclei and no associated pathology. The livers of fish fed the RO diet (Figure 3.5b) appeared more variable, and showed some associated pathology. There was an uneven deposition of fat, with some hepatocytes containing

single large vacuoles and others having a more foamy appearance of multiple vacuoles, which contained high levels of un-metabolised fat (Figure 3.5b). This appearance was more pronounced in the livers of fish fed with the LO diet where there was disruption of the normal hepatic architecture and some breakdown of hepatocytes (Figure 3.5c).

In the fish fed diets containing the mixed VO combinations (LO:RO (2:1) or the LO:RO (1:2)), the most outstanding pathology was seen in the intestine. The proximal and mid sections of fish fed the LO:RO (2:1) diet showed low mucus activity and low levels of absorptive vacuoles. All of the six intestinal sections examined from fish fed the LO:RO (1:2) diet (Figure 3.5d) showed definite mucosal fold pathology in the proximal, mid and distal regions, with areas of mucosal cell necrosis and cellular infiltration. The FO:LO:RO mixed oil diet (1:1:1) also showed signs of intestinal pathology, with cellular infiltration up the lamina propria of the distal sections (Figure 3.5e).

### 3.3.3.2 After resumption of a fish oil only diet for a further twelve weeks

Fish fed the FO diet for 62 weeks appeared to have no evidence of abnormalities detected in any of the tissues examined. The hearts appeared normal and the livers had a low fat content with evenly sized fat vacuoles giving an appearance of regular even fat deposition. The gut sections appeared normal with large numbers of mucus cells in the proximal region and the absorptive vacuoles far more prominent in the distal region causing slightly more mucosal breakdown, as the presence of the vacuoles appears to make the mucosa more

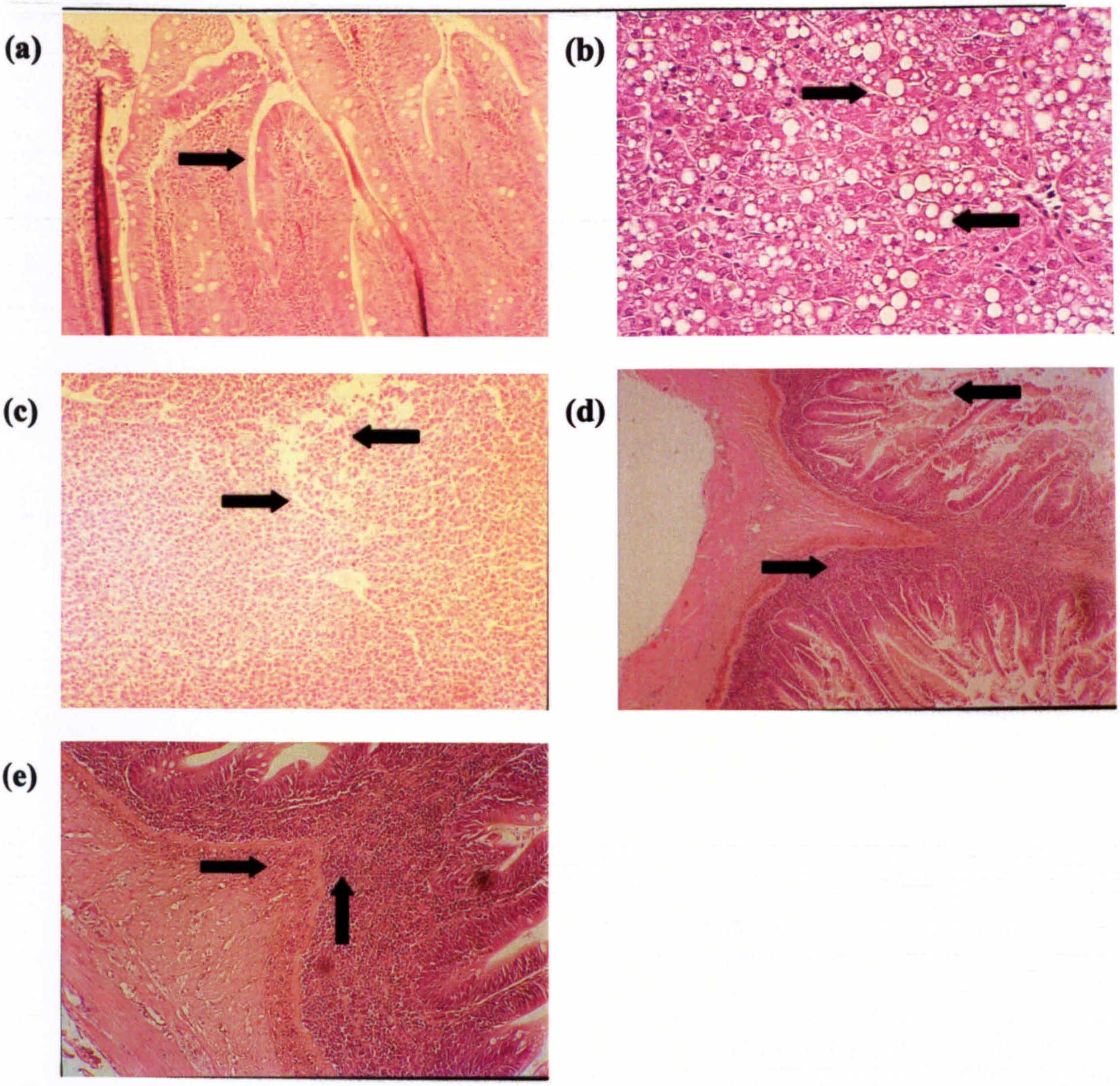



Figure 3.5 Histopathology of Atlantic salmon fed the experimental diets for 50 weeks showing (a) Proximal intestine from fish fed 100 % RO diet (mag. x 175 (b) Liver from fish fed 100 % LO diet (mag. x 175) (c) Liver from fish fed 100 % LO diet (mag. x 175) (d) Distal intestine from fish fed LO/RO (1/2) diet (mag. x 70) (e) Distal intestine from fish fed equal mixed (1:1:1) diet (mag. x 70).

vulnerable. A few of the heart samples taken from fish fed the 100 % LO diet appeared to have small areas of inflammatory cell accumulation and increased cellularity at the spongy/compact junction in the heart. The fat deposition in the liver sections of the fish previously fed the 100 % LO diet was extremely variable i.e. some cells contained very large single fat vacuoles where others contained little fat or multiple, small vacuoles. Although, the intestine had a normal appearance there was evidence of mucosal breakdown in the distal region, but this probably reflects the high level of absorptive vacuoles present. No cellular infiltration was evident in any of the intestinal sections from fish fed the 100 % LO diet. Small areas of pericarditis and inflammation were evident in the heart sections from fish previously fed the 100 % RO diet. The fat deposition in the livers of fish previously fed 100 % RO was variable and some sections showed loss of normal hepatic structure. The proximal intestine appeared to have a high mucus cell activity (Figure 3.6a) resembling that seen in fish previously fed the 100 % LO diet, and there were signs of cellular infiltration in the proximal region with breakdown of the mucosa throughout the whole length of the gut. The most outstanding finding was in the fish fed the mixed VO diets (LO:RO (2:1) (Figure 3.6b) and LO:RO (1:2)) where significant cellular infiltration could be seen and a massive inflammatory response was evident in the distal sections of the intestine. There was also definite liver pathology (Figure 3.6c and Figure 3.6d) in samples taken from fish fed the LO:RO (2:1) diet with frequent single cell necrosis, loss of structure and general degenerative change. Changes in the liver of the LO:RO (1:2) dietary group were also observed (Figure 3.6e) showing unhealthy hepatocytes and a general loss of liver structure and architecture.

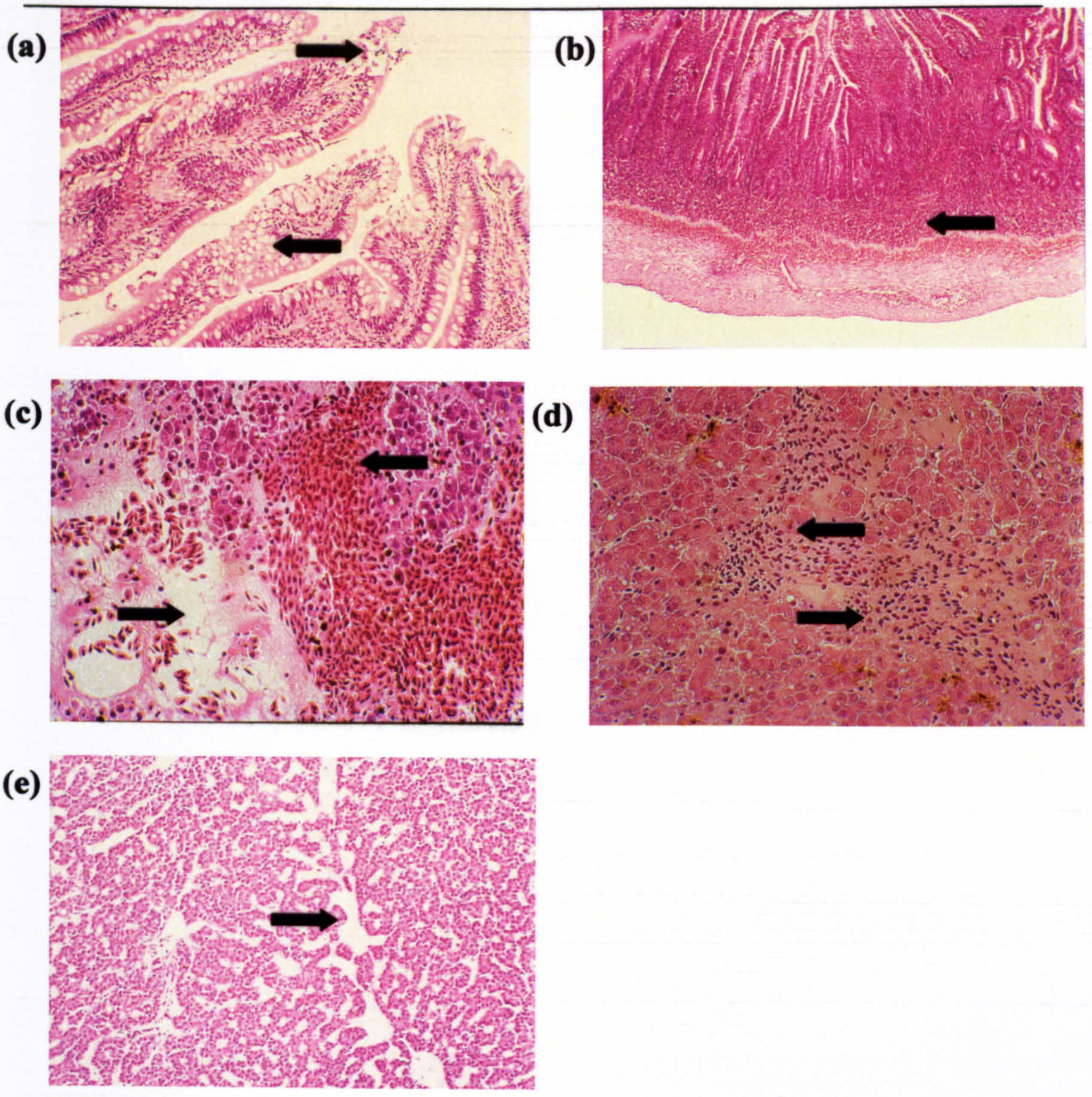



Figure 3.6 Histopathology of Atlantic salmon following resumption of the FO diet for 12 weeks showing (a) Proximal intestine of fish fed 100 % RO diet (mag. x 175) (b) Distal intestine of fish LO/RO (2/1) diet (mag. x 70) (c) Liver of fish fed LO/RO (2/1) diet (mag. x 175) (d) Liver of fish fed LO/RO (2/1) diet (mag. x 70) (e) Liver of fish fed LO/RO (2/1) diet (mag. x 175).

## 3.4 Discussion

Researchers investigating the effects of replacing FO with VO sources initially focussed on salmonid species and in particular salmon. Anadromous fish like salmon have a limited ability to convert ALA and LA, which are abundant in many VO, to their long-chain HUFA products which are essential physiological components of all cell membranes and organs. Therefore, salmon are potential candidates for replacement of dietary FO with high quality, n-3 PUFA-rich VO.

The objective of this trial was to establish if salmon could be grown on diets containing VO rather than FO without deleterious effects to their health or development. In the present study, salmon post smolts of initial weight around 120 g were grown to harvest weight of around 2 kg using diets in which the added oil component contained between 33 and 100 % of LO and/or RO. Several studies have shown that Atlantic salmon, Brook char (*Salvelinus fontinalis*) and juvenile turbot (*Scophthalmus maximus*) can be fed a variety of different dietary fats and oils, with no apparent adverse side effects (Polvi *et al.*, 1992; Bell *et al.*, 1993a; Bell *et al.*, 1993b; Guillou *et al.*, 1995; Dosanjh *et al.*, 1998). It has been documented that the different dietary fats affect the fatty acid composition of the fish, as in other animals (Marshall & Johnston, 1983; Thomassen & Røsjø, 1989; Bell *et al.*, 1993a, 2001, 2002; Rosenlund *et al.*, 2001a, b; Regost *et al.*, 2003; Bell *et al.*, 2004). However, information is still required regarding the effect of dietary fats on health and the immune status of the fish fed diets containing VO. LO is rich in ALA and is therefore a potential candidate for FO replacement. However, it also contains substantial levels of LA in a ratio of 3-4:1. ALA possesses anti-inflammatory properties due to the inhibition of ARA

synthesis and by inhibition of cyclooxygenase (Garg *et al.*, 1990). RO is also a possible candidate for FO replacement as it contains large amounts of oleic acid, as well as ALA and LA in a ratio of 1:2, which is thought to be beneficial for human health (De Lorgeril *et al.*, 1994; Ackman *et al.*, 1990).

Following 50 weeks feeding with the VO diets in the current trial, neither T-cell, nor B-cell responses to mitogen stimulation appeared to be suppressed. No change in response was observed after resumption with a FO diet for a further 12 weeks. Cultures of Atlantic salmon head kidney macrophages *in vitro* indicated that feeding with RO for 50 weeks had detrimental effects on macrophage activity. Respiratory burst activity of head kidney macrophages was significantly lowered as a result of feeding salmon with diets containing RO. With a return to a FO diet, the non-specific macrophage activity was still significantly suppressed in salmon previously fed the LO and RO diets. Montero *et al.*, (2003) carried out a similar trial, feeding gilthead seabream (*Sparus aurata*) diets containing soybean oil, RO and LO or a blend of these oils compared to a FO control diet. Significant differences were found in the number of circulating red blood cells when comparing the FO fed fish with fish fed diets containing either LO or soybean oil. Fish fed the soybean oil diet had significantly lower complement activity than fish fed the FO diet but no affect of feeding LO or RO was observed on the activity of complement. In contrast to the findings from this trial there was no effect of feeding VO diets on the macrophage respiratory burst activity measured by NBT reduction, although, the phagocytic activity of the macrophages was significantly affected in fish fed either the RO or soybean oil containing diet. Montero and colleagues (2003) concluded that the high content of either ALA or monoenes in the

cell membrane and imbalances in other fatty acids could influence the membrane's physical properties and hence the phagocytic activity. By influencing the cell membrane lipid composition and its physical properties this may have profound effects on disease resistance because many immune responses are based on leukocyte cell membrane interactions. Therefore, changes in the fatty acid composition of immunoactive cells may also change the activity of membrane associated reactions and thereby change the cellular activity. Balfry and Higgs (2001) reviewed the influence of dietary lipids on the immune system and disease resistance of finfish and summarised three possible mechanisms by which dietary fat may affect the overall immune response. Firstly, dietary lipids strongly influence the fatty acid composition of membrane phospholipids, which determine physical properties such as fluidity and permeability. Hence, the activity of membrane associated enzymes, membrane receptors, and the binding of mitogens, antigens and soluble mediators can all be affected by fatty acid composition. In addition, cyclooxygenase and lipooxygenase products of fatty acids namely, prostaglandins, thromboxanes and leukotrienes and other eicosanoid products are important intercellular signalling agents, which affect cell behaviour and cell to cell interactions. A deficiency of these compounds results in progressive impairment of function, while excessive or imbalanced production may result in a number of pathophysiological states e.g. inflammation or immunosuppression (Lands, 1986a, b). Finally, another possible mechanism by which dietary fatty acids may affect the immune system involves alteration of signal transductions, possibly due to effects on protein kinase C (Balfry & Higgs, 2001).

It is known that long chain n-3 PUFA, EPA and DHA, can influence the immune system of humans, fish and many other animals (Virella *et al.* 1989; Fujikawa *et al.* 1992; Purasiri *et al.* 1997; Wu & Chen 2001; Calder *et al.*, 1998a, 2001b, c, 2002). Changing ratios and types of n-3 PUFA can affect the capacity of immune and inflammatory cells to produce eicosanoids such as $PGE_2$ and $LTB_4$. A mammalian diet with a high n-6/n-3 ratio favours the production of $LTB_4$, which enhances cellular immune functions (Hwang 1989; Kinsella 1990). Conversely, a diet with a high n-3/n-6 ratio decreases cellular production of $PGE_2$, which dampens down cellular responses of the immune system (Meade & Mertin 1978; Goodwin & Ceuppens, 1983; Kinsella 1990). Feeding laboratory animals diets rich in FO (high n-3/n-6) results in decreased mitogen-stimulated lymphocyte proliferation, decreased natural killer cell activity, decreased cytokine production by lymphocytes and reduced capacity of macrophages to produce cytokines amongst other parameters (Calder 1998b). In short, n-6 PUFA are pro-inflammatory and n-3 PUFA are anti-inflammatory. In fish, however, the cellular requirements for n-3 and n-6 are reversed and, consequently, the diet must reflect this high n-3/n-6 requirement. The results from this study indicate that there was no statistical reduction or enhancement in T-cell or B-cell capacity when high n-3/n-6 FO was replaced with high n-6/n-3 VO. Thompson *et al.*, (1996) however did find significantly reduced B-cell function in response to *A. salmonicida* antigens following vaccination in Atlantic salmon given a high n-6/n-3 diet (sunflower oil). Bacterial challenge led Thompson *et al.*, (1996) to the conclusion that salmon fed with a high n-6/n-3 diet were less resistant to infection than those fed diets containing lipid with a low n-6/n-3. In another study by Bransden *et al.* (2003), significant differences were detected in cumulative mortalities of Atlantic salmon fed diets containing various ratios of sunflower

oil and challenged with *V. anguillarum*. No significant correlation was found however between cumulative mortality to any dietary nutrient (i.e. fatty acids, lipid, protein). Increased head kidney macrophage activity has been associated with higher levels of n-3 fatty acids in channel catfish (Blazer, 1991; Sheldon & Blazer, 1991) and rainbow trout (Ashton *et al.*, 1994).

In the present study, the most significant finding was the effect of VO on tissue pathology. Histopathological examination revealed that in salmon fed diets containing LO and RO there was a progressive loss of intestinal mucosal integrity, particularly in the distal portion and disruption of the normal hepatic architecture. It was observed in the salmon fed the LO/RO 2:1 diet that the intestine showed low mucus activity and low levels of absorptive vacuoles. This may be a consequence of the diet, with the gut becoming less responsive and having a low absorption capacity. A surprising outcome of the study was that after cessation of trial diet feeding and reversion to a normal FO diet for 12 weeks, evidence of pathological disruption was still evident.

Studies carried out by Bell *et al.*, (1991a; 1993b) revealed that Atlantic salmon fed sunflower oil containing diets developed marked cardiac histopathology with severe lesions that caused thinning of the ventricular wall and muscle necrosis. This histopathology was also present in the current study at a low level in FO fed fish and RO fed fish but was virtually absent in the salmon fed diets containing LO. The findings of Bell *et al.*, (1993b) are in agreement with the results found in this trial where the hearts from the LO fed salmon appeared to be the healthiest of all the groups examined. Grisdale-Helland *et al.*,

(2002) found no histological pathologies in heart tissue of Atlantic salmon after feeding with soybean oil for two months. In the present study a major histological difference found among fish fed the experimental diets was the occurrence of variable sized vacuoles and large amounts of lipid droplets within the hepatocytes of salmon fed both RO and LO compared to the control FO fed salmon. These findings suggest an effect of dietary lipid on the transport and metabolism of lipid in fish. Perhaps, accumulation of lipid droplets in the liver of salmon fed the RO and LO diets may be due to the use of certain fatty acids as energy sources in preference to other fatty acids, resulting in the storage of certain ones, for example, ALA and oleic acid within the lipid droplet giving the appearance of "un-metabolised fat" as outlined in the histopathology report.

Caballero *et al.*, (2002) found that feeding rainbow trout diets containing either soybean oil, RO or a palm oil/RO mix, resulted in the occurrence of numerous lipid vacuoles in the enterocytes compared to fish fed the control FO diet. Similarly, livers from these dietary groups showed large amounts of lipid droplets within the hepatocytes of the liver. It has been suggested that the accumulation of lipid in the enterocytes may be considered as a temporal storage of lipid due to insufficient lipoprotein synthesis (Watanabe, 1982; Kanazawa, 1985; Sargent *et al.*, 1989; Olsen *et al.*, 1999, 2000). Following on from these results, Caballero *et al.*, (2003) evaluated the morphological changes in the intestine of gilthead seabream (*Sparus aurata*) fed different lipid sources. Accumulation of lipid was again observed in the enterocytes of fish fed diets containing soybean oil and LO when compared to the FO control group. It was noted, that despite the large accumulation of lipid droplets no pathological damage or cellular necrosis of the intestinal epithelium

occurred. In contrast to this, Olsen *et al.*, (1999, 2000) found a significant impact on the gastrointestinal tract function and integrity when feeding large amounts of LO to Arctic char (*Salvelinus alpinus*). Feeding with LO promoted lipid droplet accumulation in the enterocytes, which resulted in epithelial damage and lipid droplet and cell debris in the intestinal lumen. Olsen *et al.*, (1999, 2000) concluded that feeding experimental diets containing high levels of PUFA and low amounts of saturated fatty acids promotes the accumulation of lipid droplets that induce a pathogenic state.

Long chain PUFA are known to play an important role in the maturation and composition of enterocytes. It has been shown in piglets with histological lesions and biochemical alterations caused by malnutrition, that the small intestine of those fed diets supplemented with long chain PUFA recovered more quickly than those on a non-supplemented diet (Lopez-Pedrosa *et al.* 1999). In this study it may be that 12 weeks feeding with normal FO diets is too short a time for such recovery of intestinal and liver integrity of the fish to be seen. However, it is known from other studies, involving alternative ingredients, that a sub-acute inflammation of the distal epithelial mucosa is rapidly evident following feeding with soybean meal for six weeks. A fully developed pathological condition was observed after 3 weeks on the experimental diets, with the first signs of morphological change observed after 2 days (Baeverflord & Krogdahl, 1996). However, this pathology resolves completely within a short time (days) of reversion to a non-soya diet.

Salmon flesh is rich in long chain n-3 PUFA such as EPA and DHA and low in n-6 PUFA such as LA (Bell *et al.* 1998a). Around 5-6 % of the total fatty acids in salmon flesh are in the form of EPA and 10-12% as DHA, which represents an intake of around 15 g of EPA

and DHA/kg of flesh. The benefits of the inclusion of FO in the human diet have been outlined in Chapter 1 and they provide a strong human nutritional case for retaining high levels of EPA and DHA and low levels of LA in farmed fish. Therefore, in conclusion, regarding VO inclusion in aquaculture feeds, oils with a high content of LA should be avoided, or at least used sparingly. In addition, if higher levels of VO are used for most of the production cycle, an appropriate period of "wash-out" with a FO-containing finishing diet should be included prior to harvesting. Bell *et al.*, (2003a) found that returning salmon, previously fed 100 % LO or RO diets, to a marine FO diet for 20 weeks prior to harvest, in an attempt to "wash-out" the effects of the VO feeding, restored the flesh n-3 PUFA levels to 80 % of values in salmon fed 100 % FO although LA and ALA remained significantly higher. Finally, it is apparent that high level replacement of FO with RO and LO has a detrimental effect on the salmon immune system and may cause an increase in pathological change. However, replacement of FO with lower levels of VO may have less detrimental effects on immune function and cellular pathology and the use of other VO blends, which may be less challenging to salmon physiology, should be thoroughly investigated. Therefore, it is suggested that future studies of such dietary replacements, at any level, should incorporate examinations of additional physiological and immunological consequences.

The preliminary study presented in Chapter 3 provided some initial findings regarding the suitability of LO and RO as replacements for FO in the diets of Atlantic salmon. The present study however only focussed on the sea water phase of Atlantic salmon production

and we therefore need to determine the effects of these oils throughout the whole production cycle from first feeding to harvest which is the topic covered in Chapter 4.

Chapter 4 – Fish oil Substitution in Atlantic salmon (*Salmo salar*) FOSIS: LINK Aquaculture – Immunological Studies

## 4.1 Introduction

From the results gained in Chapter 3 a second trial was undertaken examining the effects of replacing the FO currently used in Atlantic salmon feeds with RO and LO. The overall objective of the present study was to test the hypothesis that salmon could be grown on diets containing VO without deleterious effects on fish growth, health and development. In addition, the nutritional quality and value as an important food for consumers was taken into consideration so to establish the suitability of substituting marine FO with appropriate VO through the whole salmon production cycle. To achieve the overall objective, Atlantic salmon were grown from first feeding through to harvest on diets in which the marine FO component was replaced with a blend of LO and RO. Each oil or oil blend was fed to groups of salmon parr at first feeding through to smoltification, and each oil was fed at either low or high lipid levels (12 and 25 %). After seawater transfer the fish were fed the same oils, but with higher levels of lipid (16 and 34 %) respectively, and the fish which were fed a low lipid diet in freshwater were fed both a low and high lipid diet in sea water and vice versa as shown in Figure 4.1. In the seawater phase subgroups of fish fed the VO diets were switched to diets containing marine FO for 6 months prior to harvest in order to "wash-out" or dilute the VO fatty acids, returning the salmon flesh to a more "marine-type" fatty acid composition. This study was a collaboration between fish feed manufacturers, EWOS Innovation, Biomar and Skretting, and Marine Harvest, Uniq Prepared Foods (Annan) and the Institute of Aquaculture at the University of Stirling.

In fish, as well as other animals, alterations in dietary fatty acid composition can affect immune function. For this reason, the trial outlined above was undertaken to

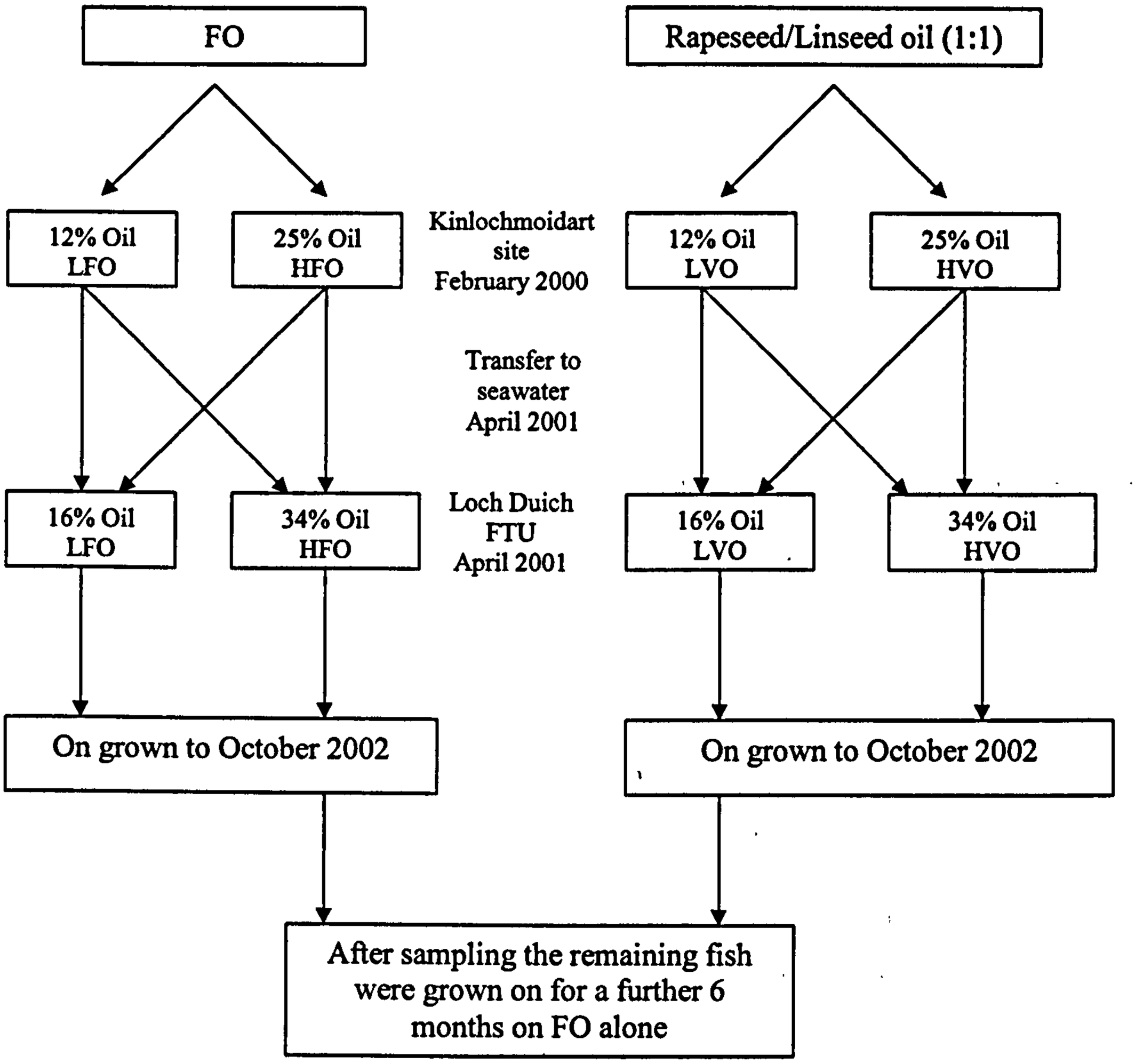



Figure 4.1 Dietary trial layout for Atlantic salmon fed either a low (12 %, 16 %) or high (25 %, 34 %) lipid level in both freshwater and seawater stages of production (February 2000 – March 2002).

FO = fish oil, LFO = low fish oil, HFO = high fish oil, LVO = low vegetable oil, HVO = high vegetable oil.

assess the effect of change in dietary fatty acids on a number of different immune functions in the experimental Atlantic salmon. The study was designed to examine the effect, if there was any, of the differing dietary ratios of n-3 and n-6 PUFA's resulting from FO and the RO/LO mix at low and high lipid levels on gut, heart muscle and liver pathology and on non-specific immune activity, such as head kidney macrophage function.

## 4.2 Materials and Methods

### *4.2.1 Dietary groups*

Atlantic salmon were hatched at Kinlochmoidart, weaned and on-grown to smolts. Salmon of approximately 30 g were housed in 8 x 5 $m^2$ tanks in fresh water at ambient temperature at Marine Harvest Ltd., Kinlochmoidart, Scotland. The tanks were assigned to 4 groups of 2 replicates and, after acclimation, fish were fed with one of the trial diets. The four diets were (1) low FO (12 %, 16 %), LFO; (2) high FO (25 %, 34 %), HFO; (3) low VO (12 %, 16 %), LVO; (4) high VO (25 %, 34 %), HVO. The four diets were formulated and prepared by EWOS Ltd. in December 1999 and delivered to the hatchery at Kinlochmoidart in February 2000. The fatty acid composition of the four diets is shown in Table 4.1. The VO was an equal blend of LO and RO by weight. These diets were fed to salmon fry in late March following yolk-sac absorption according to the plan shown in Figure 4.1 and were fed to duplicate groups of salmon parr up to smoltification. At this stage of the trial, 600 smolts were then transferred to 4 x 5 $m^2$ net pens at the Marine Harvest Ltd., Loch Duich Fish Trial Unit (FTU), Lochalsh, Scotland on 6$^{th}$ April 2001 and subjected to natural photoperiod. The distribution of the smolts into different dietary

Table 4.1 Fatty acid composition of experimental diets – sizes 1.5 and 2 mm pellets fed up to smoltification. (Values are weight % of total fatty acids).

| Fatty acid/diet | LFO | HFO | LVO | HVO |
|---|---|---|---|---|
| 14:0 | 5.1 ± 0.1 | 5.6 ± 0.1 | 3.0 ± 0.2 | 1.5 ± 0.0 |
| 16:0 | 14.4 ± 0.1 | 14.7 ± 0.1 | 11.0 ± 0.3 | 8.0 ± 0.1 |
| 18:0 | 2.0 ± 0.1 | 2.3 ± 0.0 | 2.2 ± 0.0 | 2.4 ± 0.1 |
| Total saturates[1] | 22.7 ± 0.1 | 23.6 ± 0.2 | 17.0 ± 0.7 | 12.7 ± 0.2 |
| | | | | |
| 16:1n-7 | 4.2 ± 0.2 | 4.7 ± 0.1 | 2.1 ± 0.1 | 1.2 ± 0.1 |
| 18:1n-9 | 15.2 ± 0.2 | 14.0 ± 0.2 | 24.2 ± 0.2 | 30.5 ± 1.4 |
| 18.1 n-7 | 2.5 ± 0.1 | 2.6 ± 0.1 | 3.0 ± 0.4 | 2.8 ± 0.1 |
| 20:1n-9 | 8.6 ± 0.3 | 9.5 ± 0.1 | 5.3 ± 0.1 | 3.1 ± 0.1 |
| 22:1n-11 | 11.7 ± 0.3 | 13.2 ± 0.6 | 7.3 ± 0.1 | 3.7 ± 0.1 |
| 24:1 | 1.2 ± 0.2 | 0.9 ± 0.1 | 0.8 ± 0.3 | 0.4 ± 0.1 |
| Total monounsaturates[2] | 44.7 ± 0.2 | 45.6 ± 0.5 | 42.8 ± 0.5 | 40.8 ± 0.3 |
| | | | | |
| 18:2n-6 | 5.4 ± 0.2 | 3.2 ± 0.0 | 11.3 ± 0.3 | 13.5 ± 0.0 |
| 20:4n-6 | 0.4 ± 0.0 | 0.5 ± 0.0 | 0.1 ± 0.1 | 0.1 ± 0.1 |
| Total n-6[3] | 7.0 ± 0.3 | 5.0 ± 0.2 | 12.0 ± 0.4 | 14.0 ± 0.3 |
| | | | | |
| 18:3n-3 | 2.6 ± 0.1 | 1.4 ± 0.0 | 16.7 ± 0.5 | 26.3 ± 0.2 |
| 18:4n-3 | 2.7 ± 0.0 | 3.2 ± 0.1 | 1.3 ± 0.0 | 0.7 ± 0.1 |
| 20:4n-3 | 0.8 ± 0.0 | 0.9 ± 0.6 | 0.3 ± 0.0 | 0.1 ± 0.1 |
| 20:5n-3 | 6.2 ± 0.3 | 6.7 ± 0.1 | 3.0 ± 0.1 | 1.6 ± 0.1 |
| 22:5n-3 | 1.0 ± 0.0 | 0.9 ± 0.6 | 0.3 ± 0.0 | 0.2 ± 0.0 |
| 22:6n-3 | 11.3 ± 0.2 | 11.3 ± 0.3 | 6.0 ± 0.2 | 3.3 ± 0.2 |
| Total n-3 | 24.6 ± 0.6 | 24.5 ± 0.4 | 27.6 ± 0.7 | 32.2 ± 0.2 |
| | | | | |
| n-3/n-6 | 3.5 ± 0.2 | 4.9 ± 0.2 | 2.3 ± 0.1 | 2.3 ± 0.0 |

[1]Includes 15:0 17:0, 20:0 and 22:0; [2]Includes 16:1n-9, 20:1n-7, 22:1n-9, [3]Includes 18:3(n-6), 20:2(n-6), 20:3(n-6) and 22:5(n-6). VO = RO/LO (1:1). FO = fish oil, LFO = low fish oil, HFO = high fish oil, LVO = low vegetable oil, HVO = high vegetable oil. Values are means ± SD, n = 2.

groups is shown in Figure 4.1. Fish previously fed the HFO and HVO diets were marked by adipose fin clipping, so their previous dietary history could be traced through the seawater phase before being placed in the allotted cage. Four marine on-growing diets (3 mm) were prepared by BioMar Ltd. and delivered to Marine Harvest Ltd., Loch Duich FTU in April 2001. Fish were transferred on to 6 mm pellets of the same diet, in September 2001. The fatty acid compositions of these four experimental diets (3 mm and 6 mm) are shown in Tables 4.2 and 4.3.

The diets for the final phase of the trial, including the wash out phase, (9 mm) were provided by Skretting Nutreco ARC. These diets were fed when the salmon attained an average weight of 1-1.3 kg. The principle fatty acid composition of the experimental diets used in the marine phase is shown in Table 4.4. The final wash-out FO diet was the same diet as the 9 mm HFO diet.

### *4.2.2 Pre-vaccination sampling in freshwater phase*

#### 4.2.2.1 Collection of samples

An initial sampling was performed at Kinlochmoidart (pre-vaccination sampling) in which various immune parameters were measured including; total white and red blood cell counts, haematocrit, serum lysozyme activity and head kidney macrophage activity. Tissue samples were also collected to identify any effects of oil type on the histology of the heart, liver or intestine.

Table 4.2 Fatty acid composition of experimental diets – (3 mm pellets) fed during the seawater phase (Values are weight % of total fatty acids).

| Fatty acid/diet | LFO | HFO | LVO | HVO |
|---|---|---|---|---|
| 14:0 | 5.81 | 6.49 | 2.00 | 1.02 |
| 16:0 | 16.13 | 15.98 | 9.21 | 7.23 |
| 18:0 | 2.41 | 2.52 | 2.87 | 2.78 |
| Total saturates[1] | 25.40 | 26.03 | 15.02 | 11.90 |
| 16:1n-7 | 5.61 | 6.85 | 1.78 | 0.98 |
| 18:1n-9 | 11.79 | 9.56 | 30.83 | 35.47 |
| 18.1 n-7 | 2.42 | 2.55 | 2.39 | 2.38 |
| 20:1n-9 | 8.21 | 8.80 | 2.40 | 1.78 |
| 22:1n-11 | 11.26 | 10.4 | 2.27 | 1.35 |
| 24:1 | 0.84 | 0.77 | 0.37 | 0.23 |
| Total monounsaturates[2] | 41.49 | 40.50 | 40.44 | 42.41 |
| 18:2n-6 | 3.08 | 2.13 | 13.44 | 15.37 |
| 20:4n-6 | 0.55 | 0.67 | 0.25 | 0.06 |
| Total n-6[3] | 4.32 | 3.41 | 13.92 | 15.57 |
| 18:3n-3 | 1.13 | 0.93 | 20.58 | 25.40 |
| 18:4n-3 | 3.07 | 3.24 | 0.79 | 0.41 |
| 20:4n-3 | 0.61 | 0.64 | 0.19 | 0.10 |
| 20:5n-3 | 9.15 | 10.89 | 3.07 | 1.49 |
| 22:5n-3 | 1.02 | 1.13 | 0.41 | 0.18 |
| 22:6n-3 | 11.75 | 10.88 | 4.81 | 2.19 |
| Total n-3 | 26.84 | 27.82 | 29.86 | 29.77 |
| n-3/n-6 | 6.21 | 8.16 | 2.15 | 1.91 |

[1]Includes 15:0 17:0, 20:0 and 22:0; [2]Includes 16:1(n-9), 20:1(n-7), 22:1(n-9), [3]Includes 18:3(n-6), 20:2(n-6), 20:3(n-6) and 22:5(n-6). VO = RO/LO (1:1). VO = RO/LO (1:1). FO = fish oil, LFO = low fish oil, HFO = high fish oil, LVO = low vegetable oil, HVO = high vegetable oil. Values are means ± SD, n = 2.

Table 4.3 Fatty acid composition of experimental diets – (6 mm pellets) fed during the seawater phase (Values are weight % of total fatty acids).

| Fatty acid/diet | LFO | HFO | LVO | HVO |
|---|---|---|---|---|
| 14:0 | 5.67 | 6.25 | 1.36 | 0.67 |
| 16:0 | 12.22 | 11.90 | 8.14 | 6.62 |
| 18:0 | 1.25 | 1.10 | 2.54 | 2.99 |
| Total saturates[1] | 20.07 | 20.19 | 13.14 | 11.02 |
| 16:1n-7 | 7.44 | 8.29 | 1.72 | 0.82 |
| 18:1n-9 | 11.21 | 11.07 | 30.37 | 33.56 |
| 18.1 n-7 | 2.95 | 3.16 | 2.17 | 1.90 |
| 20:1n-9 | 16.11 | 17.94 | 3.78 | 1.93 |
| 22:1n-11 | 12.81 | 13.86 | 3.20 | 1.37 |
| 24:1 | 0.87 | 0.77 | 0.42 | 0.31 |
| Total monounsaturates[2] | 53.79 | 57.71 | 42.35 | 40.32 |
| 18:2n-6 | 6.18 | 3.06 | 17.80 | 16.96 |
| 20:4n-6 | 0.29 | 0.25 | 0.15 | 0.09 |
| Total n-6[3] | 7.15 | 4.00 | 18.09 | 17.14 |
| 18:3n-3 | 1.00 | 0.75 | 18.23 | 26.93 |
| 18:4n-3 | 2.84 | 2.99 | 0.51 | 0.37 |
| 20:4n-3 | 0.39 | 0.40 | 0.14 | 0.07 |
| 20:5n-3 | 6.66 | 6.49 | 2.98 | 1.57 |
| 22:5n-3 | 0.49 | 0.46 | 0.23 | 0.13 |
| 22:6n-3 | 6.04 | 5.38 | 3.66 | 2.12 |
| Total n-3 | 17.50 | 16.55 | 25.76 | 31.19 |
| n-3/n-6 | 2.45 | 4.14 | 1.42 | 1.82 |

[1]Includes 15:0 17:0, 20:0 and 22:0; [2]Includes 16:1(n-9), 20:1(n-7), 22:1(n-9), [3]Includes 18:3(n-6), 20:2(n-6), 20:3(n-6) and 22:5(n-6). VO = RO/LO (1:1). VO = RO/LO (1:1). FO = fish oil, LFO = low fish oil, HFO = high fish oil, LVO = low vegetable oil, HVO = high vegetable oil. Values are means ± SD, n = 2.

Table 4.4 Principal fatty acid composition (% of total fatty acids) of experimental diets (9 mm) used in the final seawater phase.

| Fatty acid / diet | LFO | HFO | LVO | HVO |
|---|---|---|---|---|
| 18:1(n-9) | 11.2 | 11.1 | 30.4 | 33.6 |
| 18:2(n-6) | 6.2 | 3.1 | 17.8 | 17.0 |
| 18:3(n-3) | 1.0 | 0.8 | 18.2 | 26.9 |
| 20:5(n-3) | 6.7 | 6.5 | 3.0 | 1.6 |
| 22:6(n-3) | 6.0 | 5.4 | 3.7 | 2.1 |
| n-3 / n-6 | 2.5 | 4.1 | 1.4 | 1.8 |

LFO = low fish oil, HFO = high fish oil, LVO = low vegetable oil, HVO = high vegetable oil, n = 2.

### 4.2.2.2 Haematology and Macrophage function

Blood and head kidney were sampled from 4 fish tank$^{-1}$ giving a total of 8 fish dietary treatment$^{-1}$ following the methods outlined in Sections 2.3.3 and 2.3.4. The total number of circulating leukocytes and erythrocyte counts along with haematocrit were determined from the heparinised blood samples according to Sections 2.3.2.1, 2.3.2.2, and 2.3.2.3, respectively. Macrophages were isolated from head kidney samples from the same fish according to Sections 2.3.4 and 2.3.7.2 and respiratory burst activity was determined according to Section 2.4.1. Head kidney macrophages were used to measure the activity of phagocytosis as described in Section 2.4.2.

### 4.2.2.3 Serum Lysozyme activity

A second aliquot of blood (8 fish treatment$^{-1}$) was allowed to clot overnight and serum collected as described in Section 2.3.3. Following this, serum lysozyme activity was measured using a turbidimetric microtitre plate technique modified by Ellis (1990) from the method described in Section 2.4.4.

### 4.2.2.4 Histopathology

Samples of proximal, mid and distal intestine together with heart and liver were collected from 8 fish from each dietary group (4 fish tank$^{-1}$) for histopathological examination.

Sections were processed and examined according to Sections 2.3.5, 2.7.1, 2.7.2 and 2.7.3.

### *4.2.3 Post-vaccination sampling and challenged experimental fish with Aeromonas salmonicida*

A sub group of the fish (n = 290) from the trial conducted at Kinlochmoidart were moved to Marine Harvest's Lochailort challenge facility unit, Scotland (Figure 4.2), for further immunological analysis and experimental bacterial challenge with *A. salmonicida*. Fish were stocked in 1 m tanks supplied with disinfected freshwater. The fish were allowed to acclimate for 2 weeks before vaccinating them with an Alphaject 1200 vaccine which is a monovalent furunculosis vaccine and given 6 weeks immune induction.

Vaccinated fish were pan jetted with alcian blue (1 % w/v) on their belly centre to distinguish them from unvaccinated fish. Unvaccinated control fish were injected with PBS placebo and were pan jet marked near the anal region. During the challenge period the fish were fed the trial diets at 1.5 % bw $day^{-1}$. Flow rates were set at 15 l $min^{-1}$ and day length set at 10 h. No mortalities were recorded post-vaccination. Eighty fish (40 vaccinated and 40 un-vaccinated) were assigned to three of the treatments in duplicate apart from the HVO diet where a total of 25 vaccinated and 25 un-vaccinated fish were grouped together. This was due to an otter attack on this dietary group at the Kinlochmoidart site. As a result 30 fish from the HVO treatment had to be returned to Kinlochmoidart to compensate for this and for continuance onto the seawater phase of the overall LINK project. A post-vaccination sample was taken mid - March 2001 to examine the immunological parameters previously mentioned in Section 4.2.2, prior to challenging the fish with 15 % co-habitation (i.e. whatever the total number of cohorts to be challenged 15% of this number was added as 'injected' cohabitants. e.g. 80 fish in tank = 12

Figure 4.2 Marine Harvest, Lochailort, Scotland challenge tank facility.

challenged fish added) challenge with *A. salmonicida* (1 x $10^5$ cfu). Specific mortalities from the bacterial challenge were confirmed by streaking kidney swabs onto TSA plates. Positive cases were confirmed by the production of a diffusible brown pigment when grown in artificial culture. An overview of the dietary trial and bacterial challenge set up is shown in Figure 4.3.

### *4.2.4 Seawater sample*

The original stock of trial fish from the Kinlochmoidart site were transferred to seawater (Marine Harvest, Loch Duich, FTU, Lochalsh) in April 2001 and they were switched to diets containing either 16 % (low) or 34 % (high) oil, with fish initially fed the low oil diet switched to the higher oil level and vice versa. Fish were on-grown in the net pens until early March 2002 when they were again sampled, measuring the various immunological parameters previously mentioned in Section 4.2.2. In addition, the circulating levels of plasma $PGE_2$ from 8 fish dietary treatment$^{-1}$ were determined as described in Section 2.6. Samples of PBL from 4 fish dietary treatment$^{-1}$ were isolated from whole blood by density gradient centrifugation according to Section 2.8.1. Total lipid from the isolated PBL was extracted with chloroform:methanol (2:1 v/v) as described in Section 2.8.2 and by Folch *et al.*, (1957). Gas chromatography was used to identify the fatty acid methyl esters following the method outlined in Section 2.8.2.

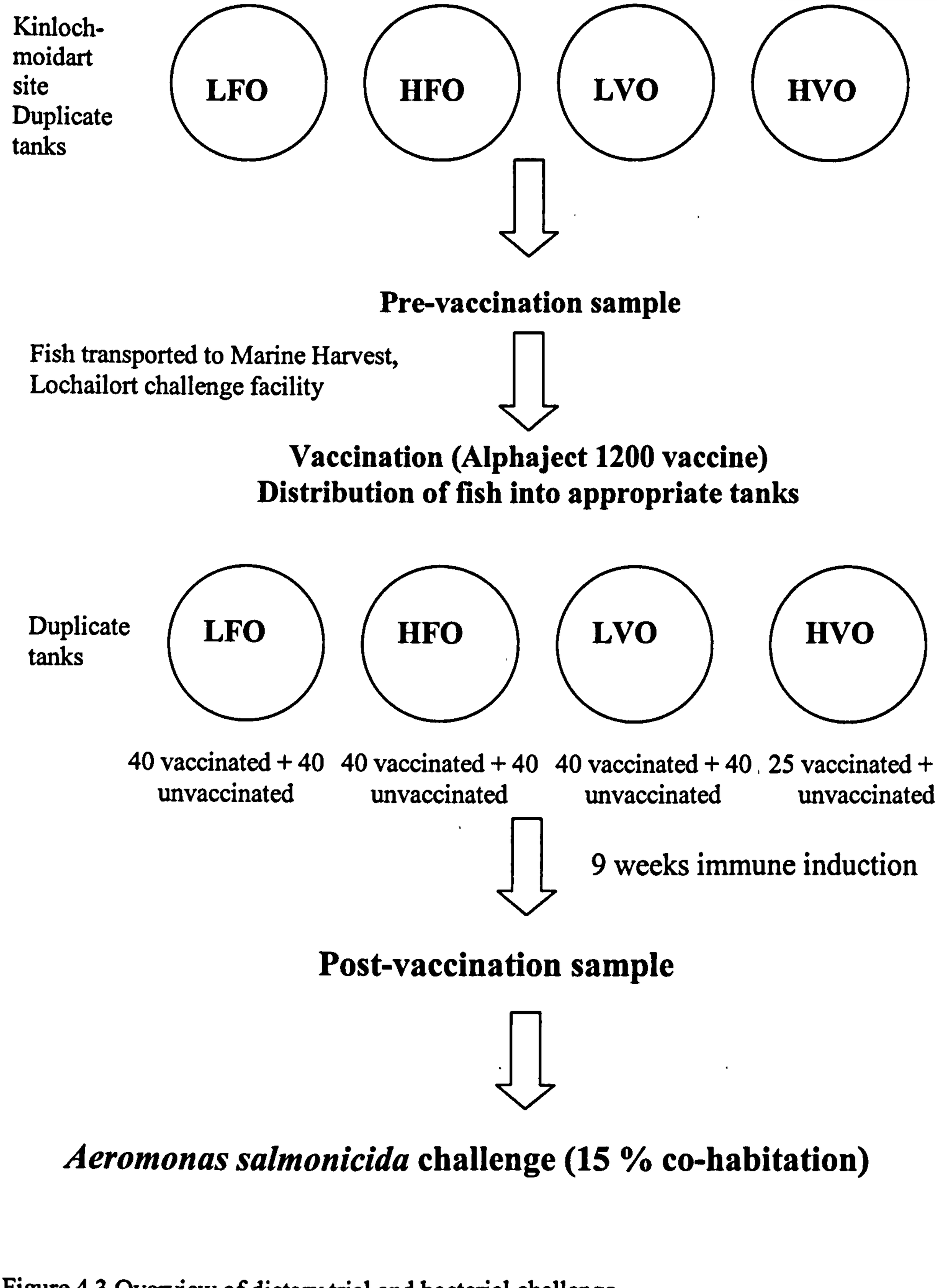



Figure 4.3 Overview of dietary trial and bacterial challenge

### *4.2.5 Statistical Analysis*

Significance of difference ($p<0.05$) between dietary treatments was determined by one-way analysis of variance (ANOVA). Differences between means were determined by Newman Keuls test. ANOVA was performed using a GraphPad Prism™ (version 3.0) statistical package. Kaplan Meir Survival Analysis was the statistical method used to analyse the data from the challenge experiments.

## 4.3 Results

### *4.3.1 Growth*

After feeding the experimental diets for 47 weeks there was no difference in growth between the fish fed the HFO and HVO diets (37 g and 37 g, respectively) but these fish were significantly larger than those fed LVO (31 g) with the smallest fish being those fed the LFO diet (29 g) (Bell *et al.*, personal communication). In February 2001 (47 weeks from on-set of feeding) 25 fish treatment$^{-1}$ were weighed individually. The low oil diets showed the lowest weights (LFO, 33.2 g; LVO, 37.8 g) compared to those fish fed with either the HFO (43.6 g) or HVO (45.8 g) diets. Significant differences were found between fish fed the high and low oil levels but no differences were found between fish fed FO or VO at the high oil inclusion level. Fish fed the LFO diet were significantly smaller than fish fed the LVO diet. During the seawater phase there were no significant differences in growth rate between the dietary treatments (LFO, 1052 g; HFO, 1294 g; LVO, 1148 g; HVO 1146g) after 33 weeks post-seawater transfer. The final weights of fish sampled at harvest (96 weeks) were significantly higher in fish fed the HVO diet (2002 g) compared to

those fed the LVO (1710 g) and LFO diet (1440 g) while fish fed the HFO diet (1880 g) were not significantly different from either the HVO or LVO fish after 96 weeks feeding.

### *4.3.2 Pre-vaccination sampling*

#### 4.3.2.1 Measurement of immune parameters

After feeding fish with the LVO diet, significantly lower haematocrit percentages were observed compared to fish given the LFO diet ($p=0.04$) (Table 4.5). Blood cell counts for each group of fish from the dietary trials are presented in Table 4.5. Fish fed both LVO and HVO diets had significantly lower leukocyte counts ($1.6 \times 10^7$ ml$^{-1}$, $1.9 \times 10^7$ ml$^{-1}$ respectively) than the LFO fed fish ($4.4 \times 10^7$ ml$^{-1}$) ($p=0.01$). Erythrocyte counts from fish fed the VO diets also showed a reduction in number when compared to the HFO fed fish. There was a statistical difference ($p=0.03$) between fish fed the HFO diet ($2.8 \times 10^9$ ml$^{-1}$) and LVO diet ($1.2 \times 10^9$ ml$^{-1}$), and fish fed the HFO and the HVO diets ($1.2 \times 10^9$ ml$^{-1}$). Feeding fish either the HVO or LVO diets, significantly depressed the activity of head kidney macrophages (17.0 and 30.9 Absorbance per $10^5$ cells x 100, respectively) compared with fish fed the HFO diet (74.9 Absorbance per $10^5$ cells x 100) (Table 4.5). In fish fed the LFO diet a significantly lower macrophage activity was found compared to fish fed the HFO ($p=0.003$). When comparing the degree of serum lysozyme it was found that there was no statistical difference between the dietary groups (Table 4.5).

Table 4.5 Haematocrit, Haematology, NBT activity and serum lysozyme activity values of fish fed the experimental diets sampled pre-vaccination.

| | LFO | HFO | LVO | HVO |
|---|---|---|---|---|
| **Haematocrit (%)** | $41.0 \pm 1.7^{a}$ | $38.4 \pm 2.0^{ab}$ | $33.2 \pm 2.2^{b}$ | $35.4 \pm 1.7^{ab}$ |
| **Total number of leukocytes ($ml^{-1}$)** | $4.4 \times 10^{7} \pm 9.5 \times 10^{6\,b}$ | $3.1 \times 10^{7} \pm 4.4 \times 10^{6\,ab}$ | $1.6 \times 10^{7} \pm 3.8 \times 10^{6\,a}$ | $1.9 \times 10^{7} \pm 5.4 \times 10^{6\,a}$ |
| **Total number of erythrocytes ($ml^{-1}$)** | $1.9 \times 10^{9} \pm 2.3 \times 10^{8\,ab}$ | $2.8 \times 10^{9} \pm 7.2 \times 10^{8\,b}$ | $1.2 \times 10^{9} \pm 2.3 \times 10^{8\,a}$ | $1.2 \times 10^{9} \pm 1.6 \times 10^{8\,a}$ |
| **Macrophage respiratory burst (Absorbance per $10^5$ cells x 100)** | $41.8 \pm 7.4^{a}$ | $74.9 \pm 17.6^{b}$ | $30.9 \pm 7.3^{a}$ | $17.0 \pm 3.1^{a}$ |
| **Serum lysozyme activity ($U\ min^{-1}\ ml^{-1}$)** | $228.1 \pm 51.4$ | $220.8 \pm 56.1$ | $321.4 \pm 88.2$ | $293.8 \pm 43.5$ |

Values are means ± SEM, n =8. Values within a row having different superscript letters are significantly different, ($p<0.05$). LFO = low fish oil, HFO = high fish oil, LVO = low vegetable oil, HVO = high vegetable oil.

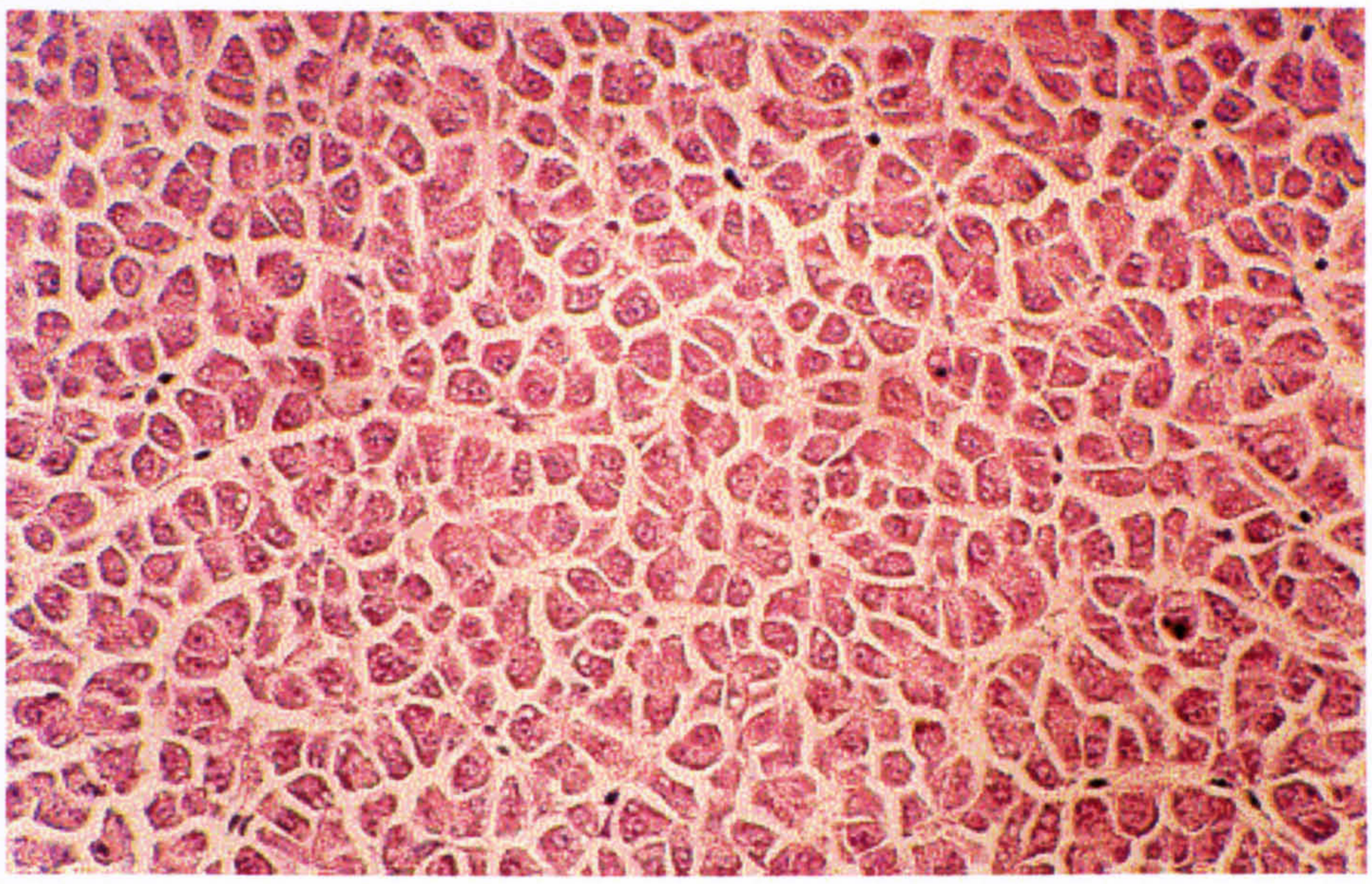
### 4.3.2.2 Pre-vaccination histopathology

All of the tissues examined from fish fed the LFO diet appeared "normal" histologically. Most heart sections had no apparent histological abnormalities, however a couple showed small areas of inflammation. The majority of livers showed a moderate to high fat content and had reasonably regular single fat vacuoles with no associated pathology (Figure 4.4a). The gut sections appeared normal with high mucus activity in the proximal and mid sections correlating with low mucus activity in the distal sections.

Hearts, livers and intestinal sections from fish maintained on the HFO diet appeared histologically normal. In the liver high fat levels were seen with evenly distributed, single, large vacuoles. A higher level of mucus activity was seen in the proximal and mid intestinal sections compared with a low level in the distal sections. Whereas the distal sections showed more vacuolation than the proximal and mid gut. A slight degree of sloughing was also observed in the mid gut.

All heart sections from fish fed the LVO diet appeared normal. The livers had a variable appearance, with moderate to high fat deposition. The fat vacuoles ranged from having a regular even distribution to having an uneven distribution with variable sized vacuoles and "foamy" hepatocytes (small, multiple vacuoles) (Figure 4.4b). One section also had the occasional inflammatory cell. The gut sections appeared normal with high mucus activity in the proximal and mid sections correlating with low mucus activity in the distal sections.

**(a)**



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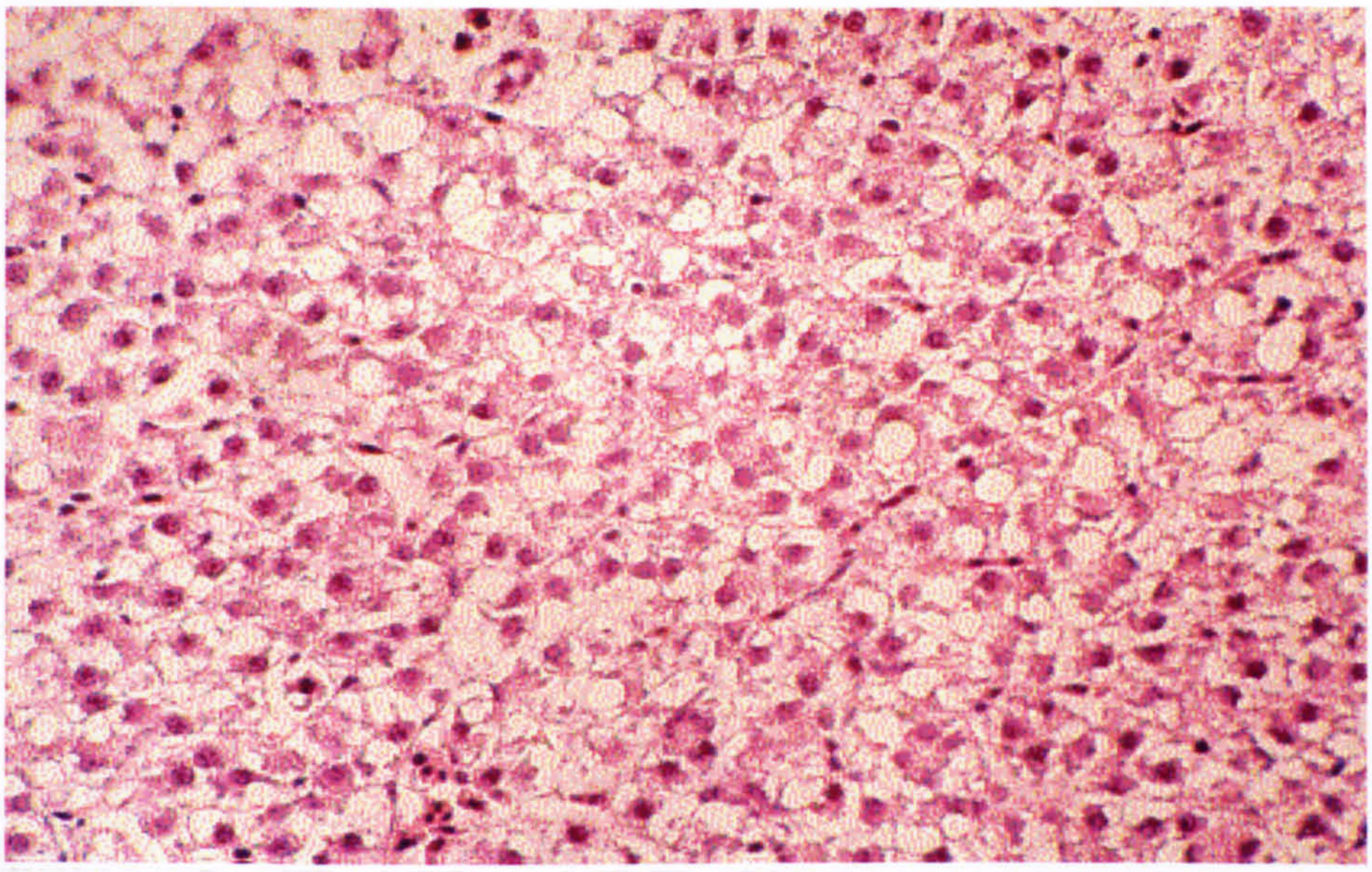


**Figure 4.4 Histopathology of Atlantic salmon fed a (a) LFO diet prior to vaccination (mag. x 175). Liver showing a normal histological appearance with regular single fat vacuoles (b) LVO diet prior to vaccination (mag. x 175). Liver showing a variable appearance, with moderate to high fat deposition and irregular shaped "foamy" hepatocytes.**

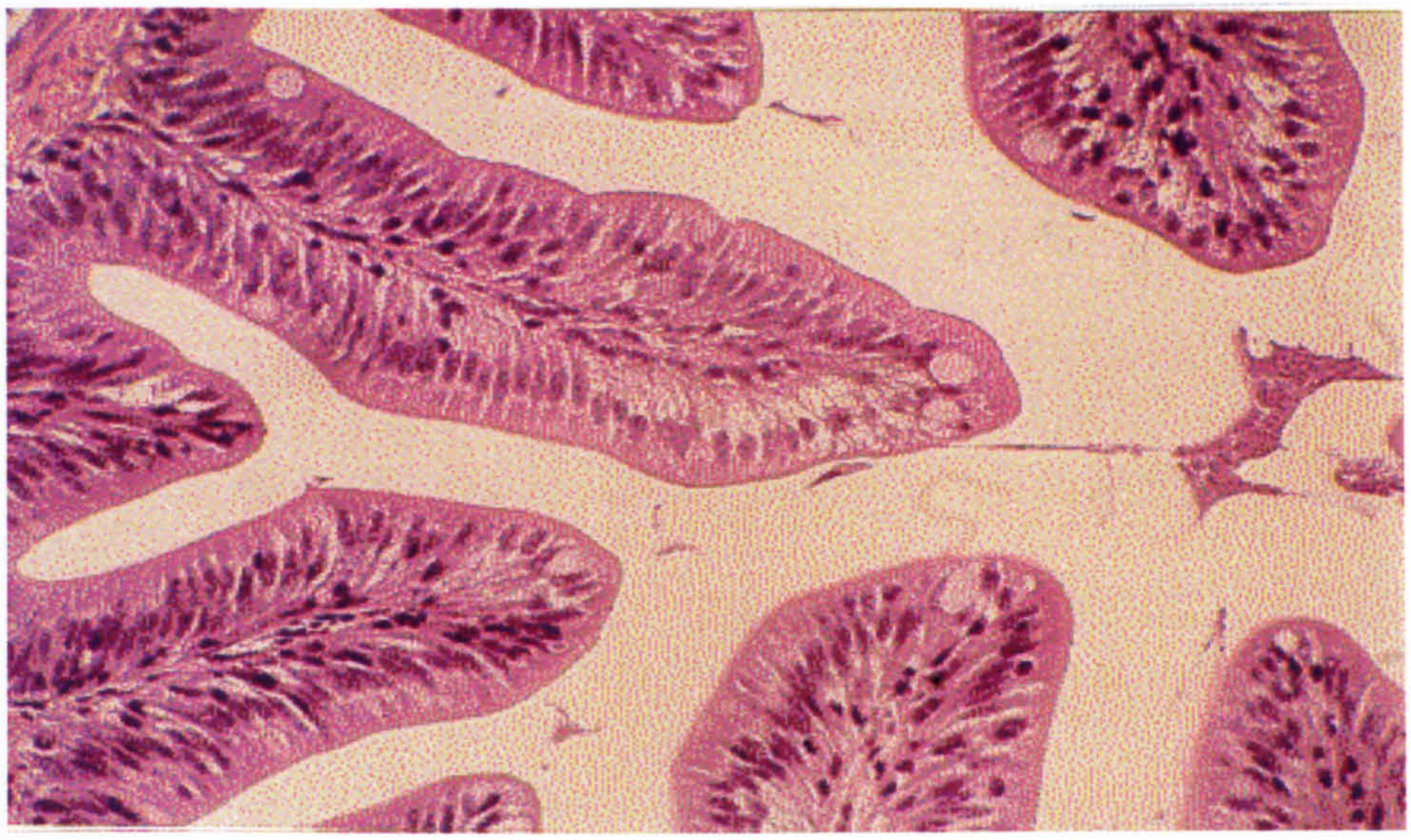
In fish fed the HVO diet all heart sections appeared normal. In general, the livers had a moderate to high fat content with evenly distributed, single vacuoles with no pathology. The gut showed low vacuolation in the proximal (Figure 4.5a) and mid regions with high vacuolation in the proximal section, but no pathology. The distal intestine showed a high degree of vacuolation and sloughing (Figure 4.5b).

### *4.3.3 Post-vaccination sampling*

#### 4.3.3.1 Measurement of immune parameters

Significant differences in fish were only found between dietary groups in their head kidney macrophage activity. No significant differences were found between groups in their haematocrit, the total number of circulating leukocyte and erythrocyte counts, and serum lysozyme activity (Table 4.6). Vaccinated fish fed the LFO diet had significantly lower macrophage activities (43.0 Absorbance per $10^5$ cells x 100) than unvaccinated fish fed the HFO diet (132.2 Absorbance per $10^5$ cells x 100). The activity of macrophages from the head kidney also appeared to be significantly depressed by the HVO diet from unvaccinated fish (48.9 Absorbance per $10^5$ cells x 100) when compared to the HFO diet (Table 4.6).

**(a)**



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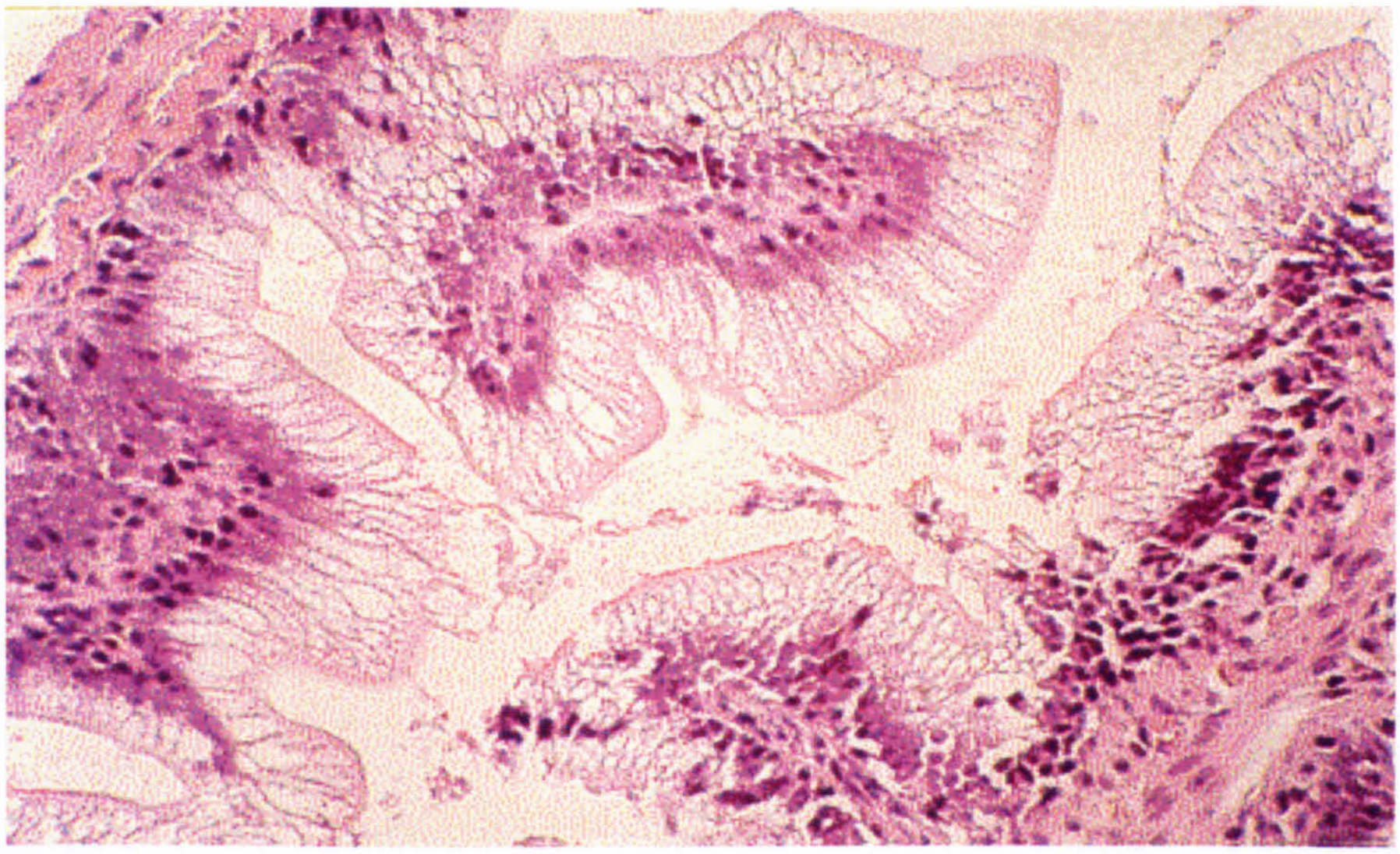

Figure 4.5 Histopathology of Atlantic salmon fed a (a) HVO diet prior to vaccination (mag. x 175). Proximal intestine showing a low degree of vacuolation but no pathology (b) HVO diet prior to vaccination (mag. x 175). Distal intestine showing a high degree of vacuolation and sloughing, but no pathological change.

Table 4.6 Haematocrit, Haematology, NBT activity and serum lysozyme activity values of fish fed the experimental diets sampled post-vaccination.

| | LFO (V) | LFO (UV) | HFO (V) | HFO (UV) | LVO (V) | LVO (UV) | HVO (V) | HVO (UV) |
|---|---|---|---|---|---|---|---|---|
| **Haematocrit (%)** | 27.3 ± 2.60 | 28.2 ± 1.49 | 32.4 ± 3.85 | 32.7 ± 2.44 | 34.2 ± 1.62 | 32.2 ± 1.14 | 34.3 ± 1.65 | 28.3 ± 2.58 |
| **Total number of leukocytes ($ml^{-1}$)** | $1.9 \times 10^7 \pm 1.5 \times 10^6$ | $1.7 \times 10^7 \pm 3.8 \times 10^6$ | $1.4 \times 10^7 \pm 3.2 \times 10^6$ | $1.5 \times 10^7 \pm 3.2 \times 10^6$ | $1.4 \times 10^7 \pm 3.0 \times 10^6$ | $1.3 \times 10^7 \pm 4.2 \times 10^6$ | $7.8 \times 10^6 \pm 6.5 \times 10^5$ | $9.0 \times 10^6 \pm 1.3 \times 10^6$ |
| **Total number of erythrocytes ($ml^{-1}$)** | $1.2 \times 10^9 \pm 2.3 \times 10^8$ | $9.7 \times 10^8 \pm 9.8 \times 10^7$ | $8.8 \times 10^8 \pm 1.5 \times 10^8$ | $1.2 \times 10^9 \pm 1.8 \times 10^8$ | $9.8 \times 10^8 \pm 1.7 \times 10^8$ | $1.1 \times 10^9 \pm 1.3 \times 10^8$ | $8.6 \times 10^8 \pm 9.9 \times 10^7$ | $8.5 \times 10^8 \pm 7.9 \times 10^7$ |
| **Macrophage respiratory burst (Absorbance per $10^5$ cells x 100)** | $43.0 \pm 8.5^{a}$ | $67.8 \pm 17.0^{ab}$ | $79.7 \pm 20.3^{ab}$ | $132.2 \pm 35.3^{b}$ | $104.3 \pm 9.7^{ab}$ | $66.6 \pm 16.4^{ab}$ | $61.1 \pm 10.6^{ab}$ | $48.9 \pm 12.1^{a}$ |
| **Serum lysozyme activity (U $min^{-1}$ $ml^{-1}$)** | 95.0 ± 55.0 | 93.0 ± 46.2 | 130.0 ± 47.6 | 358.8 ± 92.5 | 230.0 ±45.0 | 265.0 ± 72.0 | 51.7 ± 21.3 | 180.5 ± 50.5 |

Values are means ± SEM, n =8. Values within a row without a common superscript letter differ, ($p<0.05$). LFO = low fish oil, HFO = high fish oil, LVO = low vegetable oil, HVO = high vegetable oil. (V) = vaccinated, (UV) = unvaccinated.

### 4.3.3.2 Post-vaccination histopathology

None of the heart samples examined in fish fed the LFO diet showed histological abnormalities. The livers had a moderate fat content with some variability in vacuole size and distribution. Occasional single cell necrosis and accumulations of inflammatory cells were present in some of the tissues. The proximal and mid intestine had an active mucosa with high mucus activity and moderate vacuolation. The distal segment showed a high degree of vacuolation and a moderate amount of sloughing.

In general, most heart sections within the HFO dietary group gave a normal appearance with only an occasional one showing small patches of endocarditis. Many of the livers contained a low to moderate fat content that ranged from small regular vacuoles to some variable and multiple vacuoles. A few sections also appeared to have scattered single cell necrosis. The proximal and mid gut sections displayed a fair amount of sloughing and mucus activity, whereas the distal portion showed definite signs of mucosal pathology. Some distal sections were broken up perhaps as a result of the vaccination.

On the whole, there was no abnormality detected in most heart sections from fish fed the LVO feed, however a few had small areas of endocarditis (Figure 4.6a) or pericarditis. Overall the livers revealed variable fat sized vacuoles with a low fat content within the hepatocytes. Occasional single necrotic cells were evident (Figure 4.6b). The proximal and mid intestinal sections displayed high mucus activity and sloughing with a lower degree of cellular infiltration and vacuolation. In the distal segments there were visible

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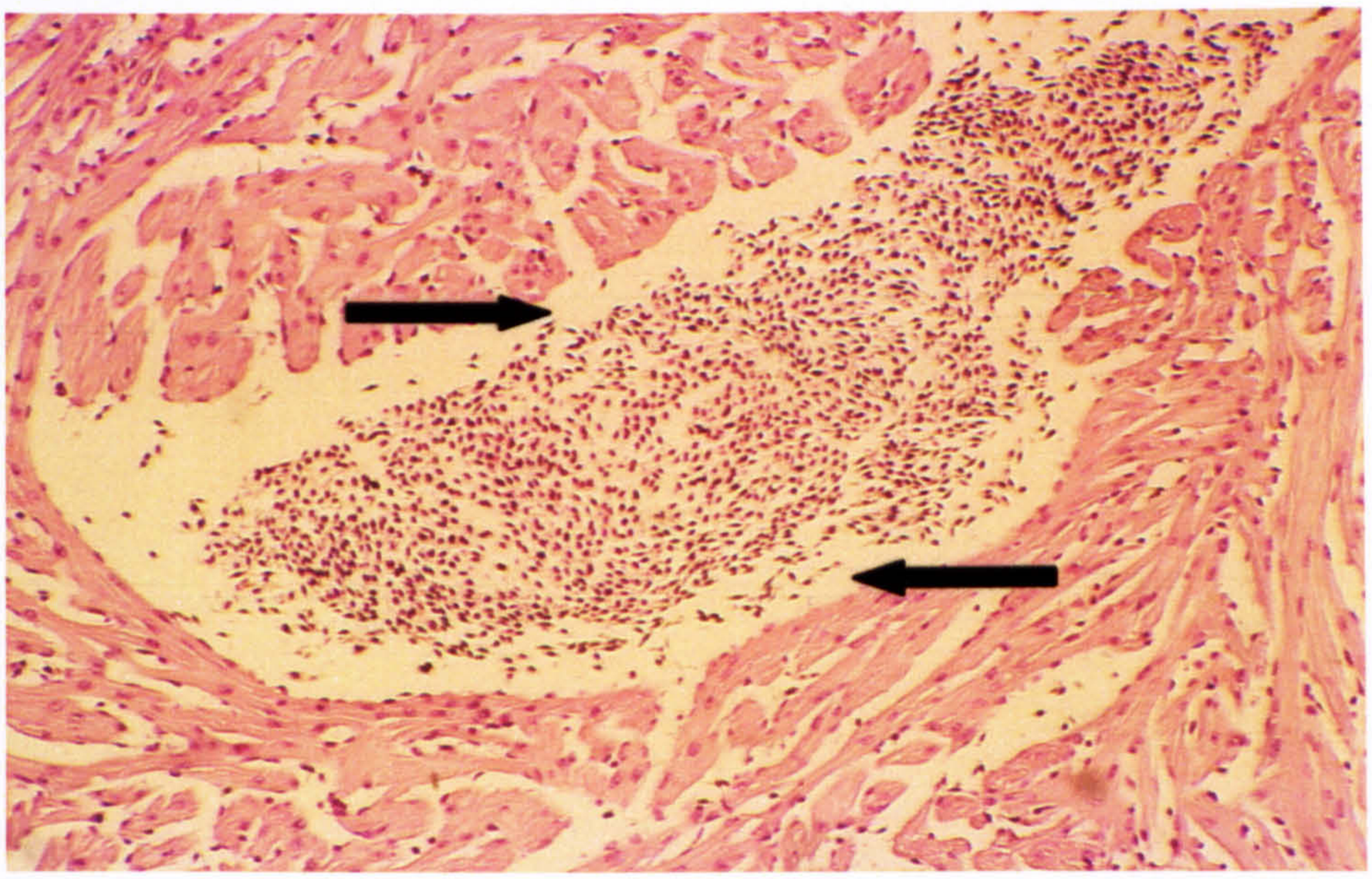

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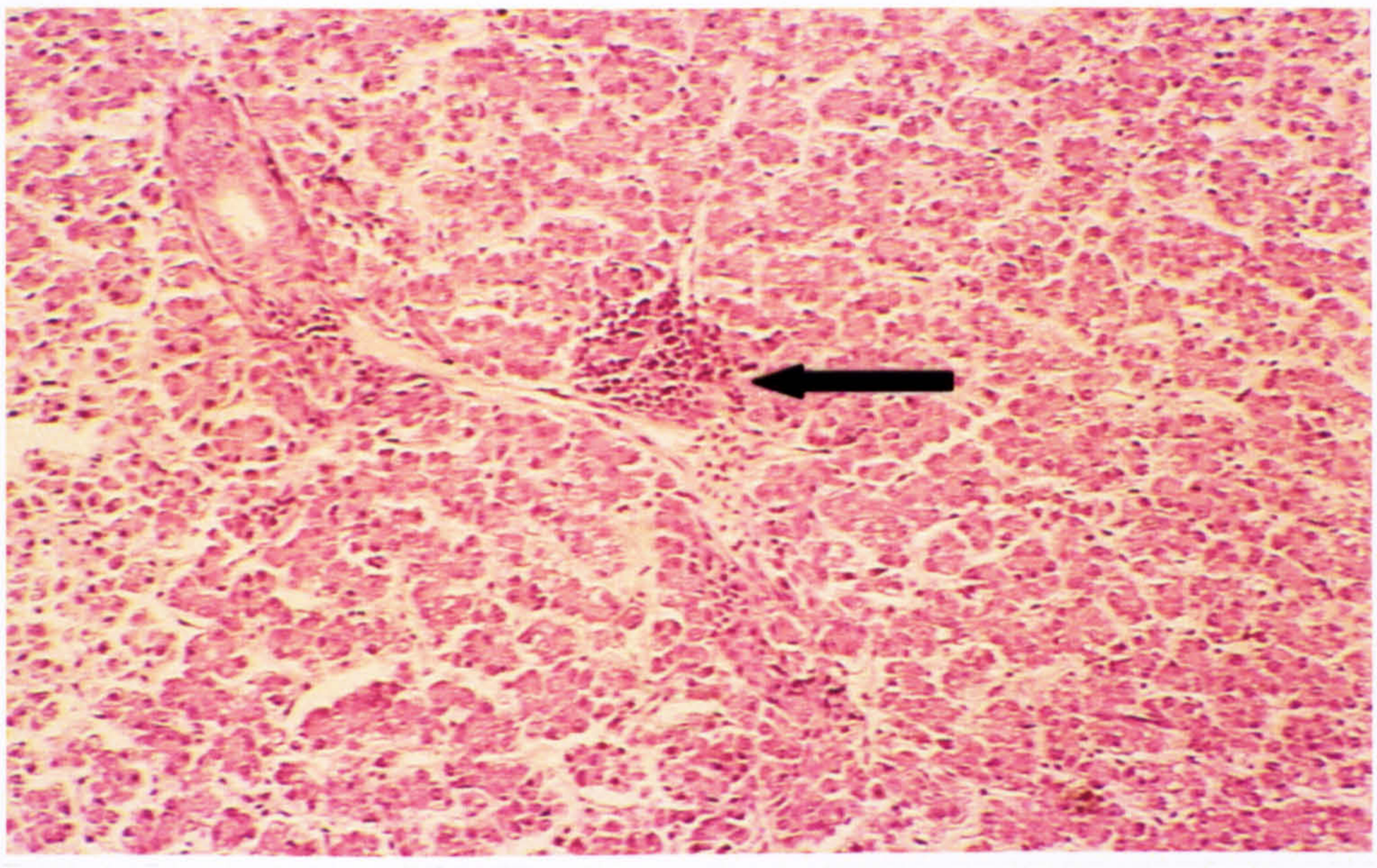

**Figure 4.6 Histopathology of Atlantic salmon fed a (a) LVO diet post-vaccination. Heart showing a small area of endocarditis (mag. x 175) (b) LVO diet post-vaccination. Liver showing the occasional single necrotic cell (mag. x 70).**

signs of pathology with a high incidence of cellular infiltration and breakdown of the mucosa (Figure 4.7a).

All the heart sections in the HVO group appeared normal, whereas the livers showed a higher level of hepatic pathology with scattered single cell necrosis and variable large vacuoles with a moderate fat content (Figure 4.7b). A few sections also displayed a loss of hepatic structure. The proximal intestine appeared to resemble an "inactive" gut with low mucus activity, vacuolation, cellular infiltration and sloughing. In contrast to this finding, the mid and distal sections showed a high degree of vacuolation and sloughing (Figure 4.8). Of the distal section examined, six out of the eight were very broken up and a detailed examination was impossible. This may have been due to a high degree of pathology or more likely in response to the vaccination.

### *4.3.4 Experimental challenge of fish with Aeromonas salmonicida*

No significant differences were found between groups of salmon fed the various experimental diets after bacterial challenge with *A. salmonicida*. The unvaccinated populations, as expected, had a high proportion of mortalities, which started to die eight days after commencing the challenge. The LFO group showed 50 % mortality at 24 days post-challenge, however, this group suffered a fungal infection during the challenge and were treated with Pyceze®. After treatment, no more deaths were recorded in this dietary group.

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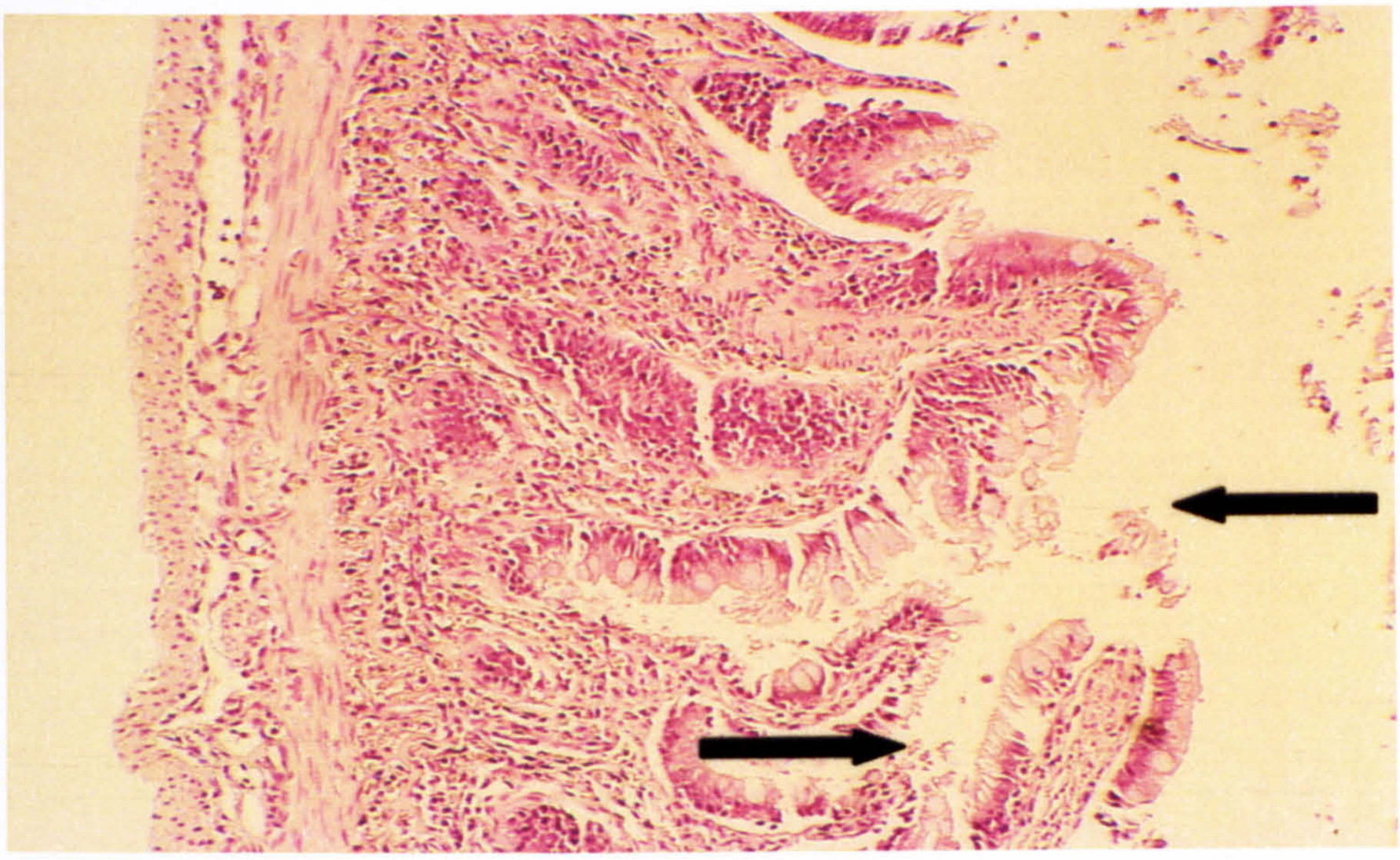


Figure 4.8 Histopathology photograph of Atlantic salmon maintained on HVO diet post-vaccination. Distal intestine showing a high degree of vacuolation and sloughing that may be in response to the vaccination (mag. x 70).

All of the other vaccinated dietary groups showed no more than a 12 % cumulative mortality up to 33 days post-challenge. The pathological symptoms observed in the fish challenged with *A. salmonicida* were minor. Slight haemorrhaging at the base of the fins, darkening of the skin and loss of appetite were observed, however, no sign of necrotic lesions was observed. The cumulative mortalities of fish challenged with *A. salmonicida* are shown in Figure 4.9.

### *4.3.5 Sampling post-seawater transfer*

#### 4.3.5.1 Measurement of immune parameters

Significant differences were found when looking at haematocrit between the different dietary treatments ($p<0.02$) (Table 4.7). The LVO group showed significantly reduced haematocrit (39.7 %) when compared to both the LFO (44.4 %) and HFO (46.4 %) dietary groups (Table 4.7). The HVO group (41.9 %) also showed significantly reduced haematocrit when compared to the HFO fed fish. No significant differences were found when comparing the total number of circulating leukocyte and erythrocyte counts between the dietary groups as shown in Table 4.7. Both the LVO and HVO fed fish showed significantly reduced macrophage activity (6.5 and 10.2 Absorbance per $10^5$ cells x 100, respectively) compared to both the LFO and the HFO groups (28.9 and 22.5 Absorbance per $10^5$ cells x 100, respectively) ($p< 0.0001$) (Table 4.7). Cellular immunity measured as the phagocytic activity of head kidney macrophages, was affected by feeding the mixed VO diets at both high and low lipid levels as shown in Table 4.7. The phagocytosis of yeast

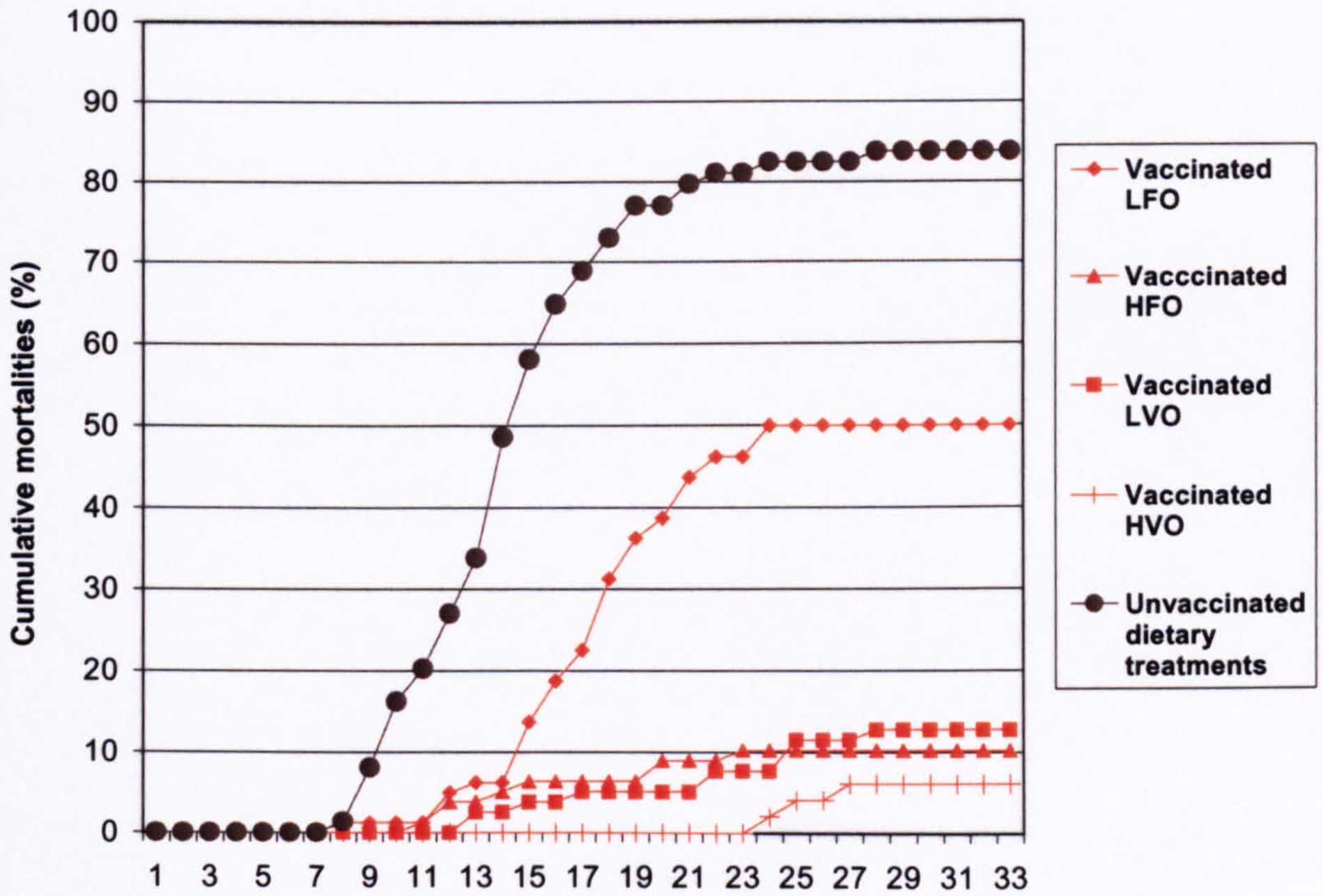



Figure 4.9 Cumulative fish mortalities against the number of days post-challenge with *Aeromonas salmonicida* (n = 290).

Table 4.7 Haematocrit, haematology, macrophage respiratory burst activity, serum lysozyme activity, phagocytic capacity and plasma $PGE_2$ values of fish fed the experimental diets sampled post-seawater transfer.

| | LFO | HFO | LVO | HVO |
|---|---|---|---|---|
| **Haematocrit (%)** | $44.4 \pm 0.9^{ac}$ | $46.4 \pm 0.88^{a}$ | $39.7 \pm 1.32^{b}$ | $41.9 \pm 1.02^{bc}$ |
| **Total number of leukocytes ($ml^{-1}$)** | $3.6 \times 10^7 \pm 2.3 \times 10^6$ | $3.6 \times 10^7 \pm 3.7 \times 10^6$ | $2.8 \times 10^7 \pm 3.5 \times 10^6$ | $2.5 \times 10^7 \pm 2.6 \times 10^6$ |
| **Total number of erythrocytes ($ml^{-1}$)** | $1.5 \times 10^9 \pm 1.2 \times 10^8$ | $1.6 \times 10^9 \pm 2.7 \times 10^8$ | $1.4 \times 10^9 \pm 1.8 \times 10^8$ | $1.9 \times 10^9 \pm 2.4 \times 10^8$ |
| **Macrophage respiratory burst activity (Absorbance per $10^5$ x 100)** | $28.9 \pm 5.1^{a}$ | $22.5 \pm 5.2^{a}$ | $6.6 \pm 1.4^{b}$ | $10.2 \pm 2.0^{b}$ |
| **Serum lysozyme activity (U $min^{-1}$ $ml^{-1}$)** | $899.4 \pm 108.1^{a}$ | $711.4 \pm 82.4^{ac}$ | $250.0 \pm 37.2^{b}$ | $416.3 \pm 114.0^{bc}$ |
| **Phagocytic capacity (%)** | $56.3 \pm 11.4^{a}$ | $48.7 \pm 17.5^{ab}$ | $34.2 \pm 14.6^{b}$ | $30.2 \pm 18.8^{b}$ |
| **Plasma $PGE_2$ (pg $ml^{-1}$)** | $1209.5 \pm 131.2^{ab}$ | $1446.9 \pm 196.0^{a}$ | $980.3 \pm 153.1^{ab}$ | $754.3 \pm 89.1^{b}$ |

Values are means ± SEM, n =8. Values within a row without a common superscript letter differ, ($p<0.05$). LFO = low fish oil, HFO = high fish oil, LVO = low vegetable oil, HVO = high vegetable oil.

particles by salmon head kidney macrophages sampled post-seawater transfer is shown in Figure 4.10 (a-d). Fish fed with the VO containing diets had significantly reduced phagocytic capacity compared to those maintained on LFO diet ($p<0.05$). Significant differences were observed when measuring salmon serum lysozyme activity. Fish fed both the LVO and HVO diets showed significantly lower lysozyme activity (250.0 and 416.3 U min $ml^{-1}$) compared to both LFO and HFO fed fish (899.4 and 711.4 U min $ml^{-1}$) ($p<0.003$) as shown in Table 4.7. In addition, fish fed with the LVO diet also had significantly reduced lysozyme activity compared to the HFO fed fish. The concentration of plasma $PGE_2$ was significantly decreased in fish fed the HVO diet, compared to those fed the HFO diet (Table 4.7).

The fatty acid compositions of the Atlantic salmon PBL fed the different dietary regimes are given in Table 4.8. After feeding salmon diets containing VO some differences in the fatty acid composition of the lipid isolated from PBL were identified. Increased levels of oleic acid, ALA, LA were observed in fish fed both VO containing diets, compared to those maintained on the FO diets, showing that the dietary lipid composition is reflected in the fatty acid composition of fish lymphocytes. Reduced levels of ARA, EPA and DHA were reported in VO fed fish compared to FO fed fish. In general, VO fed fish had higher levels of n-6 fatty acids and lower (n-3) / (n-6) ratios when compared to salmon fed the FO diets.

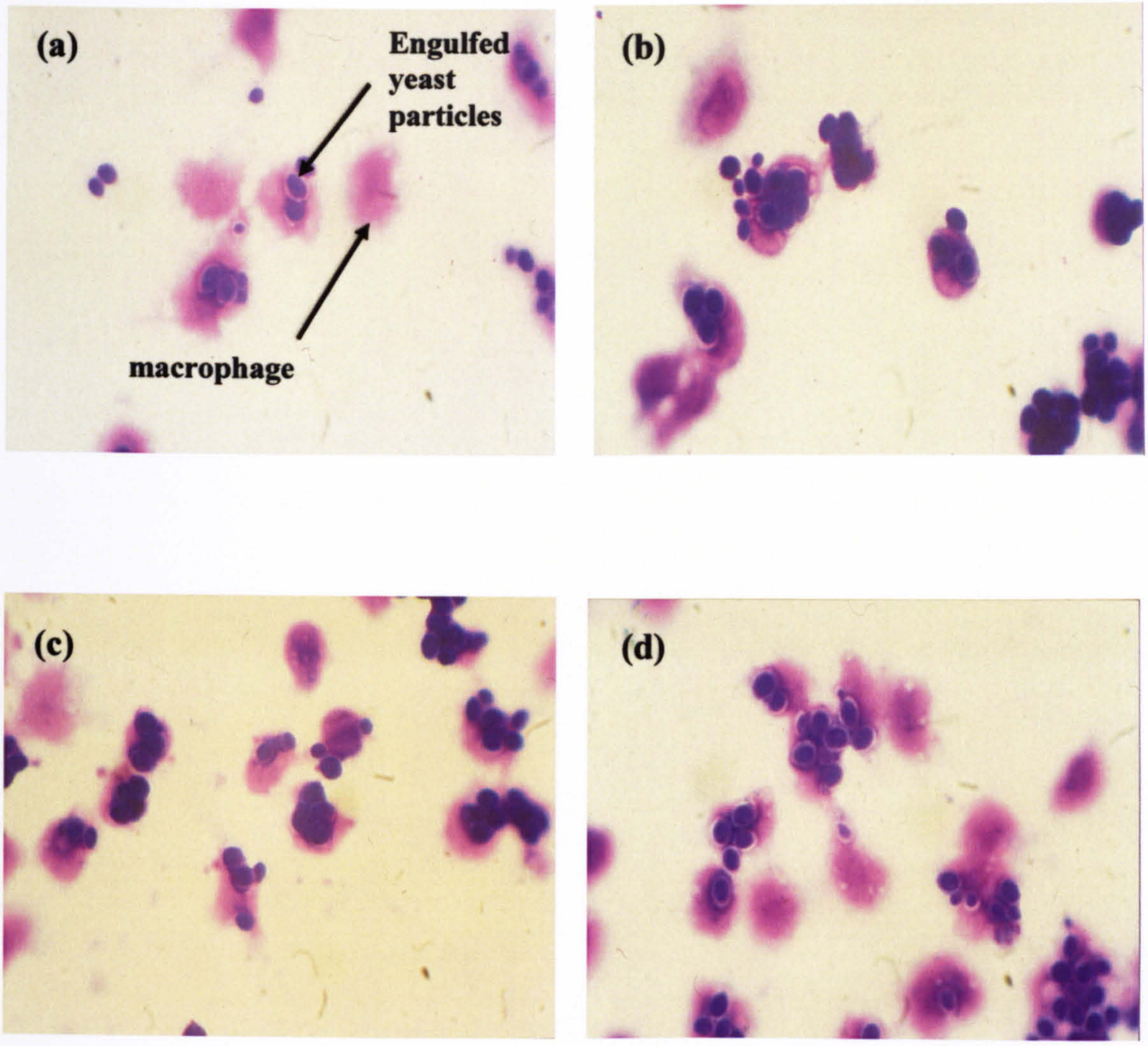



Figure 4.10 Salmon head kidney macrophages showing engulfed and partially engulfed yeast particles sampled post-seawater transfer (examined under oil immersion, total magnification x 1720).

Table 4.8 Fatty acid composition (wt %) of Atlantic salmon peripheral blood leukocytes.

| Fatty Acids | LFO | HFO | LVO | HVO |
|---|---|---|---|---|
| 14:0 | 2.0 ± 0.4 | 1.2 ± 0.9 | 0.8 ± 0.1 | 0.8 ± 0.8 |
| 15:0 | 0.7 ± 0.1 | 0.6 ± 0.1 | 0.3 ± 0.0 | 0.3 ± 0.1 |
| 16:0 | 23.5 ± 2.5$^{a}$ | 20.5 ± 3.3$^{a}$ | 16.6 ± 1.4$^{b}$ | 16.1 ± 1.3$^{b}$ |
| 18:0 | 4.9 ± 0.4$^{a}$ | 3.8 ± 0.5$^{b}$ | 4.3 ± 0.3$^{b}$ | 4.1 ± 0.2$^{b}$ |
| 20:0 | 0.2 ± 0.0 | 0.2 ± 0.0 | 0.2 ± 0.0 | 0.2 ± 0.1 |
| 22:0 | 0.3 ± 0.0 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.6 ± 0.5 |
| Total saturates | 31.6± 3.4$^{a}$ | 26.6 ± 4.9$^{ab}$ | 22.5± 1.9$^{b}$ | 22.1 ±3.0$^{b}$ |
| 16:1 (n-7) | 2.0 ± 0.1$^{a}$ | 2.7 ± 0.3$^{b}$ | 0.9 ± 0.1$^{c}$ | 0.9 ± 0.3$^{c}$ |
| 18:1 (n-9) | 12.1 ± 0.7$^{a}$ | 10.8 ± 1.1$^{a}$ | 18.1 ± 0.6$^{b}$ | 19.3 ± 1.5$^{b}$ |
| 18:1 (n-7) | 2.2 ± 0.3 | 2.4 ± 0.3 | 1.9 ± 0.1 | 2.0 ± 0.1 |
| 20:1 (n-9) | 2.0 ± 0.2$^{ab}$ | 2.5 ± 0.3$^{a}$ | 1.5 ± 0.5$^{b}$ | 1.6 ± 0.3$^{b}$ |
| 20:1 (n-7) | 0.2 ± 0.0$^{a}$ | 0.2 ± 0.0$^{a}$ | 0.1 ± 0.0$^{b}$ | 0.1 ± 0.0$^{b}$ |
| 22:1 (n-11) | 1.5 ± 0.2$^{a}$ | 2.4 ± 0.3$^{b}$ | 0.6 ± 0.1$^{c}$ | 0.6 ± 0.5$^{c}$ |
| 22:1 (n-9) | 0.3 ± 0.1 | 0.5 ± 0.2 | 0.3 ± 0.1 | 0.3 ± 0.2 |
| 24:1 | 1.3 ± 0.3$^{a}$ | 1.8 ± 0.1$^{b}$ | 1.0 ± 0.2$^{a}$ | 1.1 ± 0.4$^{a}$ |
| Total monoenes | 21.6 ± 1.9 | 23.3 ± 2.6 | 24.4 ± 1.7 | 25.9 ± 3.3 |
| 18:2 (n-6) | 5.0 ± 0.5$^{a}$ | 2.6 ± 0.6$^{b}$ | 9.4 ± 0.3$^{c}$ | 8.0 ± 0.4$^{d}$ |
| 18:3 (n-6) | 0.1 ± 0.0 | 0.1± 0.0 | 0.2 ± 0.1 | 0.2 ± 0.1 |
| 20:2 (n-6) | 0.7 ± 0.1$^{ab}$ | 0.2 ± 0.0$^{a}$ | 1.1 ± 0.3$^{b}$ | 1.0 ± 0.6$^{b}$ |
| 20:3 (n-6) | 0.2 ± 0.0$^{a}$ | 0.1 ± 0.0$^{a}$ | 0.4 ± 0.3$^{b}$ | 0.6 ± 0.1$^{b}$ |
| 20:4 (n-6) | 1.3 ± 0.2$^{a}$ | 1.3 ± 0.1$^{a}$ | 0.7 ± 0.1$^{b}$ | 0.5 ± 0.0$^{c}$ |
| 22:4 (n-6) | 0.1 ± 0.0$^{a}$ | 0.1 ± 0.0$^{a}$ | 0.1 ± 0.0$^{a}$ | 0.0 ± 0.0$^{b}$ |
| 22:5 (n-6) | 0.3 ± 0.0$^{ab}$ | 0.3 ± 0.1$^{a}$ | 0.2 ± 0.2$^{ab}$ | 0.1 ± 0.0$^{b}$ |
| Total (n-6) | 7.7 ± 0.8$^{a}$ | 4.7 ± 0.8$^{b}$ | 12.1 ± 1.3$^{c}$ | 10.4 ± 1.2$^{d}$ |
| 18:3 (n-3) | 0.6 ± 0.1$^{a}$ | 0.7 ± 0.1$^{a}$ | 5.7 ± 0.8$^{b}$ | 7.4 ± 0.5$^{c}$ |
| 18:4 (n-3) | 0.3 ± 0.0$^{a}$ | 0.4 ± 0.1$^{a}$ | 0.7 ± 0.3$^{a}$ | 1.1 ± 0.4$^{b}$ |
| 20:4 n-3 | 0.6 ± 0.1$^{a}$ | 1.0 ± 0.3$^{b}$ | 1.1 ± 0.2$^{b}$ | 1.7 ± 0.3$^{c}$ |
| 20:5 n-3 | 9.2 ± 1.1$^{ab}$ | 11.1 ± 1.6$^{a}$ | 8.5 ± 1.4$^{b}$ | 7.7 ± 1.0$^{b}$ |
| 22:5 n-3 | 1.9 ± 0.3$^{ab}$ | 2.3 ± 0.7$^{a}$ | 1.9 ± 0.1$^{ab}$ | 1.3 ± 0.2$^{b}$ |
| 22:6 n-3 | 19.8 ± 2.2$^{a}$ | 21.1 ± 2.7$^{a}$ | 17.2 ± 2.3$^{ab}$ | 14.4 ± 2.4$^{b}$ |
| Total (n-3) | 32.4 ± 3.8 | 36.6 ± 5.5 | 35.1 ± 5.1 | 33.6 ± 4.8 |
| Total PUFA | 40.1 ± 3.5 | 41.3 ± 4.5 | 47.2 ± 1.4 | 44.0 ± 2.5 |
| (n-3):(n-6) | 4.2 ± 0.8 | 7.8 ± 0.9 | 2.9 ± 0.9 | 3.2 ± 0.8 |

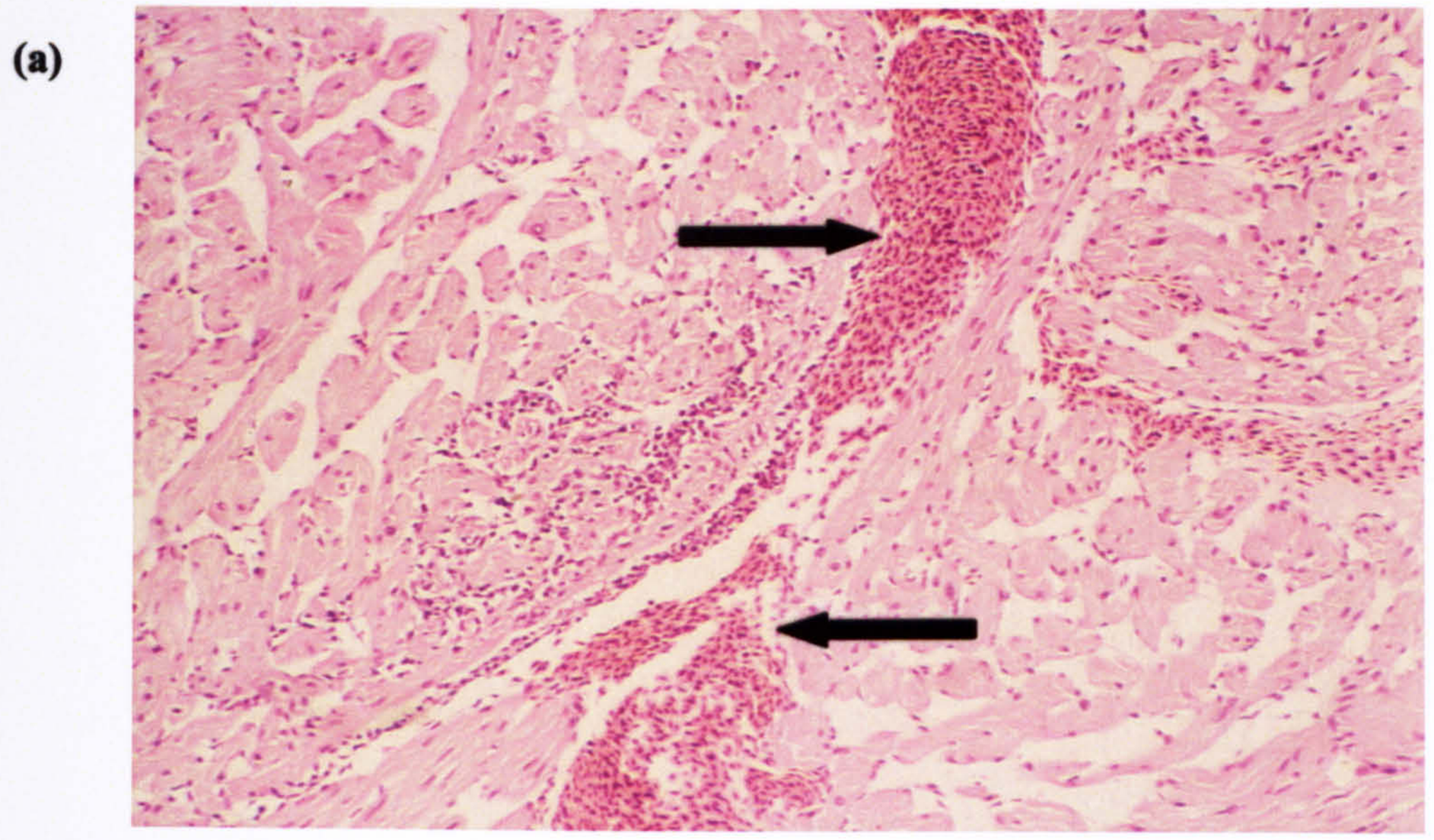
Different superscript letters within a row represent significant differences ($p<0.05$). LFO = low fish oil, HFO = high fish oil, LVO = low vegetable oil, HVO = high vegetable oil. Values are expressed as the mean percent of total fatty acids for four fish ± SD.

### 4.3.5.2 Post-seawater transfer histopathology

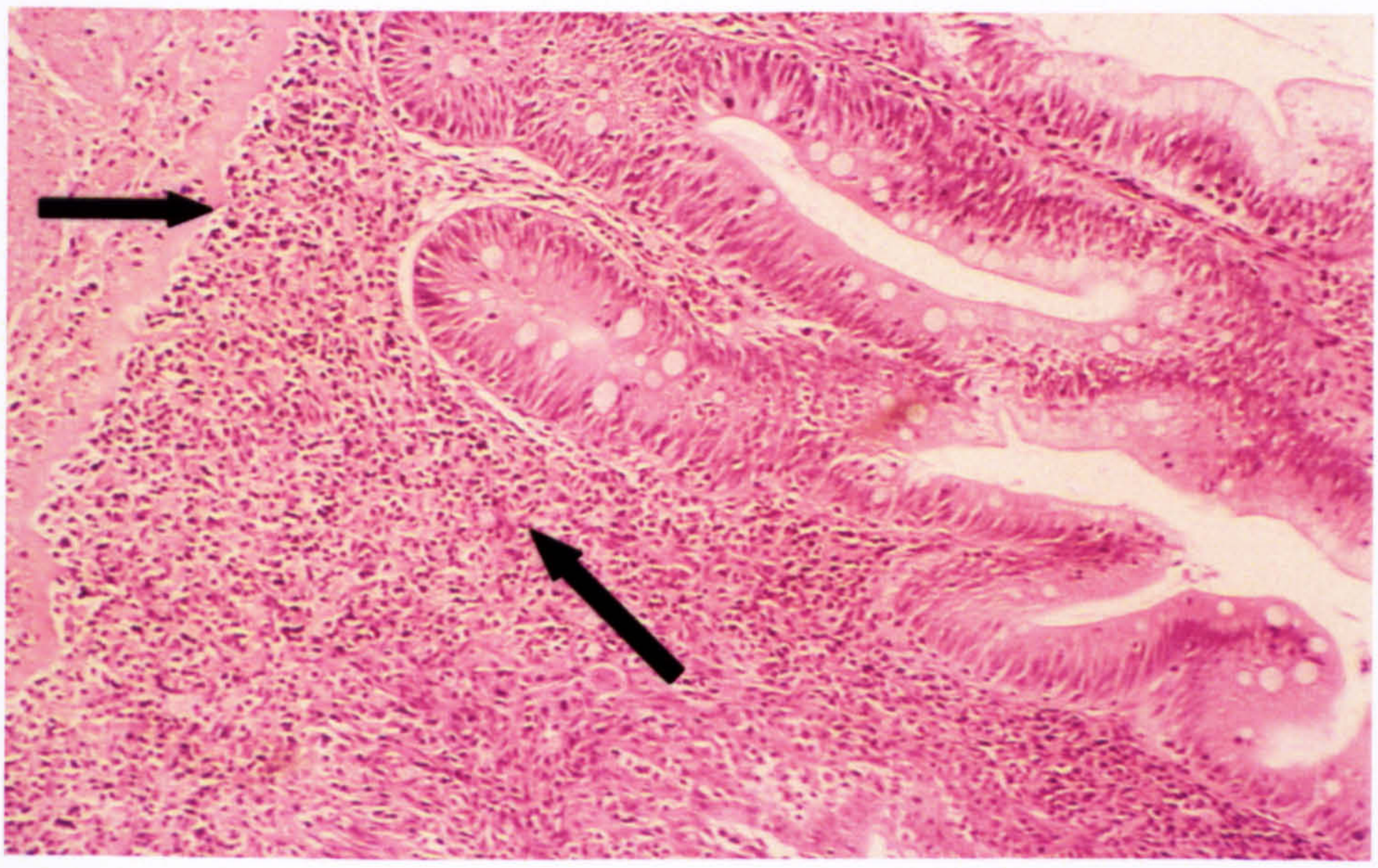
No differences in the histology samples prepared from the hearts of the different dietary groups were observed. Fish fed the HVO diet showed only some slight patches of endocarditis within the heart sections (Figure 4.11a). The livers of fish fed both the LFO and HFO diets tended to have a higher degree of fat deposition within the hepatocytes than fish fed the VO diets. Fish fed the LVO diet showed the highest amount of inflammation, pvc and necrosis out of all the groups assessed, however the degree of pathology was fairly minimal. The proximal sections of fish fed the FO diets showed high mucus activity and high vacuolation in the mid and distal gut with no cellular infiltration. The LVO fed fish showed sloughing of the mucosa and mid sections and some areas of cellular infiltration up the lamina propria in the distal sections. The distal intestine was also highly vacuolated resulting in a high degree of mucosal sloughing. The HVO fed fish showed the highest amount of cellular infiltration in the distal section compared to the other dietary groups (Figure 4.11b). An overview of the histological changes observed in fish fed the experimental diets is shown in Table 4.9.

## 4.4 Discussion

This trial was the first in which fish were fed entirely on oil from vegetable sources from first feeding to harvest. The issues of FO supply, and thereby price, necessitate research into alternative sources of oil and at the present time the Scottish aquaculture industry is considering the introduction of diets containing up to 25 % VO. While it is recognised that various VO may provide some substitution for FO many of the details require considerably



**(a)**



**(b)**

Figure 4.11 Histopathology of Atlantic salmon fed a (a) HVO diet after seawater transfer. Heart showing slight patches of endocarditis (mag x 70) (b) HVO diet after seawater transfer. Distal intestine showing a high degree of cellular infiltration (mag x 175).

Table 4.9 Overall histological changes observed in fish fed the experimental diets

| | HEART | LIVER | INTESTINE |
|---|---|---|---|
| **PRE-VACCINATION** | | | |
| LFO (12 %) | Normal | Normal – moderate fat content | High mucus activity in proximal and mid correlating with low activity in distal |
| HFO (25 %) | Normal | Normal – high fat content with large fat vacuoles | High mucus activity in anterior intestine, low in distal. Mid showed minor sloughing |
| LVO (12 %) | Normal | Variable vacuole appearance ranging from regular, even hepatocytes to "foamy" hepatocytes | Normal |
| HVO (25 %) | Normal | High fat content but no associated pathology | Low vacuolation in proximal and mid. High vacuolation in distal |
| **POST-VACCINATION** | | | |
| LFO (12 %) | Normal | Normal, moderate fat, some variability in vacuole size | High mucus activity in proximal and mid. Highly vacuolated distal, sloughing of mucosa |
| HFO (25 %) | Normal | Low-moderate fat content, small regular vacuoles | Sloughing of proximal and mid mucosa – response to vaccine? |
| LVO (12 %) | Normal – few showed signs of endo - and pericarditis | Low fat , variable sized vacuoles, occasional scn | Sloughing of proximal and mid , cellular infiltration. Definite pathology in distal |
| HVO (25 %) | Normal | Hepatic pathology, scn, large variable sized vacuoles, moderate fat | Inactive appearance to proximal (low mucus, and vacuoles) Mid and distal showed high degree of vacuolation and sloughing – response to vaccine? |
| **POST-SEAWATER TRANSFER** | | | |
| LFO (16 %) | Normal | Normal – Higher fat content than VO | High mucus activity in proximal and high vacuolation in mid and distal. No cellular infiltration |
| HFO (34 %) | Normal | Similar fat levels to fish fed LFO | Normal except for slight cellular infiltration in mid |
| LVO (16 %) | Normal | Highest levels of inflammation, pvc and necrosis | Sloughing in proximal and mid. Highly vacuolated distal with signs of cellular infiltration and sloughing of mucosa |
| HVO (34 %) | Slight endocarditis | Slight inflammation and slight necrosis | Highest degree of cellular infiltration and sloughing in distal intestine |

LFO = low fish oil, HFO = high fish oil, LVO = low vegetable oil, HVO = high vegetable oil.

more research, particularly those related to fish health and final product quality, especially those pertaining to human health. The present study followed the performance throughout the production cycle and allowed the end product to be evaluated for consumer acceptability as described by Dr Gordon Bell (personal communication). From the results of this study, new information has already been made available to improve the understanding of fatty acid metabolism in salmon and the relationship between dietary composition, growth performance and quality (Tocher *et al.*, 2004). The issue of food quality and safety, particularly with regard to levels of n-3 HUFA and organic contaminants, principally PCB and dioxins, are currently pertinent. There have been considerable changes in the permissible levels of dioxins in fish flesh. These changes have arisen due to a number of EU committee reports (SCF, SCAN and SCOOP) suggesting reductions of recommended daily intake values for dioxins and PCBs. When quoted in terms of Toxic Equivalents (ngTEQ / kg) European FO are in the range 0.7-20 (Average 4.8) while VO are in the range 0.1-1.5 ngTEQ / kg (Ave 0.2). For this reason feeding VO in place of FO for all, or part, of the salmon life cycle should considerably reduce the dioxin TEQ in salmon.

In the present study, when measuring haematocrit from fish fed the various dietary treatments, it was found that feeding fish the LVO diet prior to vaccination resulted in reduced haematocrit compared with fish fed the LFO treatment. A similar case was reported after the fish had been transferred to seawater, where both the LVO and HVO fed fish showed significantly reduced haematocrit compared to the FO fed fish. Wintrobe in 1934, first introduced the concept of haematocrit values, when measuring the ratio of

erythrocytes to plasma in blood samples and found that the haematocrit value was uniform among vertebrates, but the number of erythrocytes were more variable. Young (1949), found that repeated, haematocrits of individual fish, (opaleye, *Girella nigricans*) during several months showed that the values varied considerably and the value always became lowered when a fish lost appetite or became diseased. The haematocrit findings from the present trial are in contrast to findings by Bell *et al.*, (1996a) in which no differences in haematocrit were found after feeding salmon diets containing LO or sunflower oil.

With regards to the total number of circulating leukocytes and erythrocytes the only differences noted between fish fed the experimental diets was found when the fish were sampled prior to vaccination. Significantly lower numbers of leukocytes were found in fish fed both VO containing diets compared to fish fed the LFO diet. Puchkov (1964) stated that the numbers of leukocytes in the same species of fish will vary greatly with age, season and sexual maturation. Likewise, feeding both VO diets at low and high lipid levels, reduced the number of erythrocytes in the circulation when compared to fish fed the HFO diet. It has been found that erythrocyte counts in fishes are low compared with those of mammals (Mott, 1957) and that some degree of inverse correlation exists between the erythrocyte cell size and the total count in freshwater teleosts (Smith *et al.*, 1952). Reports published by Montero *et al.*, (2003) suggested that feeding diets containing either LO or soybean oil resulted in reduced numbers of circulating erythrocytes in sea bream. However, when feeding sunflower oil to salmon, Thompson *et al.*, (1996) found no significant differences in blood cell numbers between dietary groups of salmon fed diets high in either n-3 (FO) or n-6 (sunflower oil) fatty acids.

The production of oxygen intermediates during respiratory burst and the process of phagocytosis are of vital bactericidal importance (Jorgensen *et al.*, 1993). Therefore, by increasing the action of macrophages this may increase resistance to bacterial pathogens. Macrophages act as antigen presenting cells to lymphocytes, and if feeding VO depresses or enhances macrophage activity it may in turn alter antibody responses and affect vaccine efficacy. In the present study, one of the major outstanding findings was the effect of VO substitution in the diet on head kidney macrophage function. At every sample time point it was noted that VO inclusion in the diet had a significant effect on the macrophage respiratory burst response. The effects of dietary oils on macrophage dependent humoral and cellular immunity may be produced by an imbalance in the fatty acid composition of membrane phospholipids, affecting the physical properties of the membrane (i.e. fluidity and permeability), and the activity of membrane-associated receptors, which may be a reason for a decrease in macrophage activity (Kelley, 1996; Miles & Calder, 1998; Calder, 1998a, b; Montero *et al.*, 2003). It is also interesting that Waagbϕ *et al.*, (1993a) carried out a nutritional study in which they demonstrated that salmon fed diets high in n-3 PUFA showed reduced intracellular bacterial killing of head kidney macrophages compared to fish fed lower levels of n-3 PUFA. On the contrary, other reports show positive effects of n-3 fatty acids on the immune response of fish. Sheldon and Blazer (1991) showed that dietary lipids were beneficial to macrophage function in channel catfish, with enhanced intracellular killing correlated to increased levels of (n-3) PUFA. The authors found that channel catfish macrophages showed higher bactericidal activity with menhaden fish oil (rich in n-3 HUFA) in the diet than with beef tallow (mainly saturated fatty acids) or soybean oil (containing LA and ALA).

In the present experiment, the phagocytic activity of head kidney macrophages was significantly affected by feeding both the low and high VO diets containing equal quantities of RO and LO compared to those fish maintained on the LFO diet. It is likely that changes in the fatty acid composition of the macrophage cell membrane, resulting in a sub-optimal fatty acid composition, could in turn influence membrane physical properties and, hence, the phagocytic activity. Studies by Waagbo *et al.*, (1995), Farndale *et al.*, 1999 and Montero *et al.*, (2003) confirm that the nature of the dietary oils determines the fatty acid profile of macrophages in many fish species. Alterations in cell membrane phospholipid fatty acid composition can also have direct effects on the activities of membrane-bound enzymes and the control of ion permeability (Stubbs & Smith, 1984). From the available literature, many authors have reported differences in the macrophage function of fish species fed various dietary oils. Bell *et al.*, (1996a) reported that salmon fed diets containing LO showed no significant difference between fish fed a FO diet when measuring head kidney phagocytosis. However, Montero *et al.*, (2003) found that feeding RO or soybean oil reduced the phagocytic capacity of seabream macrophages to phagocytose *V. anguillarum*. As they measured the fatty acid composition of total lipid from the head kidney macrophages the authors concluded that the high content of either ALA or monoenes, especially oleic acid in the cell membrane, may cause the differences in phagocytic activity observed.

The only significant difference in serum lysozyme activity was found when fish were sampled after transfer to seawater. The reduction in serum lysozyme activity in fish fed the VO containing diets coincides with a reduction in macrophage activity. Therefore, as well

as a reduction in the respiratory burst activity of the head kidney macrophages, caused by VO inclusion in the diet, it also seems to affect the macrophages ability to produce lysozyme, perhaps due to membrane fatty acid alterations to membrane structure and function. Although no significant differences were found, Montero *et al.*, (2003) reported that seabream fed a FO diet had higher serum lysozyme activity than fish fed diets containing either LO, RO or soybean oil. Bell *et al.*, (1996a) also found no differences in lysozyme concentration between groups of salmon fed FO, LO or sunflower oil diets.

$PGE_2$ is produced by most cells in almost every tissue but it has important roles in modulating inflammation, reproduction, mucosal integrity and immune functions. In mammals optimum immune function requires a $PGE_2$ concentration of 1 nM with immune suppression arising when concentrations are above 10 nM (Kinsella *et al.*, 1990). $PGE_2$ is a primary product of ARA metabolism in many cells. Like most eicosanoids, it does not exist preformed in any cellular reservoir. When cells are activated or free ARA is supplied, $PGE_2$ is synthesised *de novo* and released into the extracellular space. *In vivo*, $PGE_2$ is rapidly converted to an inactive metabolite (13, 14-dihydro-15-keto $PGE_2$) by the prostaglandin 15-dehydrogenase pathway. In the present study, the concentrations of plasma $PGE_2$ were significantly decreased in fish fed the HVO diet compared to those fed the HFO diet. This reduction in $PGE_2$ can be related to the reduction in ARA found in the cellular membrane. This finding is further supported by the fact that the PBL fatty acid composition of fish fed the HVO diet had significantly reduced levels of ARA, which in turn can result in decreased production of $PGE_2$. No other significant differences were found between dietary groups. In a study by Bell *et al.*, (1996a, b) salmon fed diets

containing LO showed a reduction in the production of $LTB_4$ and $PGE_2$ by stimulated head kidney macrophages compared to those fed FO or sunflower oil. Fish fed sunflower oil had similar levels of $PGE_2$ to those fish fed the FO diet. These results support the findings from this trial where feeding salmon diets containing LO/RO (1:1) caused a significant reduction in non-specific immune parameters including reduced macrophage respiratory burst activity. However, earlier studies by Bell *et al.*, (1993a, b) showed that there were no dietary induced changes of $PGE_2$ in circulating plasma of salmon fed diets containing either FO, LO or sunflower, yet in stimulated blood cells the levels of $PGE_2$ were significantly decreased in fish given LO compared to those given both sunflower oil or FO diets.

With regard to the fatty acid composition of PBL from fish fed the VO diets, some distinct features were evident. The fatty acid composition of the PBL resembled that of the dietary treatment which is in accordance with reports from Meade and Mertin, (1978) and Johnston, (1992). Significantly increased levels of oleic acid, LA and ALA were present in the PBL of fish fed the VO containing diets compared to fish fed the FO diets. The levels of ARA and EPA were found to be decreased in fish fed the VO diets compared to those fed FO containing diets. The reduced levels of ARA found in the cellular membrane contributes to the reduced levels of circulating $PGE_2$ reported. Salmon maintained on the VO diets showed significantly higher levels of total n-6 fatty acids and reduced levels of total saturated fatty acids. Thompson *et al.*, (1996) reported differences in the fatty acid composition of salmon leukocytes fed either a FO or sunflower oil diet. In addition Bell *et al.*, (1993b) findings are similar to those found in the present study. Salmon fed diets

containing LO increased the LA, 20:2(n-6) and 20:3(n-6) but decreased ARA compared to FO fed fish. Other studies have since examined the effects that different levels of dietary fatty acids have on the fatty acid composition of tissues and cells and the subsequent effects on eicosanoid production. Bell *et al.*, (1994) showed that the lipid composition of juvenile turbot, could be modified by changes in the dietary lipid composition.

Furunculosis is a disease of salmonids caused by the bacterium *A. salmonicida* which can affect all life stages of the fish in fresh- and sea-water. Outbreaks generally occur as water temperature rises above 10 °C, but cold water forms of the disease have been recorded. Furunculosis may be acute, with diseased fish showing few symptoms, or chronic where external signs may include large swellings or furuncles, haemorrhaging in the gills at the base of the fins and loss of appetite and skin darkening. Internally, there may be haemorrhaging in the body cavity, heart and liver. Enlargement of the spleen and inflammation of the intestine may also occur. All salmonids appear susceptible to furunculosis, and the causative bacterium can also cause disease in other freshwater and marine fish species. The disease is transmitted horizontally from infected or carrier fish and via contaminated water. Treatment with antibiotics is often effective if administered early, but problems of antibiotic resistance may occur, therefore vaccines against this disease are now widely available and these are proving effective in reducing the overall impact of the disease in salmon farming. In the present study, vaccination and challenge were used as *in vivo* indicators of the influence of different dietary oils on the innate and adaptive immunity in Atlantic salmon. No outstanding findings were taken from the *A. salmonicida* bacterial challenge. The unvaccinated populations, as expected, had a high

proportion of mortalities, which started to die eight days post challenge. All other vaccinated dietary groups showed no more than a 12 % cumulative mortality up to thirty three days post challenge. This suggests that despite the differences in the non-specific immune parameters, the health of the VO-fed fish was not compromised as a result of pathogenic challenge.

Blazer and Wolke (1984) were among the first to observe the importance of diet in the immune response and disease resistance of fish. Blazer *et al.*, (1989) found that channel catfish also showed differences in immune responses after they were fed a control laboratory prepared diet or a commercially prepared diet. The authors noted that the major differences between the diets, that probably accounted for the dissimilar immune response, were the total lipid content and the ratio of the n-3 and n-6 fatty acids. The inclusion of 7 % LO in diets of catfish reduced fish survival to infection with *Edwarsiella ictaluri* at a temperature of 28 °C, as well as the ability of fish to produce antibodies (Fracalossi & Lovell, 1994). In a study by Kiron *et al.*, (1995) the lack of n-3 HUFA in rainbow trout diet decreased the resistance of fish to IHN virus.

Bransden *et al.*, (2003) detected significant differences in cumulative mortalities of salmon fed diets containing either FO or sunflower oil after challenge with *E. ictaluri*. From the results obtained by Bransden and colleagues it remains unclear as to why some of the experimental diets improved disease resistance in salmon while others did not. The results suggest that perhaps an optimal dietary n-3 / n-6 ratio exists for disease resistance. Bransden *et al.*, (2003) noted that disease susceptibility was not correlated with any fatty

acid or nutrient. Salmon fed the diet containing 4 % sunflower oil recorded the highest mortality at the end of the challenge, with a cumulative mortality of 78 %. The results obtained from this dietary group were statistically different to all other dietary treatments with the exception of fish fed the 20 % sunflower oil diet, which had a cumulative mortality of 56 %. The lowest mortalities were recorded in salmon fed a diet containing 10 % sunflower oil.

Differences in survival after bacterial infection are again probably related to the cell membrane structure. The structure may be affected by dietary fatty acid composition including the vitamin E content and water temperature. It has been proposed by Salte *et al.*, (1988) that high levels of n-3 PUFA can increase disease resistance in salmon suffering from cold-water vibriosis by increasing the strength of the blood cell membrane. The effect of n-3 PUFA on osmotic haemolysis of erythrocytes is also supported by findings from a study by Erdal *et al.*, (1991). The lack of n-3 HUFA in the diet of rainbow trout decreased the resistance of the fish to IHN virus (Kiron *et al.*, 1995) and when Fracalossi & Lovell, (1994) fed diets containing LO (high n-3 PUFA) to catfish it resulted in reduced fish survival to an infection with *E. ictaluri* at a temperature of 28 °C, as well as the ability of the fish to produce antibodies.

One of the most significant differences between fish maintained on either a FO containing or VO containing diet was the changes in histological appearance of the tissues especially the liver. The major differences between fish fed with either oil type was the variability of vacuole size within the hepatocytes. Most VO fed fish showed a high degree of variability

with vacuoles ranging from small, single ones, to multiple, large vacuoles containing varying amounts of lipid. In general, hepatocytes of fish fed the lower dietary lipid levels showed a greater number of small lipid droplets in comparison to fish fed the higher lipid levels showing a higher lipid droplet diameter. These findings are supported by the results of Caballero *et al.*, (1999) who found similar results when feeding seabream three different dietary lipid levels. At any of the time points sampled there were no significant differences between the dietary groups with regard to heart histology. Only minor changes were observed in some groups but this was not considered as a major histological change. On the contrary, Bell *et al.*, (1991a) reported that Atlantic salmon fed a sunflower oil containing diet developed cardiac lesions ranging from mild to severe. Olsen *et al.*, (1999) reported that Arctic char fed LO showed a tendency to accumulate lipid droplets within the cytoplasm which was not observed in fish fed soybean lecithin, suggesting that the accumulation could be due to insufficient synthesis of phospholipids required for lipoprotein synthesis. The pathological effects observed in the present trial poses the question is there a level at which the dietary ratio of n-6 / n-3 PUFA becomes high enough that it causes pathological change in the fish.

This study provided data confirming that sustainable alternatives to marine FO are suitable for use in salmon culture. It is likely that the Scottish salmon industry will approve limited replacement of FO with VO, probably RO, in the near future. There is no doubt that this study provided a considerable amount of new knowledge on the mechanisms and effects of FO replacement, with VO, on production of Atlantic salmon from first feeding to harvest. However, the diets used in the present study were extreme examples of FO replacement

where 100 % of the added oil was replaced with VO. It is likely that when routine inclusion of VO becomes commonplace in Scotland, as it is presently in Norway, that the inclusion levels will be at around 25 - 30 % of the added oil. Further investigation of the effects of lower levels of VO inclusion should be conducted in the future especially with regard to possible perturbation of immune function. The trials conducted with VO replacing FO, to date, have tended to use single oils, such as RO, soybean oil, LO and palm oil. As our knowledge of this area increases it is likely that the choice of oils will be optimised such that blends of different oils used are likely to vary with different species, different developmental stages and different seasons and temperatures.

# Chapter - 5 Researching Alternatives to FO in Aquaculture (RAFOA) - Part I (Scotland, Norway, Spain)

## 5.1 General Introduction

The effects on fish health of replacing FO with VO in Atlantic salmon have been previously discussed in Chapters 3 and 4. The present chapter is part of a European Union funded (QLRT-2000-30058) programme, Researching Alternatives to Fish Oil in Aquaculture (RAFOA), including partners in Scotland, Norway, Spain and France investigating FO replacement in the diets of Atlantic salmon (*S. salar*), rainbow trout (*O. mykiss*), European sea bass (*D. labrax*) and gilthead seabream (*Sparus aurata*). In this chapter the effects of FO replacement with LO, RO and olive oil (OO) on the immune response of fish will be examined from three of the international dietary trials. Two trials examined the effects of FO replacement in salmon (Scotland and Norway) and one examined the effects in the marine species, sea bass (Spain). Participants in Scotland and Norway (Institute of Aquaculture, University of Stirling and National Institute of Nutrition and Seafood Research (NIFES)) were involved in investigating the effects of FO substitution in Atlantic salmon. The Universidad De Cadiz (UCA) examined the effects of VO in sea bass.

### 5.1.1 *Sea bass (Dicentrarchus labrax L.)*

Sea bass (*Dicentrarchus labrax* L.) are highly valued and cultured widely in Mediterranean countries. Physically the body of a sea bass is covered by large regular scales, and its colour varies considerably, ranging from dark grey, blue or green on the back to a white or pale yellow belly. The head in young bass appears quite pointed, but becomes blunter in older fish and their eyes are relatively large for marine fish. Bass can tolerate a wide range of temperatures with wild adult populations withstanding ranges of between 2 °C to 32 °C

(Barnabe 1990). Adult bass spawn in the seas around February to July and the eggs hatch between 4 and 9 days after fertilisation, depending on the sea temperature. During the following 2 - 3 months, the larvae drift from the open sea inshore towards the coast and eventually into estuaries. The bass spend the next 4 - 5 years in these sheltered habitats before they mature and migrate towards the open oceans. During the spawning season, a mature female bass can produce between a quarter and half a million eggs per kg of her total body weight.

In general, the lipids of marine fish are characterised by high n-3 PUFA while their freshwater counterparts have less n-3 PUFA but more n-6 PUFA (Sheridan, 1989). The knowledge of sea bass's nutritional requirements is still incomplete compared to other fish species such as salmonids and carps (Oliva-Teles, 2000). Commercial feeds for sea bass are currently highly energetic, with lipid levels around 25 % compared to 12 % some years ago (Izquierdo *et al.*, 2003). It is just recently that research has been published on the use of vegetable VO in these oils (Alexis, 1997; Robin, 1998). Diets for sea bass have partially replaced the FO with VO such as OO, RO, LO, soyabean and sunflower (Alexis, 1997; El-Kerdawy & Salama, 1997).

Several authors reported data on the immune function of this species (Scapigliati *et al.*, 1995, 1996, 2002; dos Santos *et al.*, 1997; Abelli *et al.*, 1996, 1997). Despite the fact that our knowledge of the fish immune system is continuously increasing, the biology of cellular reaction is, at present, largely unknown mainly due to the lack of specific markers for leucocytes. Bearing this in mind, sea bass is the only marine species for which B-cell and T-cell markers are available (Scapigliaiti *et al.*, 1995; 1996). The main pathological

conditions affecting sea bass in aquaculture are vibrosis (Dec *et al.*, 1990), pasteurellosis (Bakopoulous *et al.*, 1997a) and virosis (Skliris *et al.*, 1999). To study humoral reactions of the sea bass, some monoclonal antibodies have been prepared against immunoglobulins and immunoglobulin bearing cells (Scapigliati *et al.*, 1999).

### 5.1.2 *Aquaculture of sea bass*

The increasing price of wild fish has encouraged investment in sea bass farming. On a commercial scale, eggs are firstly incubated in submerged net cones, and air or water is arranged to flow upwards from the base to ensure that eggs remain in suspension (Barnabe, 1990). The bass larvae hatch after 5 - 9 days, depending on the water temperature, and are transferred to rearing tanks which have a continuous flow of fresh seawater. Bass larvae are weaned from live foods to more cost-effective pelleted foods from 30 days onwards (Barnabe, 1990).

### 5.1.3 *Aims*

The overall aims of the project were to replace as much FO as possible with VO in the diets currently used in farmed fish feeds, without significantly compromising the health, welfare and growth performance of the fish, or its health promoting properties, taste and other characteristics acceptable to the consumer and processor. Secondly, to underpin the findings by advancing current understanding of fish lipid nutrition. In an initial 12 month trial, fish were fed the experimental blends of FO and VO prior to attaining market size.

The effects of dietary change on Atlantic salmon immune function and tissue histology by replacing FO with LO and OO in Scottish fish farms are presented in Trial 1. Trial 2 presents the effects of substituting FO with RO and OO in Atlantic salmon farmed in Norway and finally, Trial 3 investigates the effects of replacing FO with LO, RO and OO in sea bass farmed in Spain. In assessing how dietary change influences the health of fish, any alterations in immune function resulting from changes in cellular fatty acid composition, arising from VO incorporation *in vivo*, are also presented in this chapter.

## 5.2 Materials and Methods

### 5.2.1 *Trial 1 RAFOA Scotland – Replacement of FO with LO and OO in Atlantic salmon*

#### 5.2.1.1 Dietary Groups

Atlantic salmon smolts of average weight 75 g were randomly stocked into 6 x 5 $m^2$ net pens at Loch Duich Fish Trials Unit (FTU), Marine Harvest Ltd., in early March 2001. Salmon were grown for 12 months prior to market size until the average weight of the fish was around 2 kg. The experimental diets were prepared by Skretting (Nutreco, ARC) and feeding of the experimental regimes started on the 15$^{th}$ May 2001. The experimental diets contained 45 % protein and 30 % oil with the added oil being either 100 % FO (capelin oil), or LO in five combinations of FO/LO (75 %/ 25 %; 50 %/ 50 %; 25 %/ 75 % and 0 %/ 100 %). A diet containing 50 % FO and 50 % OO was also included in the dietary regime. The formulations of the dietary treatments fed to fish are given in Table 5.1, while the proximate and fatty acid compositions of the experimental diets are shown in Table 5.2.

The fish were fed the experimental diets for 40 weeks with the experiment terminating in March 2002.

### 5.2.2 *Trial 2 RAFOA Norway – Replacement of FO with RO and OO in Atlantic salmon*

#### 5.2.2.1 Dietary Groups

Atlantic salmon (*Salmo salar*) with an initial mean weight of 142 g were divided into 7 net pens (125 $m^3$) with 600 fish in each pen at the research station in Gildeskal Research Station, Inndyr, near Bodϕ in Norway (Figure 5.1). The project started in May 2001 and ran until March 2002 when a small group of fish from each dietary treatment were individually marked and fed a 100 % FO diet until August 2002. In each of the 7 net pens fish were given one of the following dietary regimes: 100 % FO (in duplicate), or RO in five combinations of FO / RO (100 % / 0 %; 75 % / 25 %; 50 % / 50 %; 25 % / 75 %; 0 % / 100 %) or a diet containing 50 % FO and 50 % OO. The diets for Atlantic salmon contained 45 % protein, 30 % oil, 6 % moisture and 7 % ash. The control diet contained capelin oil and the same basal diets used in Trial 1 (RAFOA Scotland) were used with RO replacing LO. The fatty acid composition of the experimental diets are shown in Table 5.3. The diets were fed to satiation by hand and fish exposed to natural light.

Table 5.1. Formulation (g $kg^{-1}$) of experimental diets used in Trial 1.

| | Oils (%) | | | | | |
|---|---|---|---|---|---|---|
| Component | LO:FO (0:100) | LO:FO (25:75) | LO:FO (50:50) | LO:FO (75:50) | LO:FO (100:0) | OO:FO (50:50) |
| [1]Fishmeal | 338 | 338 | 338 | 338 | 338 | 338 |
| [2]Maize gluten | 200 | 200 | 200 | 200 | 200 | 200 |
| [3]Soya (Hi Pro) | 100 | 100 | 100 | 100 | 100 | 100 |
| [4]FO | 258 | 193.5 | 129 | 64.5 | 0 | 129 |
| [5]Linseed oil/Olive oil | 0 | 64.5 | 129 | 193.5 | 258 | 129 |
| [6]Micronutrients | 25 | 25 | 25 | 25 | 25 | 25 |

[1]Scandinavain LT-fish meal (Nordsildmel, Norway).

[2]Cargill/ADM, Decatur, Illinois.

[3]Soybean meal (Denofa, Fredrikstad, Norway).

[4]Capelin oil (Nordsildmel, Norway) supplemented with 200ppm BHT.

[5]Crude E.C.C. linseed oil (N.V. Oliefabriek Lictervelde, Belgium) supplemented with 500ppm Ronoxan A (Roche, Switzerland).

[6]Vitamin, mineral and carotenoid pigment premix formulated to Nutreco specification (Farmix, Trouw Nutrition, The Netherlands).

FO = fish oil, LO = linseed oil, OO = olive oil.

Table 5.2 Proximate (% weight of diets) and fatty acid compositions (% of total fatty acids by weight) of the experimental diets used in Trial 1. Data are presented as mean (n=2).

| Component | LO:FO (0:100) | LO:FO (25:75) | LO:FO (50:50) | LO:FO (75:50) | LO:FO (100:0) | OO:FO (50:50) |
|---|---|---|---|---|---|---|
| Protein | 44.1 | 44.1 | 44.2 | 44.4 | 43.6 | 44.6 |
| Lipid | 28.9 | 28.9 | 30.2 | 29.5 | 31.1 | 29.9 |
| Ash | 6.5 | 6.9 | 7.2 | 7.5 | 7.6 | 6.9 |
| Moisture | 5.9 | 6.3 | 5.7 | 6.1 | 5.6 | 6.3 |
| Fatty acid | | | | | | |
| 14:0 | 6.3 | 4.7 | 3.4 | 2.0 | 0.4 | 3.3 |
| 16:0 | 12.1 | 10.6 | 9.3 | 8.1 | 6.9 | 11.9 |
| 18:0 | 1.1 | 1.7 | 2.1 | 2.7 | 3.1 | 2.0 |
| Total saturates[1] | 19.9 | 17.2 | 15.1 | 13.0 | 10.5 | 17.4 |
| 16:1 n-7 | 8.1 | 6.1 | 4.2 | 2.3 | 0.5 | 4.5 |
| 18:1 n-9 | 11.9 | 13.6 | 15.1 | 16.0 | 17.0 | 37.8 |
| 18:1 n-7 | 3.3 | 2.6 | 2.2 | 1.6 | 1.0 | 2.8 |
| 20:1 n-9 | 17.9 | 13.1 | 9.0 | 5.0 | 1.1 | 9.0 |
| 22:1 n-11 | 13.3 | 10.1 | 7.1 | 4.3 | 1.1 | 7.2 |
| 22:1 n-9 | 2.1 | 1.5 | 1.0 | 0.5 | 0.1 | 0.9 |
| Total monoenes[2] | 58.4 | 48.4 | 39.6 | 30.5 | 21.1 | 63.3 |
| 18:2 n-6 | 4.2 | 7.4 | 9.8 | 12.3 | 15.1 | 8.2 |
| 20:4 n-6 | 0.2 | 0.2 | 0.1 | 0.1 | 0.1 | 0.1 |
| Total n-6 PUFA[3] | 5.0 | 8.0 | 10.2 | 12.6 | 15.2 | 8.4 |
| 18:3 n-3 | 0.9 | 14.0 | 25.6 | 37.8 | 50.4 | 1.6 |
| 18:4 n-3 | 2.9 | 2.1 | 1.6 | 0.9 | 0.2 | 1.5 |
| 20:5 n-3 | 5.9 | 4.6 | 3.5 | 2.2 | 1.0 | 3.5 |
| 22:6 n-3 | 5.0 | 4.0 | 3.4 | 2.4 | 1.5 | 3.2 |
| Total n-3 PUFA[4] | 15.7 | 25.6 | 34.6 | 43.7 | 53.3 | 10.3 |
| Total PUFA | 21.7 | 34.4 | 45.3 | 56.5 | 68.5 | 19.3 |

[1]Totals include 15:0, present at up to 0.3 %; [2]Totals include 16:1(n-7), 20:1(n-11), 20:1(n-7) and 24:1, present at up to 0.5 %; [3]Totals include 18:3(n-6), 20:1(n-6) and 20:3(n-6), present at up to 0.2 %; [4]Totals include 20:3(n-3), 20:4(n-3) and 22:5(n-3), present at up to 0.4 %. PUFA. FO = fish oil, LO = linseed oil, OO = olive oil.

Figure 5.1 Fish pens at the research station in Gildeskal, Inndyr, near Bodϕ in Norway.

Table 5.3 Dietary fatty acid composition (% fatty acid per weight (w/w)) of the experimental diets used in Trial 1. Data are presented as mean (n=2). (When data are below 0.1. it is denoted by - ). FO = fish oil, RO = rapeseed oil, OO = olive oil.

| | RO:FO (0:100) | RO:FO (25:75) | RO:FO (50:50) | RO:FO (75:25) | RO:FO (100:0) | OO:FO (50:50) |
|---|---|---|---|---|---|---|
| 14:0 | 6.7 | 5.0 | 3.6 | 2.1 | 0.4 | 3.4 |
| 16:0 | 11.6 | 10.0 | 8.9 | 7.5 | 5.7 | 11.7 |
| 18:0 | 1.0 | 1.2 | 1.4 | 1.6 | 1.7 | 1.9 |
| Total saturates[1] | 20.4 | 17.4 | 14.9 | 12.5 | 9.1 | 18.0 |
| 16:1n-7 | 8.0 | 6.1 | 4.4 | 2.6 | 0.6 | 4.5 |
| 18:1n-9 | 11.2 | 22.5 | 32.4 | 42.4 | 53.6 | 37.3 |
| 18:1n-7 | 3.4 | 3.3 | 3.4 | 3.4 | 3.2 | 2.8 |
| 20:1n-9 | 17.1 | 13.3 | 9.8 | 6.1 | 2.1 | 9.0 |
| 22:1n-11 | 13.3 | 10.1 | 7.3 | 4.3 | 1.0 | 5.4 |
| 22:1n-9 | 2.0 | 1.6 | 1.3 | 0.9 | 0.5 | 1.1 |
| Total monoenes | 57.1 | 58.4 | 59.6 | 60.4 | 61.1 | 61.2 |
| 18:2n-6 | 3.5 | 7.6 | 11.5 | 15.4 | 19.5 | 7.7 |
| 20:4n-6 | 0.3 | 0.3 | 0.1 | - | - | 0.2 |
| Total n-6 | 4.1 | 8.1 | 11.5 | 15.4 | 19.5 | 7.9 |
| 18:3n-3 | 1.1 | 3.0 | 4.7 | 6.6 | 8.6 | 2.6 |
| 18:4n-3 | 2.8 | 2.2 | 1.6 | 0.9 | 0.2 | 1.5 |
| 20:5n-3 | 5.9 | 4.5 | 3.4 | 2.1 | 0.7 | 3.2 |
| 22:6n-3 | 4.6 | 3.7 | 3.0 | 2.1 | 1.0 | 2.9 |
| Total n-3 | 15.8 | 14.2 | 13.3 | 11.9 | 10.6 | 10.9 |
| Total PUFA | 19.9 | 22.3 | 24.8 | 27.3 | 30.1 | 18.8 |

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

### 5.2.3 *Trial 3 RAFOA Spain – Replacement of FO with LO, RO and OO in European sea bass*

#### 5.2.3.1 Dietary Groups

European sea bass of average weight 90 g were purchased from MARESA in Huelva, Spain and stocked in 5000 L conical tanks at 100 fish per tank (stocking density approximately 2 kg $m^{3\,-1}$) at the facilities of ACUINOVA Andalucia in San Fernando, Cadiz. (Figure 5.2). The experimental diets were prepared by the Nutreco Aquaculture Research Centre, Stavanger, Norway and were fed *ad libitum* to satiation with mechanical belt feeders. Sea bass were fed diets containing various blends of FO and VO including RO, LO and OO. The experimental diets contained approximately 22 % oil. The control FO diet contained 100 % anchovy oil and the added oil combinations for the experimental diets were as follows: RO diet, 40 % FO and 60 % RO; LO diet, 40 % FO and 60 % LO and OO diet, 40 % FO and 60 % OO. Diet formulations and total fatty acid compositions are given in Tables 5.4 and 5.5, respectively. Fish were sampled after 34 weeks feeding the experimental diets.

### 5.2.4 *Collection of samples for immunological studies*

In all three dietary trials, fish were individually sampled from each cage/tank (8 fish dietary $treatment^{-1}$). The immunological studies were designed to examine the effects, if any, of the differing concentrations of VO in the diet on gut, heart and liver pathology, the non-

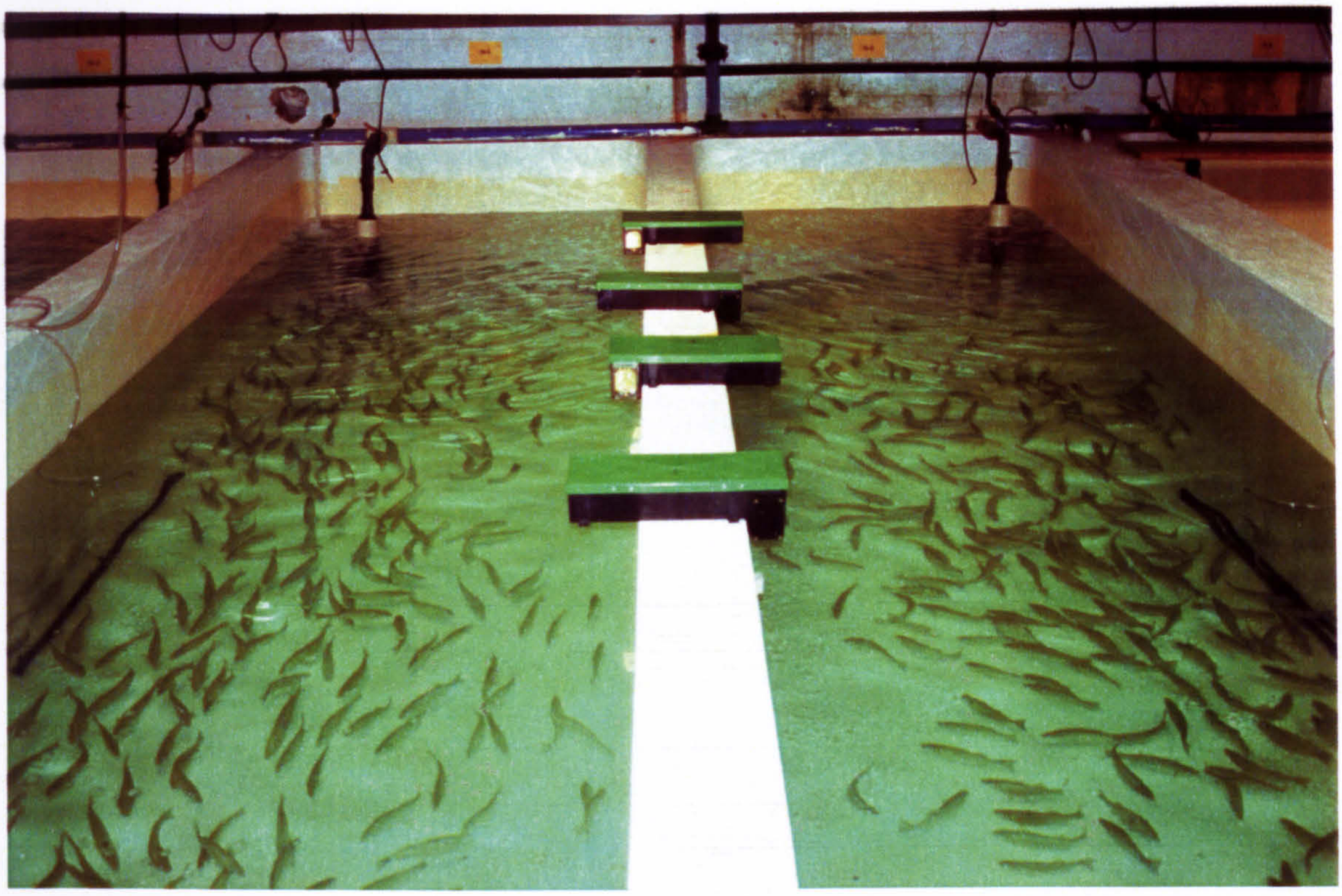


Figure 5.2 Tank facilities of ACUINOVA Andalucia in San Fernando, Cadiz containing the experimental sea bass in the final month prior to sampling.

Table 5.4 Composition of experimental diets (g $kg^{-1}$ feed) for Trial 3.

| Diets | 100 % FO | 60 % RO | 60 % LO | 60 % OO |
|---|---|---|---|---|
| Components | | | | |
| Fish meal [1] | 381.3 | 381.3 | 381.3 | 381.3 |
| Maize gluten [2] | 259.8 | 259.8 | 259.8 | 259.8 |
| Wheat [3] | 157.2 | 157.2 | 157.2 | 157.2 |
| Oil | 176.7 | 176.7 | 176.7 | 176.7 |
| Premixes [4] | 25.0 | 25.0 | 25.0 | 25.0 |
| Composition (%) of added oil | | | | |
| FO (FO) [5] | 100 | 40 | 40 | 40 |
| Rapeseed oil (RO) [6] | 0 | 60 | 0 | 0 |
| Linseed oil (LO) [7] | 0 | 0 | 60 | 0 |
| Olive oil (OO) [8] | 0 | 0 | 0 | 60 |
| Gross composition (dry mass %) | | | | |
| Crude protein | 47.5 | 48.5 | 50.1 | 48.7 |
| Crude lipids | 23.7 | 23.2 | 23.7 | 25.5 |

[1] Scandinavian LT-fish meal (Nordsildmel, Norway).

[2] Cargill, Staley, USA

[3] Statkorn, Oslo, Norway.

[4] Vitamin and mineral premix added min. to NRC recommendations.

[5] Anchovy oil (Denofa, Fredrikstad, Norway) supplemented with 200 ppm BHT.

[6] Crude rapeseed oil (Oelmühle Hamburg, Germany) no antioxidant added.

[7] Crude E.C.C. linseed oil (N.V. Oliefabriek Lictervelde, Belgium) supplemented with 500ppm Ronoxan A (Roche, Basel, Switzerland).

[8] Crude olive oil

FO = fish oil, RO = rapeseed oil, LO = linseed oil, OO = olive oil.

Table 5.5 Total lipid content (% of dry mass) and fatty acid composition (mass % of total fatty acids) of the experimental diets for sea bass. Results are means ± SD (n = 3).

| Dietary treatments | FO | RO | LO | OO |
|---|---|---|---|---|
| Total lipid | 23.7 ± 3.4 | 23.2 ± 2.7 | 23.7 ± 3.5 | 25.5 ± 2.9 |
| Fatty acid | | | | |
| 14:0 | 6.6 ± 0.3 $^{a}$ | 3.8 ± 0.6 $^{b}$ | 3.0 ± 0.5 $^{b}$ | 4.5 ± 1.1 $^{ab}$ |
| 16:0 | 16.2 ± 0.3 $^{a}$ | 10.3 ± 0.1 $^{c}$ | 10.5 ± 0.1 $^{c}$ | 13.1 ± 0.2 $^{b}$ |
| 18:0 | 3.2 ± 0.1 $^{a}$ | 2.4 ± 0.1 $^{c}$ | 3.1 ± 0.0 $^{a}$ | 2.7 ± 0.0 $^{b}$ |
| Total saturates[1] | 27.3 ± 0.2 $^{a}$ | 18.7 ± 0.9 $^{c}$ | 17.6 ± 0.7 $^{c}$ | 21.6 ± 1.1 $^{b}$ |
| 16:1n-7 | 8.2 ± 0.2 $^{a}$ | 6.9 ± 0.2 $^{b}$ | 7.2 ± 0.1 $^{b}$ | 7.4 ± 0.2 $^{b}$ |
| 18:1n-9 | 13.4 ± 0.2 $^{d}$ | 34.4 ± 1.3 $^{b}$ | 15.6 ± 0.1 $^{c}$ | 37.2 ± 0.6 $^{a}$ |
| 20:1n-9 | 3.2 ± 0.1 $^{a}$ | 2.7 ± 0.1 $^{ab}$ | 2.1 ± 0.0 $^{b}$ | 1.9 ± 0.5 $^{b}$ |
| 22:1n-11 | 3.1 ± 0.3 $^{a}$ | 2.1 ± 0.1 $^{bc}$ | 1.8 ± 0.1 $^{c}$ | 2.4 ± 0.1 $^{b}$ |
| Total monoenes[2] | 33.7 ± 0.3 $^{c}$ | 48.9 ± 0.9 $^{b}$ | 29.4 ± 0.3 $^{d}$ | 52.0 ± 0.9 $^{a}$ |
| 18:2n-6 | 5.7 ± 0.2 $^{d}$ | 12.3 ± 0.3 $^{a}$ | 10.3 ± 0.1 $^{b}$ | 9.0 ± 0.2 $^{c}$ |
| 20:4n-6 | 0.8 ± 0.0 $^{a}$ | 0.4 ± 0.0 $^{b}$ | 0.4 ± 0.0 $^{b}$ | 0.4 ± 0.0 $^{b}$ |
| Total n-6 PUFA[3] | 8.4 ± 0.4 $^{d}$ | 13.4 ± 0.2 $^{a}$ | 11.4 ± 0.0 $^{b}$ | 10.2 ± 0.2 $^{c}$ |
| 18:3n-3 | 0.8 ± 0.5 $^{c}$ | 4.4 ± 0.2 $^{b}$ | 25.9 ± 0.4 $^{a}$ | 1.2 ± 0.0 $^{c}$ |
| 18:4n-3 | 1.6 ± 0.4 $^{a}$ | 0.9 ± 0.1 $^{b}$ | 1.0 ± 0.0 $^{b}$ | 0.9 ± 0.0 $^{b}$ |
| 20:5n-3 | 10.9 ± 0.2 $^{a}$ | 5.0 ± 0.3 $^{b}$ | 5.2 ± 0.1 $^{b}$ | 4.7 ± 0.2 $^{b}$ |
| 22:6n-3 | 9.2 ± 0.0 $^{a}$ | 5.0 ± 1.5 $^{b}$ | 4.4 ± 0.1 $^{b}$ | 4.3 ± 0.2 $^{b}$ |
| Total n-3 PUFA[4] | 26.7 ± 1.2 $^{b}$ | 16.9 ± 1.5 $^{c}$ | 38.5 ± 0.5 $^{a}$ | 12.8 ± 0.4 $^{d}$ |
| Total PUFA | 35.1 ± 0.8 $^{b}$ | 30.3 ± 1.4 $^{c}$ | 50.1 ± 0.6 $^{a}$ | 23.1 ± 0.6 $^{d}$ |

Values bearing different superscript letters within a row are significantly different ($P<0.05$). [1]Totals include 15:0, present at up to 0.3 %; [2]Totals include 16:1(n-7), 20:1(n-11), 20:1(n-7) and 24:1, present at up to 0.5 %; [3]Totals include 18:3(n-6), 20:1(n-6) and 20:3(n-6), present at up to 0.2 %; [4]Totals include 20:3(n-3), 20:4(n-3) and 22:5(n-3), present at up to 0.4 %. PUFA. FO = fish oil, RO = rapeseed oil; LO = linseed oil; OO = olive oil.

specific immune capacity of the fish, such as head kidney macrophage activity, total blood counts, haematocrit, lysozyme activity and prostaglandin concentration.

### 5.2.4.1 Haematology and Macrophage function

Heparinised blood was obtained by caudal sinus puncture with 5 ml heparinised vacutainers as outlined in Section 2.3.2 from 8 fish from each cage/tank and stored on ice until required. Haematocrit was measured by microcentrifugation (10,000 x g, 4 min) as described in Section 2.3.2.3 The total number of circulating erythrocytes and leucocytes were determined on diluted blood by counting with an Improved Neubauer haemocytometer following the methods given in Sections 2.3.2.1 and 2.3.2.2. Head kidneys were collected from the same fish as described in Sections 2.3.4. and 2.3.7.2 for use in the NBT reduction assay (Section 2.4.2) and measurement of head kidney macrophage phagocytic capacity (Section 2.4.3). It was not possible to measure macrophage activity in Trial 2 using the NBT reduction assay due to the location of the trial site in Norway, lack of laboratory equipment on site (e.g. cooled incubator) and the allocated time for the sampling schedule. Therefore, the ability of salmon head kidney macrophages to phagocytose yeast cells was measured as an indicator of macrophage function.

### 5.2.4.2 Serum lysozyme activity

From the same fish sampled (8 dietary treatment$^{-1}$), a second aliquot of blood was allowed to clot at 4 °C overnight as described in Section 2.3.3. Serum was then separated by

centrifugation and lysozyme activity was assayed by a modified turbidimetric microtitre plate technique, measuring the lytic activity of the fish serum against *Micrococcus lysodeikticus* according to Section 2.4.4.

### 5.2.4.3 Measurement of $PGE_2$

From the remaining heparininsed blood samples, plasma was collected according to Section 2.3.3 and stored at –20 $^0$C until further analysis. The frozen acidified plasma samples were thawed and centrifuged at 12,000 x g for 2 min to remove any precipitate. The supernatants were extracted using octadecyl silyl ($C_{18}$) 'Sep-Pak' mini-columns by the method of Powell (1982) and as described in detail by Bell *et al.*, (1994) and outlined in Section 2.6. Quantification of $PGE_2$ was performed using EIA kits, according to the manufacturers protocol.

### 5.2.4.4 Fatty acid analysis of peripheral blood leukocytes (PBL)

Samples of PBL from 4 fish dietary treatment$^{-1}$ were isolated from whole blood (Section 2.8.1) by density centrifugation on Histopaque gradients. Total lipid from PBL was extracted with a chloroform:methanol (2:1 v/v) mixture as described by Folch *et al.*, (1957) and in Section 2.8.2. Fatty acid methyl esters were obtained by transmethylation as described by Christie (1982) and identified using gas chromatography.

### 5.2.4.5 Histopathology

Samples of proximal, mid and distal intestine were collected from 8 fish from each

dietary group together with heart and liver for histopathological examination to identify any effects of oil type on fish histology. Processed sections were prepared according to Sections 2.3.5, 2.7.1 and 2.7.2 and random slides assessed "blind" externally by a fish veterinarian of the Fish Vet. Group, Inverness according to Section 2.7.3. to eliminate any bias in interpretation.

### 5.2.5 *Statistical analysis*

All data are presented as means ± SEM (n = 8) unless stated otherwise. Significant differences between dietary treatments were determined by ANOVA using $p<0.05$ as a cut off for significance. Differences between means were determined by Newman-Keuls post-test.

## 5.3 Results

### 5.3.1 *Trial 1 RAFOA Scotland – Replacement of FO with LO and OO in Atlantic salmon*

#### 5.3.1.1 Growth, tissue lipid content and flesh fatty acid compositions

According to Dr Gordon Bell and colleagues (personal communication), no significant differences in growth were observed with any of the dietary treatments used. They found no differences in the muscle lipid content of salmon fed the LO-containing diets compared to those fed the FO diet. The liver lipid content of salmon fed the 100 % LO feed was significantly higher than all other treatments and the value for salmon fed the 75 % LO

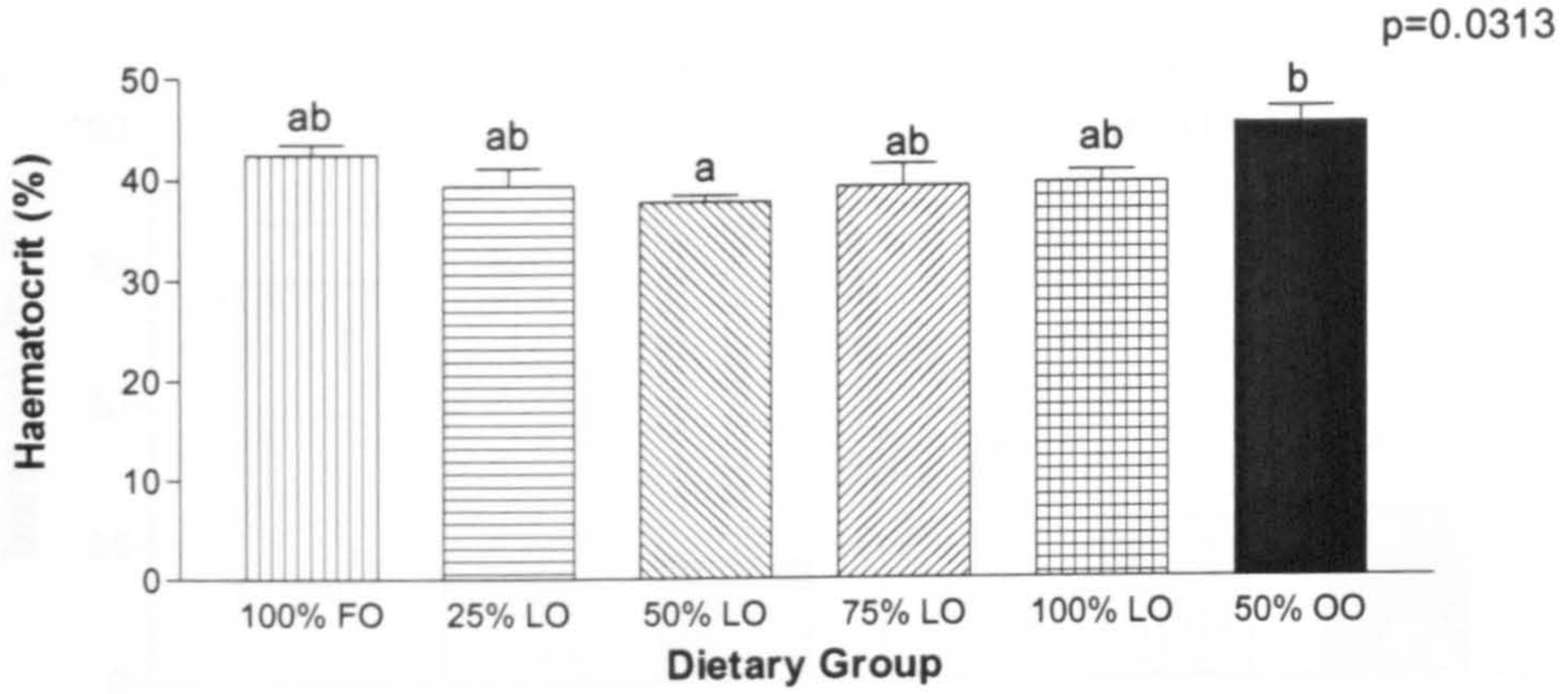
feed was higher than in fish fed the FO, 25 % LO and 50 % OO feeds. Flesh DHA and EPA levels were reduced as the level of VO in the feed increased, such that values in salmon fed 100 % VO were around 30 - 40 % of values in fish fed the 100 % FO feed.

### 5.3.1.2 Haematology and Macrophage function

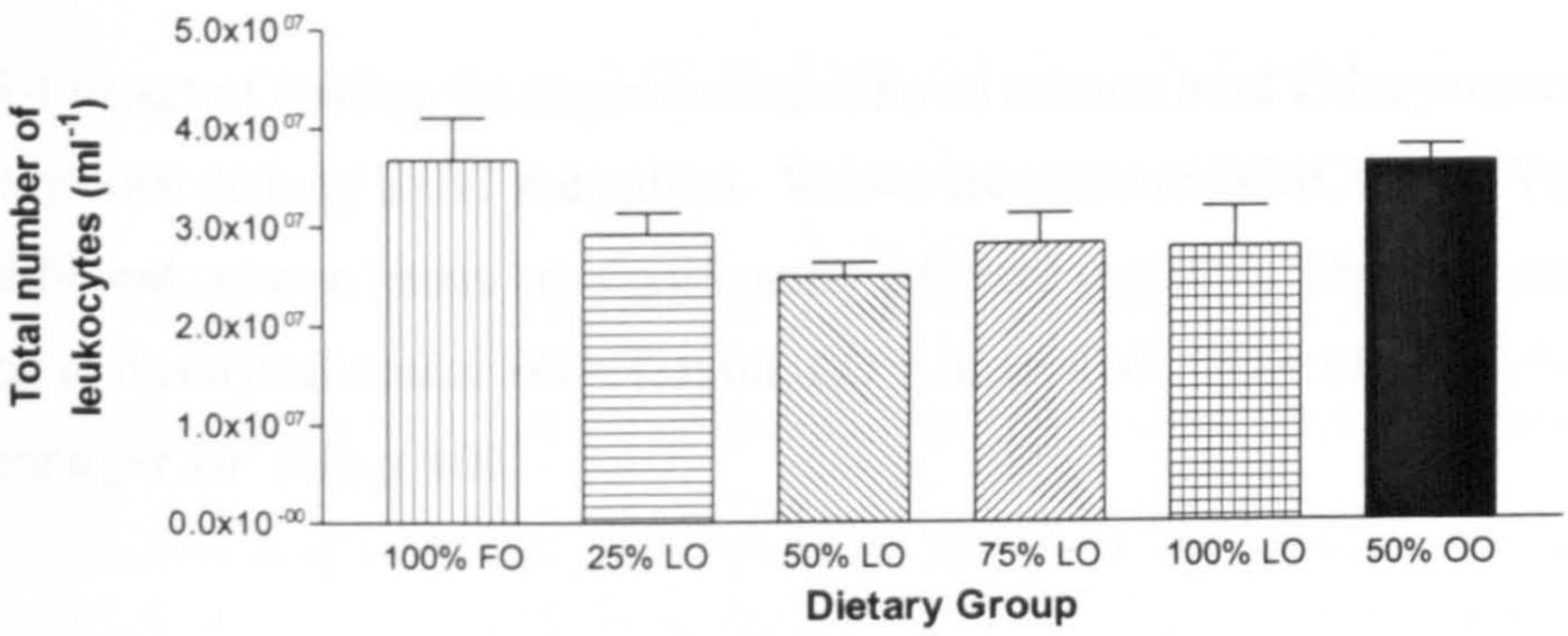
Haematocrits were measured from the same blood samples as used to determine total blood counts. Statistical differences were found when comparing the 50 % LO treatment with the 50 % OO group. The 50 % LO dietary group had significantly lower ($p< 0.05$) (37.6 %) haematocrit when compared to the 50 % OO group (45.2 %). Figure 5.3a shows the effect of feeding VO on haematocrit value. Although all the LO groups showed reduced leukocyte numbers when compared to the FO control diet no statistical differences were found (Figure 5.3b). The OO group had approximately the same number of total leukocytes when compared with the FO control group. No significant differences were found between the dietary groups when measuring the total number of erythrocytes (Figure 5.3c).

Overall the VO containing diets reduced the macrophage activity of the fish as measured by the reduction of NBT compared to that of fish fed the FO diet (Figure 5.4). The fish fed the 50 % LO diet (21.9 Absorbance per $10^5$ cells x 100) had significantly lower macrophage activity than the 100 % FO diet (72.4, Absorbance per $10^5$ cells x 100) ($p<0.01$) and the 25 % LO diet (59.5, Absorbance per $10^5$ cells x 100) ($p<0.05$). In addition, the 100 % LO group (29.3, Absorbance per $10^5$ cells x 100) also showed a lower average macrophage activity than the control (100 % FO) ($p<0.01$). The 50 % OO fed fish

(a)




(b)




(c)

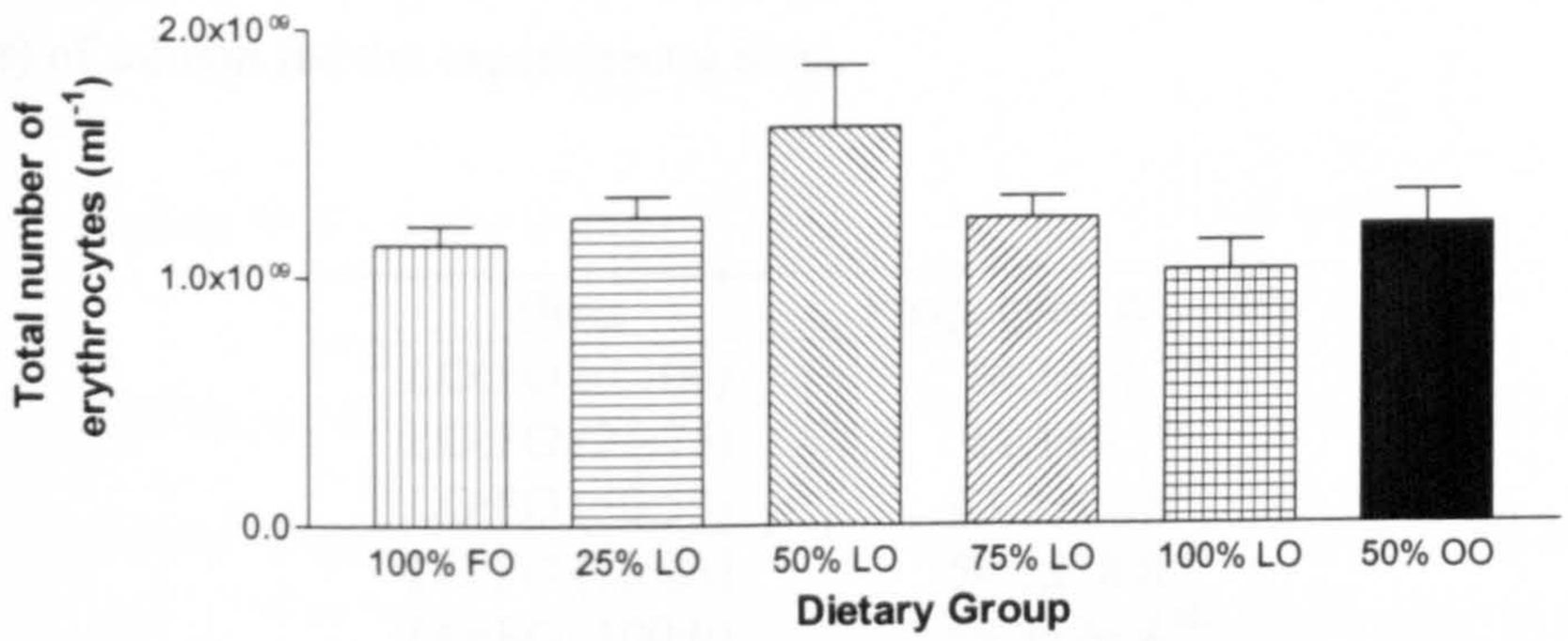



Figure 5.3 Effect of feeding the experimental diets to salmon on (a) % haematocrit (b) the total number of circulating leukocytes (c) the total number of circulating erythrocytes. Values are means ± SEM, n = 8. Values having different column letters are significantly different ($p<0.05$). Diets are coded according to dietary oil source FO = fish oil, LO = linseed oil, OO = olive oil.

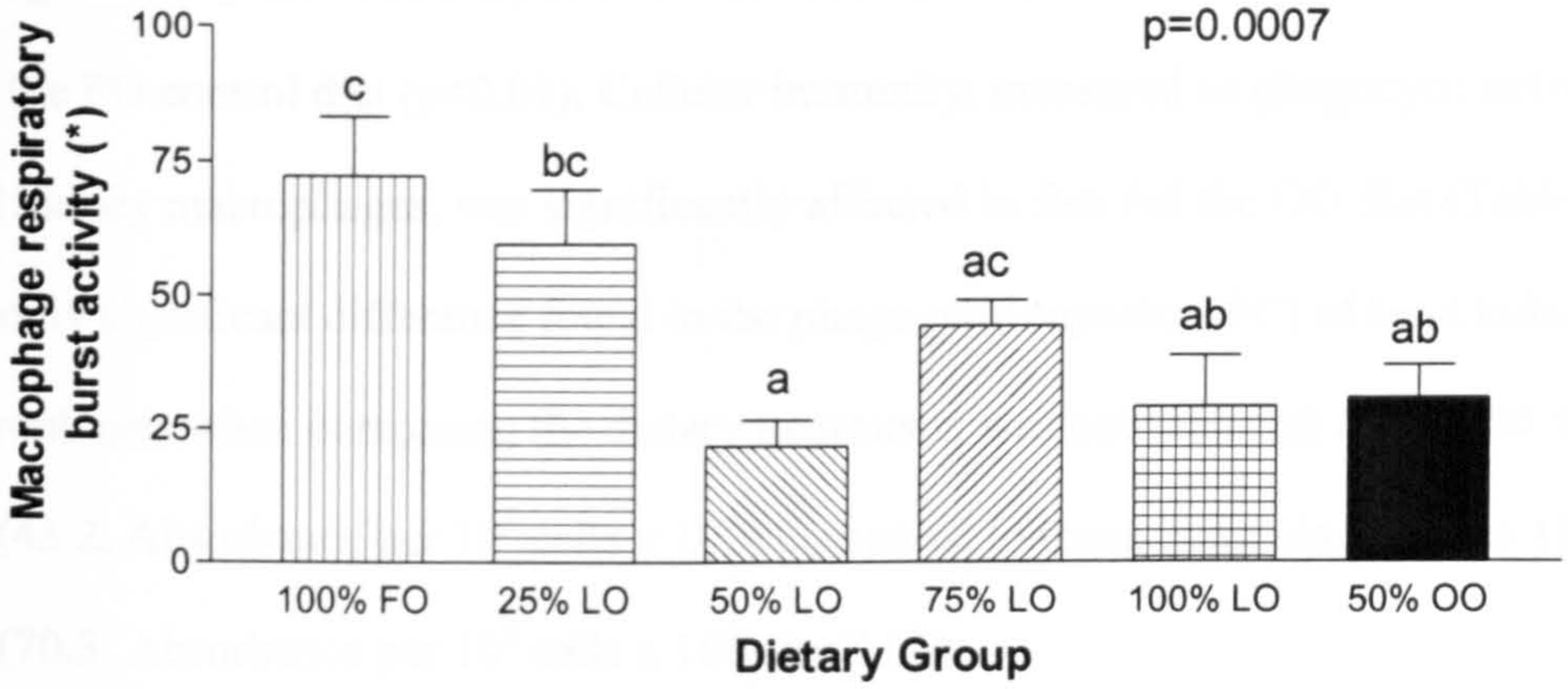



Figure 5.4 Effect of feeding the experimental diets on salmon head kidney macrophage respiratory burst activity (NBT reduction). Values are means ± SEM, n = 8. Values having different column letters are significantly different ($p<0.05$). Diets are coded according to dietary oil source FO = fish oil, LO = linseed oil, OO = olive oil. * = Absorbance per $10^5$ cells x 100.

Table 5.6 Phagocytic capacity (% of macrophages which have phagocytosed yeast particles) of salmon fed the experimental diets.

| Diets | Phagocytic capacity |
|---|---|
| LO:FO (0:100) | $70.3\pm14.2^{a}$ |
| LO:FO (25:75) | $61.1\pm9.7^{ab}$ |
| LO:FO (50:50) | $63.4\pm13.6^{ab}$ |
| LO:FO (75:25) | $54.1\pm18.8^{ab}$ |
| LO:FO (100:0) | $55.8\pm24.4^{ab}$ |
| OO:FO (50:50) | $43.2\pm10.9^{b}$ |

Values are means ± SEM, n = 8. Values in the same column with different superscript letters are significantly different ($p<0.05$). Diets are coded according to dietary oil source FO = fish oil, LO = linseed oil, OO = olive oil.

had significantly lower activity of NBT reduction (30.7, Absorbance per $10^5$ cells x 100) than the FO control diet ($p<0.01$). Cellular immunity, measured as phagocytic activity of head kidney macrophages, was significantly affected in fish fed the OO diet (Table 5.6). The only significant difference found in the phagocytic capacity (PC) of head kidney macrophages when comparing the dietary treatments was between fish fed the 50 % OO diet (43.2, Absorbance per $10^5$ cells x 100) compared to those maintained on the 100 % FO diet (70.3, Absorbance per $10^5$ cells x 100) ($p<0.05$).

#### 5.3.1.3 Serum lysozyme activity

Regarding the humoral immunology of the experimental fish, diets containing 100 % LO showed significantly lower serum lysozyme activity (146.3 U $ml^{-1}$ $min^{-1}$) compared to fish fed the 50 % LO diet (605.0 U $ml^{-1}$ $min^{-1}$) and the 75 % LO diet (560.0 U $ml^{-1}$ $min^{-1}$). No other significant differences were found when measuring the serum lysozyme of fish fed the experimental diets (Figure 5.5a)

#### 5.3.1.4 Plasma $PGE_2$ concentration

There were significant dietary-induced changes in levels of the eicosanoid $PGE_2$ in circulating plasma (Figure 5.5b). $PGE_2$ was significantly decreased in fish given the 25 % (277.8 pg $ml^{-1}$), 50 % (285.0 pg $ml^{-1}$), and 100 % LO (235.0 pg $ml^{-1}$) diets compared to those fish given either the FO or OO diets.

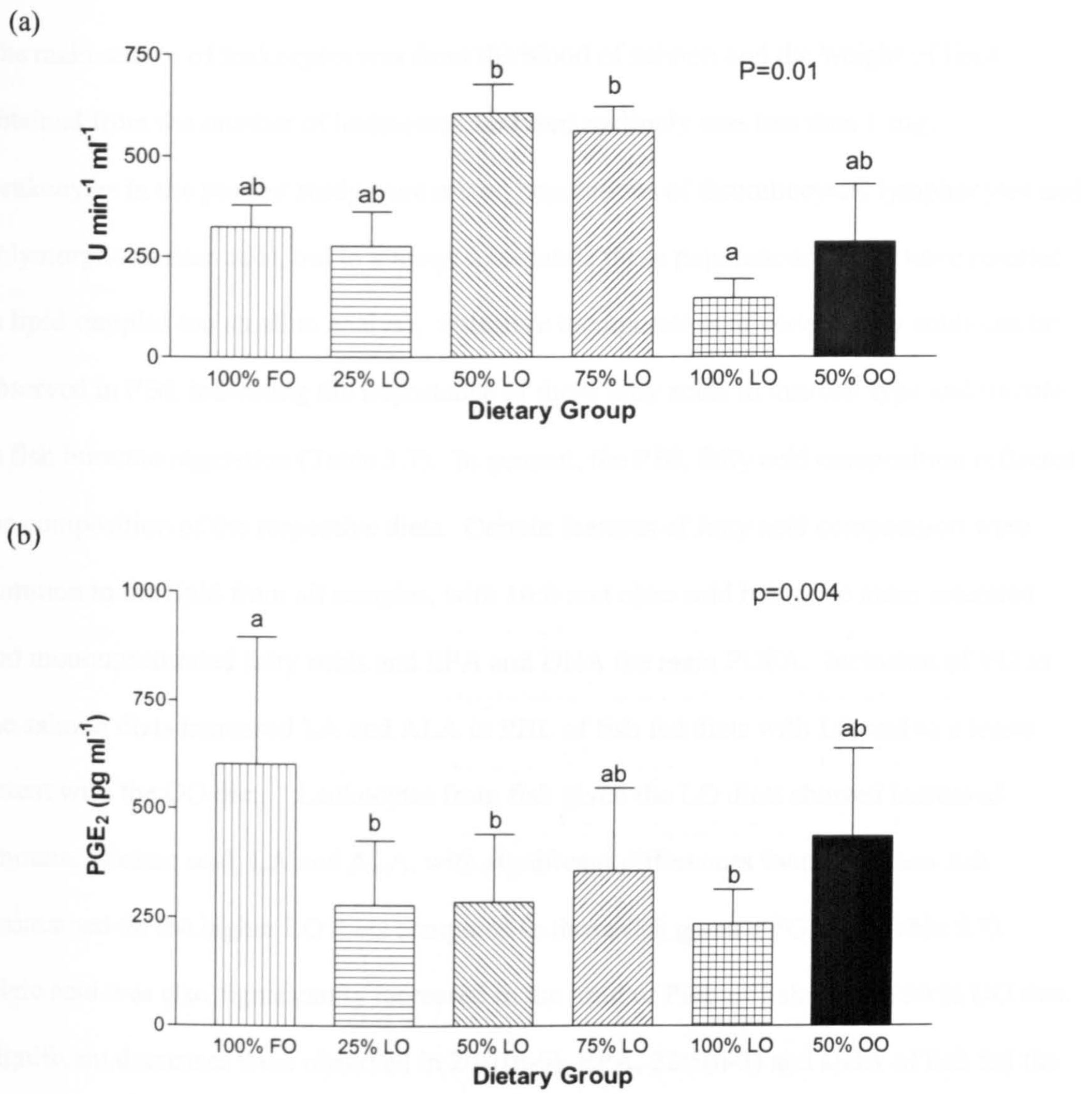



Figure 5.5 Effect of feeding the experimental diets to salmon on (a) serum lysozyme activity (b) plasma $PGE_2$ levels. Values are means ± SEM, n = 8. Values having different column letters are significantly different ($p<0.05$). Diets are coded according to dietary oil source FO = fish oil, LO = linseed oil, OO = olive oil.

### 5.3.1.5 Fatty acid composition of peripheral blood leukocytes

The main source of leukocytes was from the blood of salmon and the weight of lipid obtained from the number of leukocytes analysed routinely was less than 1 mg. Leukocytes in the present study were mixed populations of thrombocytes, lymphocytes and polymorphonuclear cells, but to attempt to separate these populations would have resulted in lipid samples too small to analyse. Selective incorporation of certain fatty acids can be observed in PBL indicating the importance of these fatty acids in this cell type and its role in fish immune regulation (Table 5.7). In general, the PBL fatty acid composition reflected the composition of the respective diets. Certain features of fatty acid composition were common to the lipid from all samples, with 16:0 and oleic acid being the main saturated and monounsaturated fatty acids and EPA and DHA the main PUFA. Inclusion of VO in the salmon diets increased LA and ALA in PBL of fish fed diets with LO and to a lesser extent with the OO diet. Leukocytes from fish given the LO diets showed increased amounts of oleic acid, LA and ALA, with significant differences found between fish maintained on the higher LO diets compared to those fish given a FO diet (Table 5.7). Oleic acid was also significantly increased in the lipid of PBL of fish fed the 50 % OO diet. Significant decreases were observed in 20:1(n-9), EPA, 22:5(n-3) and DHA of fish fed the VO diets compared to those fed the FO diet. Feeding LO at 75 % and 100 % inclusion significantly reduced the total amount of monoenes and increased significantly the total amount of n-6 fatty acids.

Table 5.7 Fatty acid composition (wt %) of Atlantic salmon peripheral blood leukocytes. Values are expressed as the mean % of total fatty acids for four fish ± SD.

| Fatty Acids | LO:FO (0:100) | LO:FO (25:75) | LO:FO (50:50) | LO:FO (75:50) | LO:FO (100:0) | OO:FO (50:50) |
|---|---|---|---|---|---|---|
| 14:0 | 2.2±0.2 $^{a}$ | 1.2±0.1 $^{bc}$ | 2.2±0.4 $^{a}$ | 1.9±0.6 $^{ac}$ | 1.3±0.2 $^{c}$ | 2.3±0.7 $^{a}$ |
| 15:0 | 0.5±0.3 $^{ab}$ | 0.3±0.0 $^{a}$ | 0.8±0.3 $^{ab}$ | 1.0±0.4 $^{b}$ | 0.6±0.3 $^{ab}$ | 0.6±0.2 $^{ab}$ |
| 16:0 | 18.5±2.5 $^{ab}$ | 15.7±0.9 $^{b}$ | 19.4±2.0 $^{ab}$ | 22.2±3.5 $^{a}$ | 20.7±1.8 $^{a}$ | 23.1±2.5 $^{a}$ |
| 18:0 | 3.3±0.7 $^{a}$ | 3.4±0.5 $^{a}$ | 5.0±0.7 $^{a}$ | 7.4±1.3 $^{b}$ | 6.8±1.5 $^{b}$ | 6.3±1.8 $^{b}$ |
| 20:0 | 0.2±0.1 $^{ac}$ | 0.1±0.0 $^{a}$ | 0.2±0.1 $^{ab}$ | 0.4±0.2 $^{b}$ | 0.2±0.0 $^{ab}$ | 0.4±0.1 $^{bc}$ |
| 22:0 | 0.2±0.1 $^{a}$ | 0.4±0.3 $^{a}$ | 0.5±0.1 $^{a}$ | 0.9±0.4 $^{b}$ | 0.3±0.0 $^{a}$ | 0.5±0.1 $^{a}$ |
| Total saturates | 24.9±3.7 $^{ac}$ | 28.0±3.1 $^{ab}$ | 20.9±1.4 $^{c}$ | 33.8±4.6 $^{b}$ | 29.9±3.2 $^{ab}$ | 33.2±5.1 $^{b}$ |
| 16:1 n-7 | 2.2±0.5 $^{a}$ | 1.8±0.3 $^{a}$ | 1.7±0.3 $^{a}$ | 1.0±0.6 $^{bc}$ | 0.8±0.1 $^{b}$ | 1.5±0.1 $^{ac}$ |
| 18:1 n-9 | 10.4±1.1 $^{a}$ | 11.6±1.0 $^{a}$ | 10.8±0.5 $^{a}$ | 12.9±0.7 $^{b}$ | 12.5±0.5 $^{b}$ | 15.1±1.4 $^{c}$ |
| 18:1 n-7 | 3.1±0.2 $^{a}$ | 2.4±0.2 $^{b}$ | 3.1±0.2 $^{a}$ | 1.9±0.3 $^{bc}$ | 1.7±0.2 $^{c}$ | 2.3±0.4 $^{b}$ |
| 20:1 n-9 | 5.5±1.1 $^{a}$ | 3.6±0.2 $^{bd}$ | 3.6±0.6 $^{bd}$ | 0.9±1.2 $^{c}$ | 1.1±0.1 $^{c}$ | 3.5±0.5 $^{d}$ |
| 20:1 n-7 | 0.4±0.1 $^{a}$ | 0.3±0.1 $^{bc}$ | 0.3±0.1 $^{ac}$ | 0.1±0.1 $^{b}$ | 0.1±0.0 $^{b}$ | 0.4±0.0 $^{ac}$ |
| 22:1 n-11 | 2.0±0.7 $^{a}$ | 1.6±0.1 $^{ac}$ | 1.1±0.7 $^{bc}$ | 0.4±0.0 $^{b}$ | 0.4±0.0 $^{b}$ | 1.2±0.2 $^{bc}$ |
| 22:1 n-9 | 0.6±0.2 | 0.7±0.4 | 0.7±0.7 | 0.4±0.5 | 0.2±0.0 | 0.6±0.3 |
| 24:1 n-9 | 1.3±0.4 | 1.4±0.3 | 1.2±0.2 | 0.9±0.3 | 0.8±0.2 | 1.2±0.5 |
| Total monenes | 25.6±3.7 $^{ac}$ | 22.5±1.4 $^{a}$ | 23.0±1.0 $^{a}$ | 18.6±1.4 $^{c}$ | 17.4±0.5 $^{b}$ | 25.8±2.8 $^{a}$ |
| 18:2 n-6 | 2.7±0.9 $^{a}$ | 3.3±2.1 $^{ac}$ | 3.9±0.4 $^{ac}$ | 5.2±0.9 $^{bc}$ | 6.5±0.6 $^{b}$ | 3.8±0.7 $^{ac}$ |
| 18:3 n-6 | 0.1±0.0 $^{a}$ | 0.1±0.0 $^{a}$ | 0.2±0.1 $^{a}$ | 0.1±0.0 $^{a}$ | 0.2±0.1 $^{a}$ | 0.5±0.5 $^{b}$ |
| 20:4 n-6 | 0.9±0.1 | 0.8±0.2 | 1.0±0.3 | 0.6±0.0 | 0.6±0.2 | 0.8±0.1 |
| 20:2 n-6 | 0.5±0.1 $^{a}$ | 0.9±0.1 $^{b}$ | 0.6±0.0 $^{a}$ | 0.6±0.2 $^{a}$ | 1.0±0.1 $^{b}$ | 0.5±0.1 $^{a}$ |
| 20:3 n-6 | 0.2±0.1 $^{a}$ | 0.4±0.0 $^{ac}$ | 0.2±0.1 $^{a}$ | 0.4±0.1 $^{bc}$ | 0.6±0.1 $^{b}$ | 0.4±0.1 $^{bc}$ |
| 22:4 n-6 | 0.1±0.0 $^{ab}$ | 0.0±0.0 $^{a}$ | 0.1±0.0 $^{ab}$ | 0.2±0.2 $^{b}$ | 0.1±0.0 $^{ab}$ | 0.1±0.0 $^{ab}$ |
| 22:5 n-6 | 0.2±0.0 | 0.2±0.0 | 0.1±0.0 | 0.1±0.1 | 0.1±0.1 | 0.2±0.0 |
| Total n-6 | 40.7±1.5 $^{a}$ | 34.9±4.3 $^{ac}$ | 48.3±3.2 $^{b}$ | 33.0±6.7 $^{a}$ | 31.9±2.7 $^{a}$ | 29.4±7.0 $^{c}$ |
| 16:4 n-3 | 0.2±0.1 $^{ab}$ | 0.3±0.1 $^{ab}$ | 0.3±0.3 $^{ab}$ | 0.1±0.0 $^{a}$ | 0.2±0.2 $^{ab}$ | 0.5±0.2 $^{b}$ |
| 18:3 n-3 | 0.3±0.1 $^{a}$ | 6.1±1.2 $^{b}$ | 2.4±0.2 $^{c}$ | 5.0±1.3 $^{b}$ | 8.8±1.3 $^{d}$ | 0.3±0.1 $^{a}$ |
| 20:4 n-3 | 0.8±0.0 $^{ae}$ | 1.4±0.1 $^{bd}$ | 0.8±0.1 $^{ae}$ | 1.3±0.2 $^{ad}$ | 2.4±0.6 $^{c}$ | 0.5±0.2 $^{e}$ |
| 20:5 n-3 | 10.9±1.5 $^{b}$ | 10.7±1.5 $^{b}$ | 7.5±1.8 $^{a}$ | 5.8±1.6 $^{a}$ | 6.9±0.7 $^{a}$ | 7.0±2.9 $^{a}$ |
| 22:5 n-3 | 2.5±0.4 $^{a}$ | 2.5±05 $^{a}$ | 1.6±0.5 $^{ac}$ | 1.2±0.1 $^{bc}$ | 1.4±0.1 $^{bc}$ | 1.7±0.8 $^{ac}$ |
| 22:6 n-3 | 21.7±1.4 $^{a}$ | 20.1±0.8 $^{ac}$ | 15.9±2.2 $^{ac}$ | 11.0±3.1 $^{b}$ | 13.0±1.2 $^{bc}$ | 13.7±3.3 $^{bc}$ |
| Total n-3 | 36.1±1.6 $^{a}$ | 28.9±4.0 $^{b}$ | 42.7±1.7 $^{c}$ | 25.8±6.1 $^{b}$ | 23.0±2.5 $^{b}$ | 23.4±7.3 $^{b}$ |
| Total PUFA | 76.8±3.0 $^{a}$ | 63.8±8.3 $^{ad}$ | 91.1±4.6 $^{b}$ | 58.8±12.7 $^{cd}$ | 54.9±5.2 $^{cd}$ | 52.8±14.3 $^{cd}$ |
| (n-3)/(n-6) | 0.9±0.0 $^{a}$ | 0.8±0.0 $^{ae}$ | 0.9±0.0 $^{a}$ | 0.8±0.0 $^{be}$ | 0.7±0.0 $^{c}$ | 0.8±0.1 $^{de}$ |

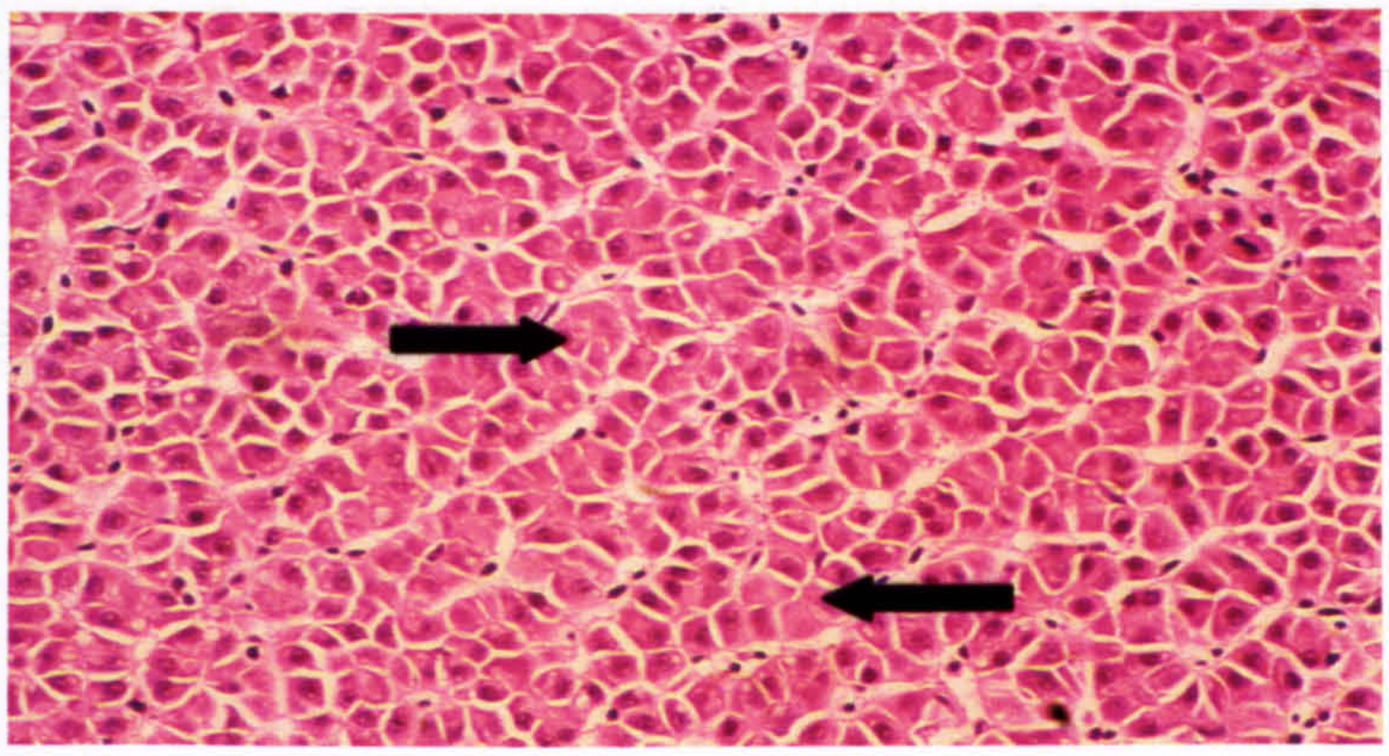
Values are means ± SD, n = 4. Values in the same row with different superscript letters are significantly different ($p<0.05$). Diets are coded according to dietary oil source FO = fish oil, LO = linseed oil, OO = olive oil.

### 5.3.1.6 Histopathology

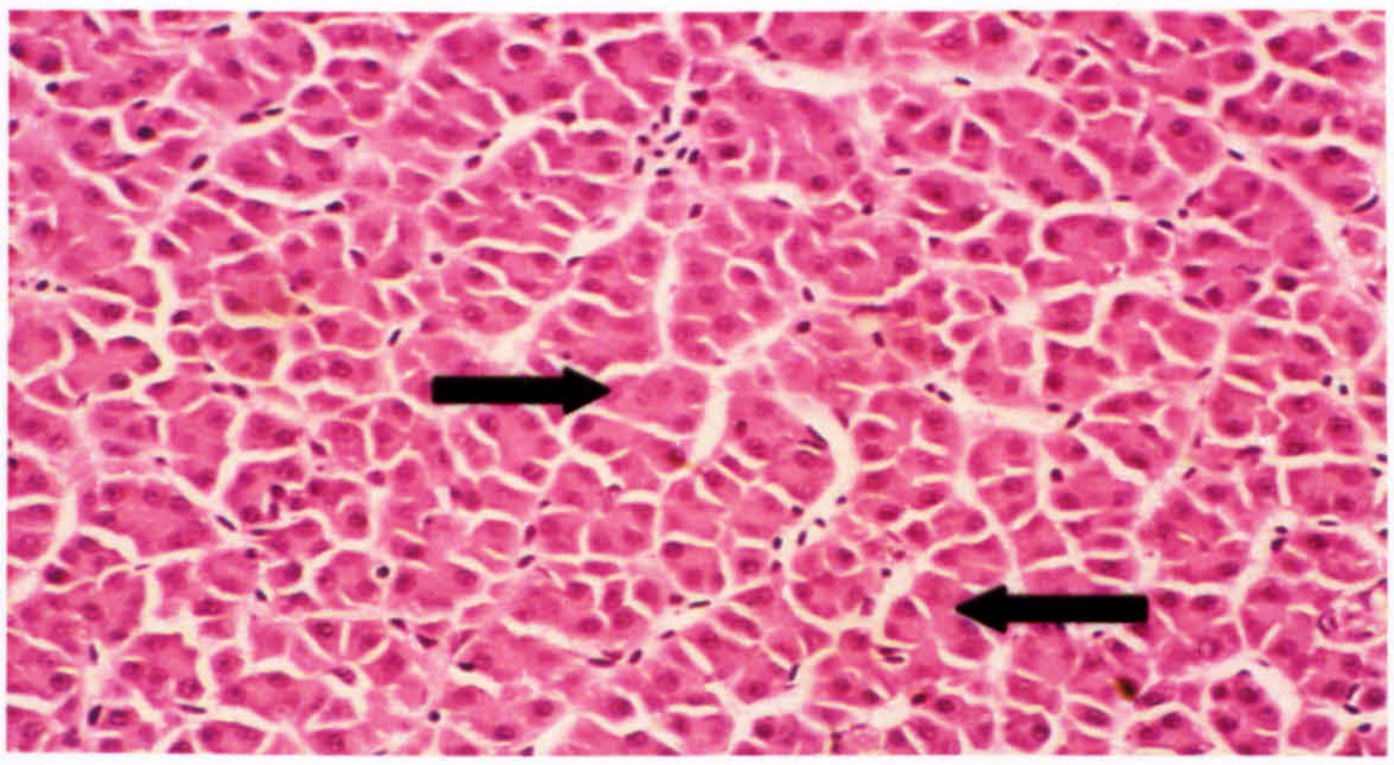
Fat levels within the livers tended to be fairly normal with moderate amounts in fish fed the 25 % LO, 75 % LO, 100 % LO and 50 % OO diets. Levels were quite low in the 100 % FO fed fish as shown in Figure 5.6a and in fish maintained on the 50 % LO diet (Figure 5.6b). Fat was fairly evenly distributed throughout the livers and mostly in small single vacuoles particularly in the 100 % LO fed fish, which showed some variability of fat distribution within the organ. This was not considered to be particularly abnormal, although the higher levels of fat tended to cause some loss in the regular structure of the liver with a variable hepatocyte size. The liver of some fish fed the 100 % LO diet were typical of this, with some extremely swollen hepatocytes evident and also some much smaller cells (Figure 5.6c). Peri vascular cuffing was not seen in the majority of the fish examined.

The proximal intestine in the majority of fish appeared normal with moderate mucus cells, few absorptive vacuoles and no significant inflammation and sloughing of the mucosal membrane. Mid intestinal sections were more variable with fish fed the 25 %, 50 % and 100 % LO diets showing some signs of minor cellular infiltration. A section of intestine from one fish fed the 100 % LO diet, showing some cellular infiltration in the mid gut section is illustrated in Figure 5.7a. The distal intestines were more variable with fish fed the 25 % and 50 % LO diets having less prominent absorptive vacuoles tending to suggest a 'less active' gut (Figure 5.7b) and combined with lower levels of fat in the livers of these fish would suggest that these fish were performing less well than the others. Cellular infiltration in the distal intestine also appeared to be more prominent in fish fed the 25 %

(a)



(b)



(c)

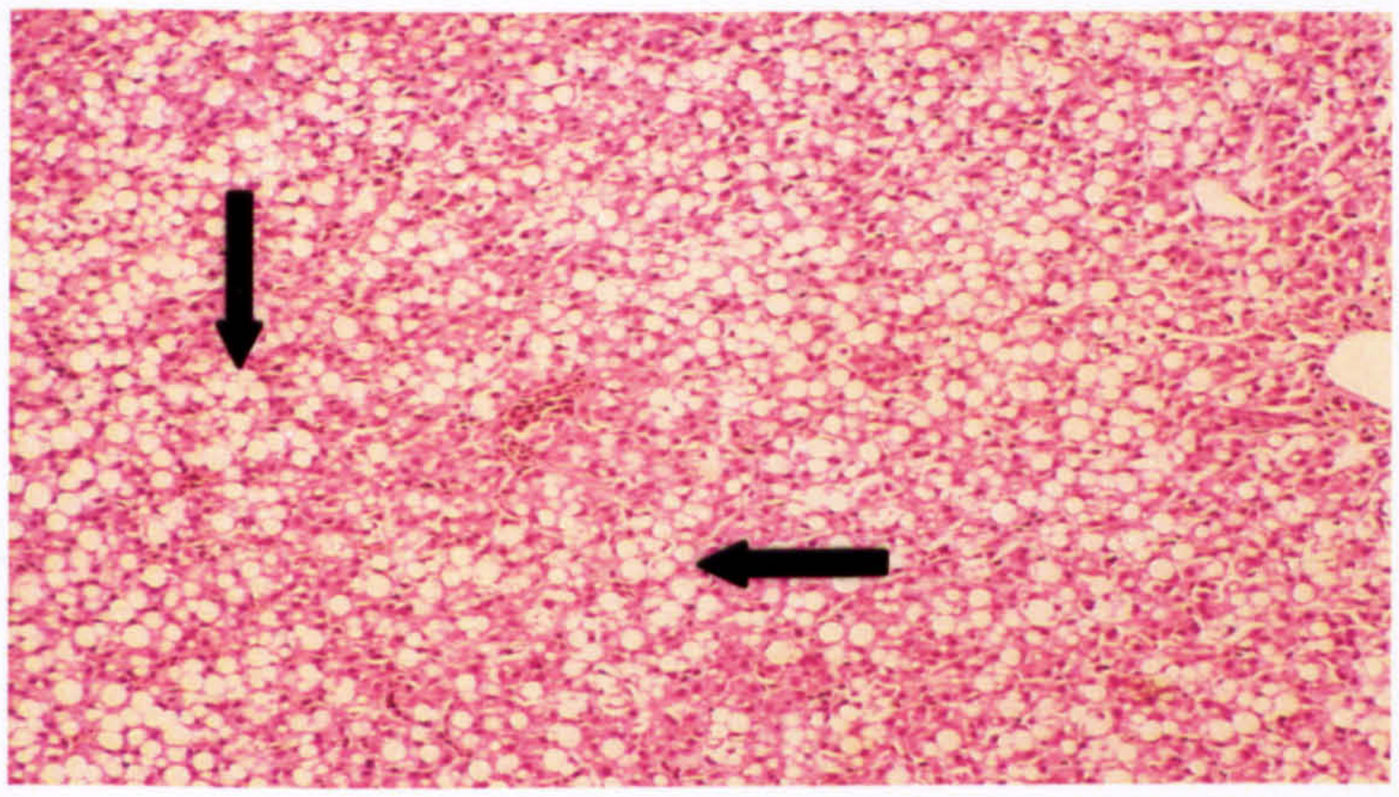


Figure 5.6 Histopathology of Atlantic salmon fed a (a) 100 % FO diet. Liver showing a low fat content with small single vacuoles within the hepatocytes (mag. x 430) (b) 50% LO diet. Liver showing low levels of fat within the hepatocytes (mag. x 430) (c) 100% LO diet. Liver showing some variability in fat distribution resulting in swollen hepatocytes (mag. x 175).

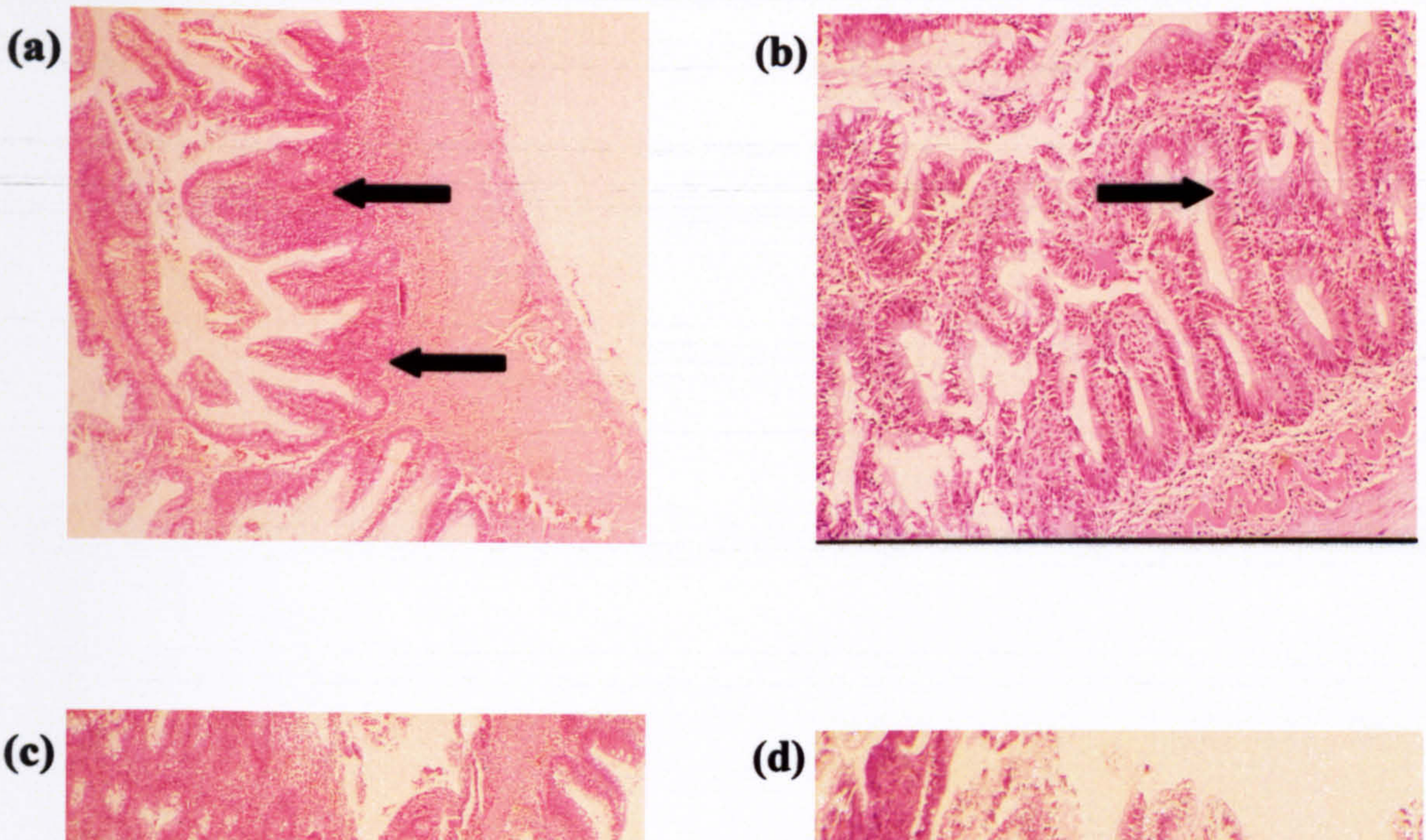



Figure 5.7 Histopathology of Atlantic salmon fed a (a) 100 % LO diet. Mid intestine showing signs of cellular infiltration (mag. x 70) (b) 25 % LO diet. Distal intestine showing low numbers of absorptive vacuoles suggesting a "less active" gut (mag. x 175) (c) 25 % LO diet. Distal intestine showing signs of cellular infiltration (mag. x 175) (d) 50 % OO diet. Distal intestine showing a small amount of cellular infiltration (mag. x 70).

LO (Figure 5.7c), 50 % LO and 50 % OO (Figure 5.7d) diets. With regard to the histology of the heart sections sampled from the dietary groups, the only minor pathological change was small patches of endocarditis from fish fed the OO diet (Figure 5.8). These changes were so minimal that it is doubtful if they were of any clinical significance to the overall health of the fish.

### 5.3.2 *Trial 2 RAFOA Norway – Replacement of FO with RO and OO in Atlantic salmon*

#### 5.3.2.1 Growth, tissue lipid content and flesh fatty acid compositions

Torstensen *et al.*, (2004) found no major differences in growth between dietary groups, but did find a trend of decreased growth in the fish fed the 100 % RO diet. However, after the fish were returned to a 100 % FO diet for a final 6 month wash-out period, fish previously fed the 100 % RO had the highest mean fish weight of all the experimental groups. The authors reported that in fish fed the 100 % RO diet the total lipid content of white muscle was decreased compared to all other dietary groups after 22 weeks feeding. When sampled after 42 weeks, no differences in muscle lipid content were found between the dietary groups. Flesh oleic acid was shown to have increased 3-fold from the 100 % FO to 75 % RO fed fish, and the increase was 5-fold from 100 % FO to 100 % RO.

#### 5.3.2.2 Haematology and Macrophage function

Haematocrits were measured from the same blood samples used for total blood counts.

The group fed the 100 % RO diet had significantly lower haematocrit percentages

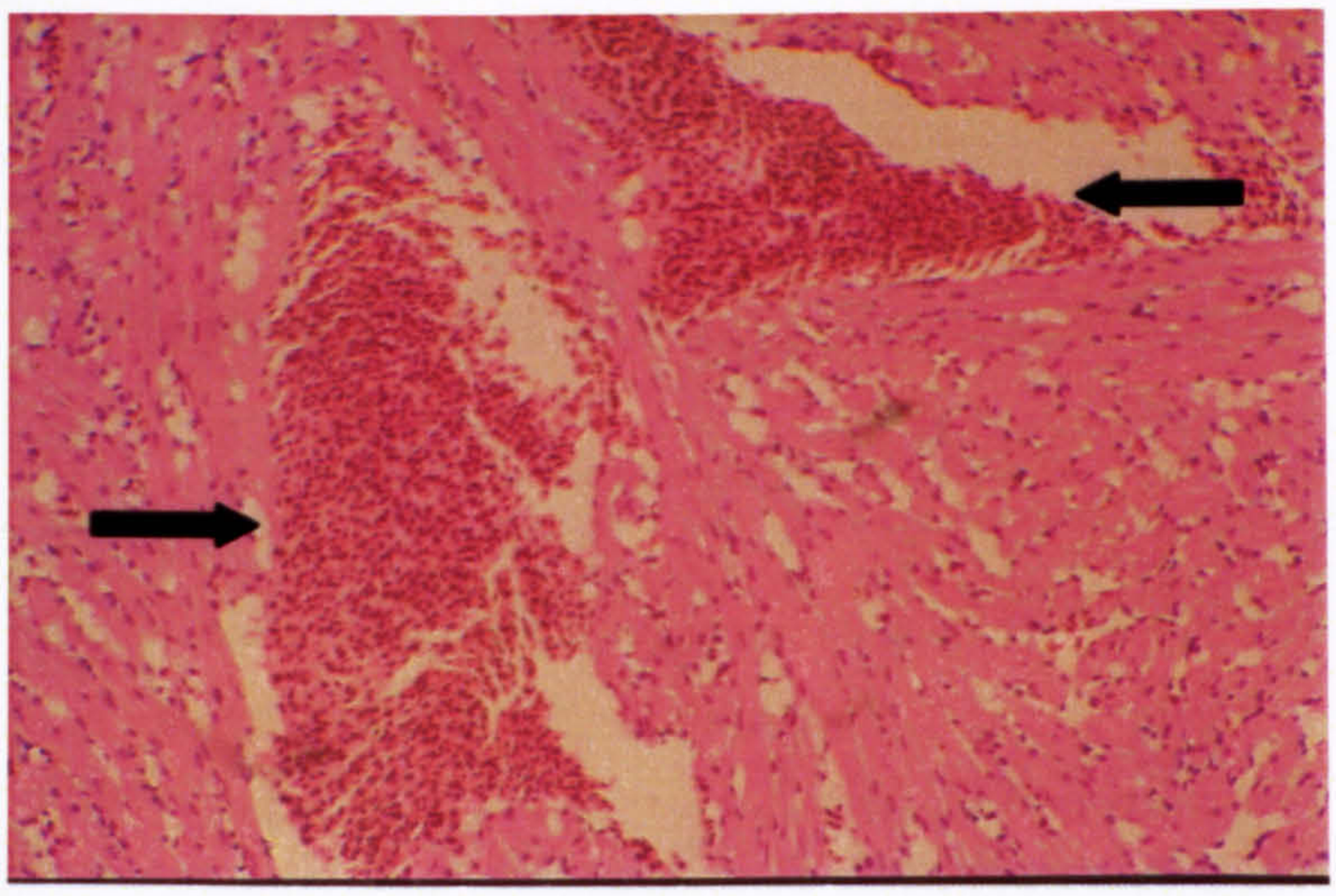


Figure 5.8 Histopathology of Atlantic salmon fed a 50 % OO diet. Heart showing minor signs of endocarditis (mag. x 175).

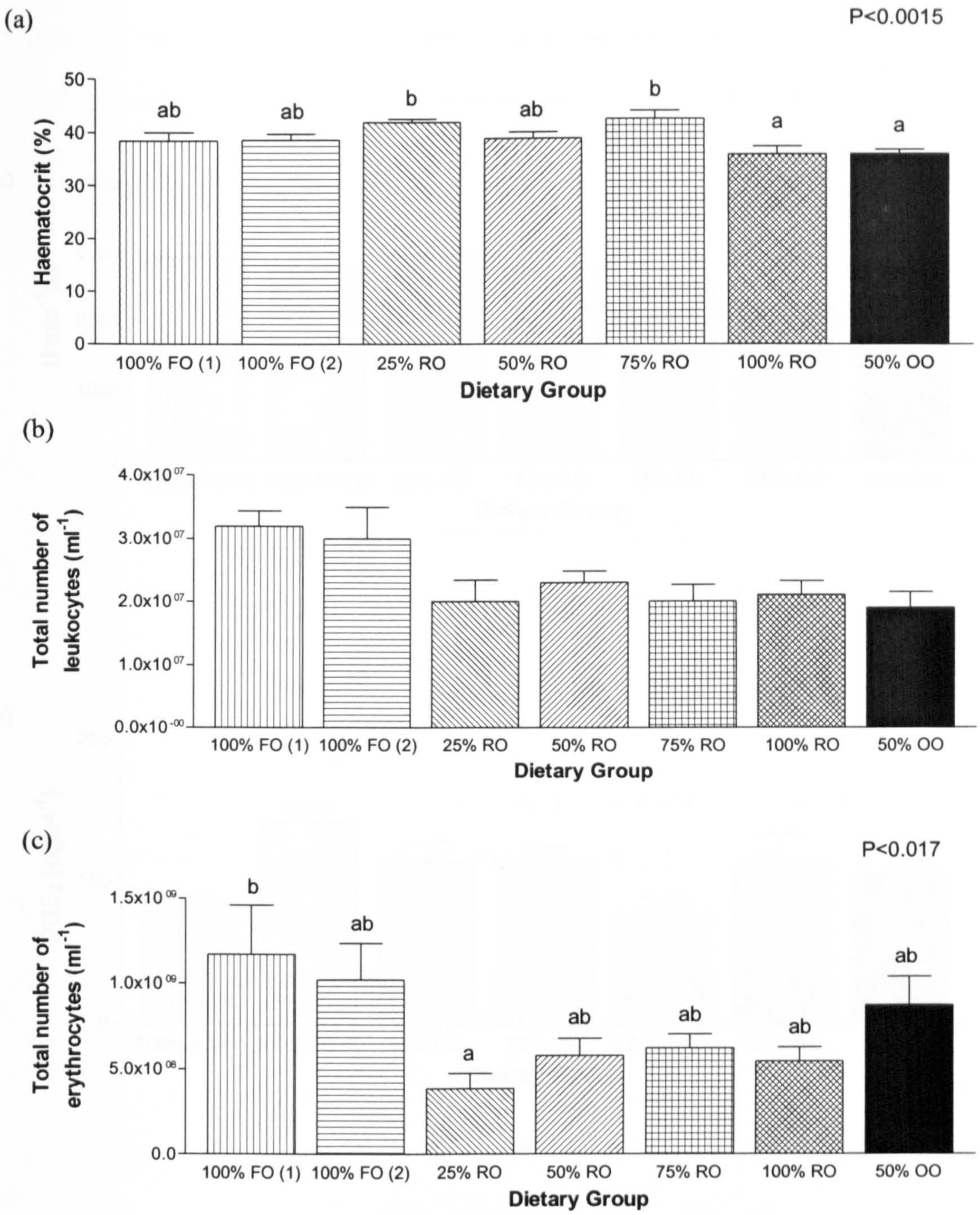
(p=0.0015) than fish given both the 25 % and 75 % RO diets (Figure 5.9a). Statistical differences were also found when comparing the 50 % OO diet with the 25 % and 75 % RO groups (Figure 5.9a). Although all the VO groups showed reduced leukocyte numbers when compared to the FO (control) diets there were no statistical differences found (Figure 5.9b). Erythrocyte counts from all fish fed the VO diets also showed a reduction in number when compared to the FO diets (Figure 5.9c). There was a statistical difference (p=0.017) found when comparing the 25 % RO group with one of the FO control groups (control 1). The phagocytic capacity of head kidney macrophages from fish fed the experimental diets are shown in Table 5.8. The phagocytic ability of salmon macrophages was significantly affected by feeding both RO and OO diets compared to fish fed the FO diet.

#### 5.3.2.3 Serum lysozyme activity

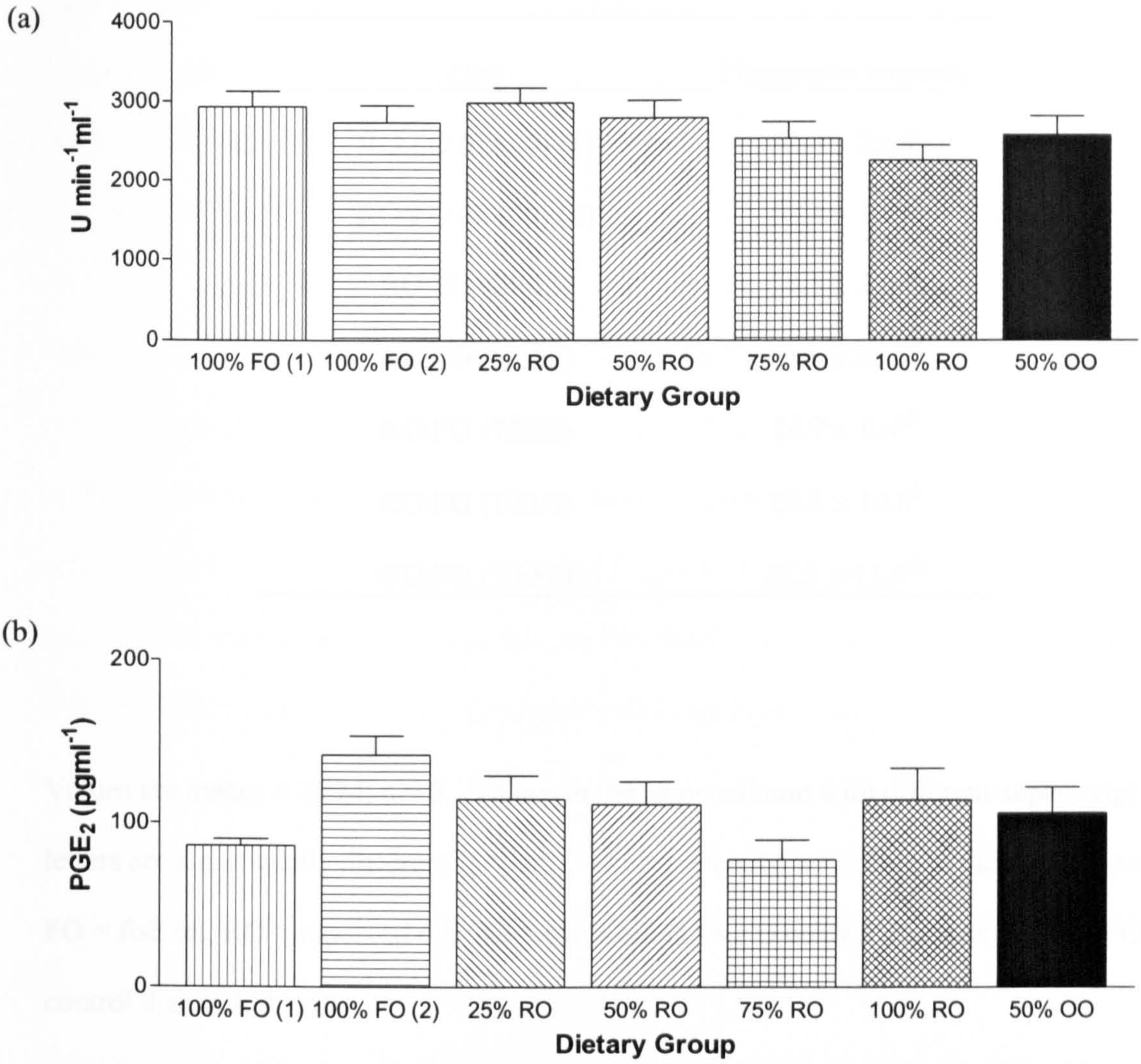
No effect of dietary VO was observed on Atlantic salmon serum lysozyme activity (Figure 5.10a). Although with increasing percentages of dietary RO the lysozyme activity decreased, but not significantly.

#### 5.3.2.4 Plasma $PGE_2$ concentration

No significant differences were found when measuring the plasma concentration of $PGE_2$ of fish fed the experimental diets (Figure 5.10b).




Figures 5.9 Effects of feeding the experimental diets to salmon on (a) % haematocrit (b) total number of circulating leukocytes (c) total number of circulating erythrocytes. Values are means ± SEM, n = 8. Values having different column letters are significantly different ($p<0.05$). Diets are coded according to dietary oil source FO = fish oil, RO = rapeseed oil, OO = olive oil. (1) = FO control diet cage 1, (2) = FO control diet cage 2.




Figures 5.10 Effects of feeding the experimental diets to salmon on (a) serum lysozyme activity (b) plasma $PGE_2$ concentration. Values are means ± SEM, n = 8. Diets are coded according to dietary oil source FO = fish oil, RO = rapeseed oil, OO = olive oil. (1) = FO control diet cage 1, (2) = FO control diet cage 2.

Table 5.8 Phagocytic capacity (% of macrophages which have phagocytosed yeast particles) of salmon head kidney macrophages fed the experimental diets.

| Diet | Phagocytic capacity |
|---|---|
| RO:FO (0:100) (1) | $58.4 \pm 22.6^{c}$ |
| RO:FO (0:100) (2) | $62.4 \pm 14.8^{c}$ |
| RO:FO (25:75) | $50.5 \pm 21.4^{a}$ |
| RO:FO (50:50) | $39.3 \pm 28.6^{ab}$ |
| RO:FO (75:25) | $26.7 \pm 9.4^{ab}$ |
| RO:FO (100:0) | $20.2 \pm 10.6^{b}$ |
| OO:FO (50:50) | $28.3 \pm 11.6^{ab}$ |

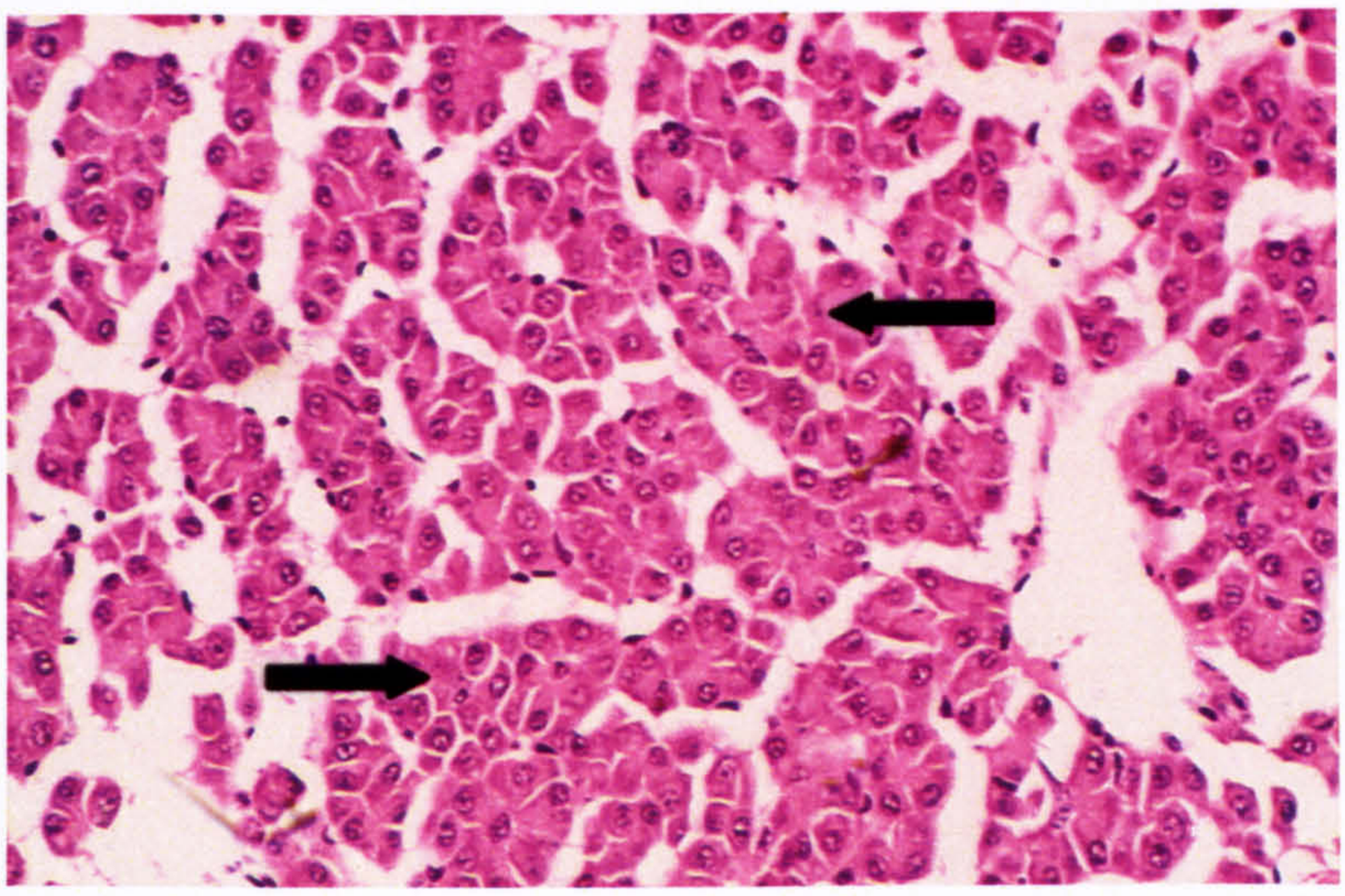
Values are means ± SEM, n = 8. Values in the same column with different superscript letters are significantly different ($p<0.05$). Diets are coded according to dietary oil source FO = fish oil, RO = rapeseed oil, OO = olive oil. (1) = FO control diet tank 1, (2) = FO control diet tank 2.

### 5.3.2.5 Histopathology

Overall the livers from each dietary group had a low fat content, some were so low that scores of zero were reported. The fat was generally organised into small individual fat vacuoles with little variation in the distribution of the fat throughout the organ. In general, the higher the RO content in the diet the higher degree of fat evident in the livers e.g. the 100 % RO diet gave the highest fat content scores. A liver section from a fish maintained on the 100 % FO diet can be seen in Figure 5.11a with a low fat content within the hepatocytes. There was some indication of inflammation in fish fed the 50 % RO group but nothing significant that would indicate severe pathology (Figure 5.11b). Fish fed the 50 % RO diet also showed the highest pvc scores indicating collections of inflammatory cells around the vessels. The 100 % FO and the 25 % RO fed fish revealed some single cell necrosis within the liver, but as this appears quite regularly in fish the low scores reported indicate that there is no significant pathology present in the livers.

In all hearts examined no abnormalities were detected. All the dietary groups sampled appeared to have a high amount of mucus cell activity in the proximal intestine. There was no difference in the degree of vacuolation between the groups. The 100 % RO group revealed the highest cellular infiltration score in the proximal intestine but this was still relatively low in comparison to other trial results. Sloughing of the mucosa was minimal in all groups. The only noticeable finding in the mid intestines was that fish fed the VO had a more "active" mucus cell activity compared to that of the FO control groups. There were no great variations in vacuolation, cellular infiltration and breakdown of the mucosa between dietary treatments. The distal gut from the VO fed fish had a higher number of

**(a)**



**(b)**

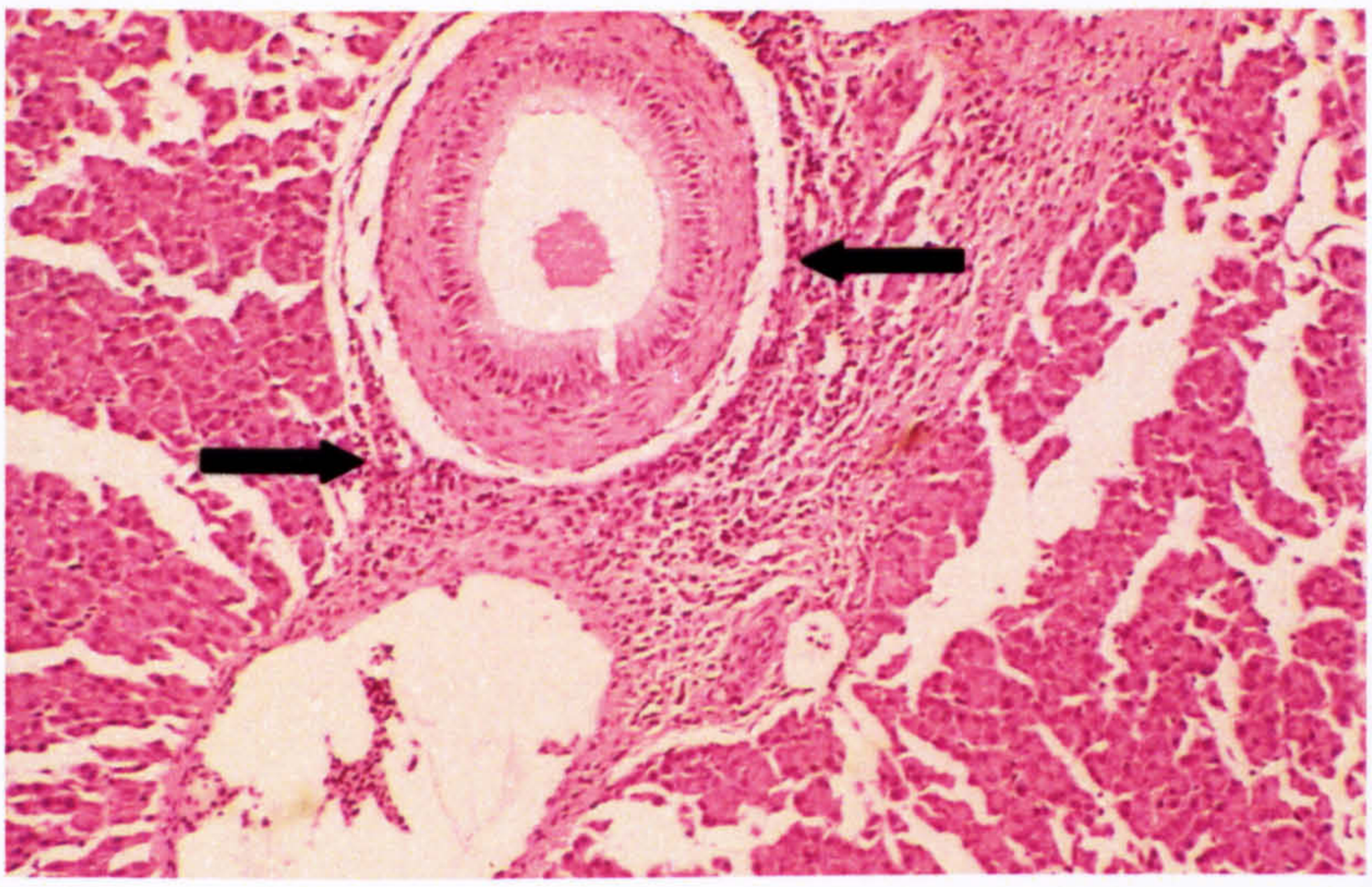


**Figure 5.11 Histopathology of Atlantic salmon fed a (a) 100 % FO diet. Liver showing a low fat content (mag. x 175) (b) 50 % RO diet. Liver showing single necrotic cells and some slight inflammation (mag. x 175).**

vacuolated cells and much more breakdown of the mucosal folds compared to the control groups. The distal intestine from a fish fed with the 25 % RO diet showing a highly vacuolated mucosa but no sloughing can be seen in Figure 5.12a. Cellular infiltration was most evident in the 75 % RO group (Figure 5.12b), however, overall the scores were lower than found in other trials where FO was replaced by RO in diets for Atlantic salmon (Chapter 3).

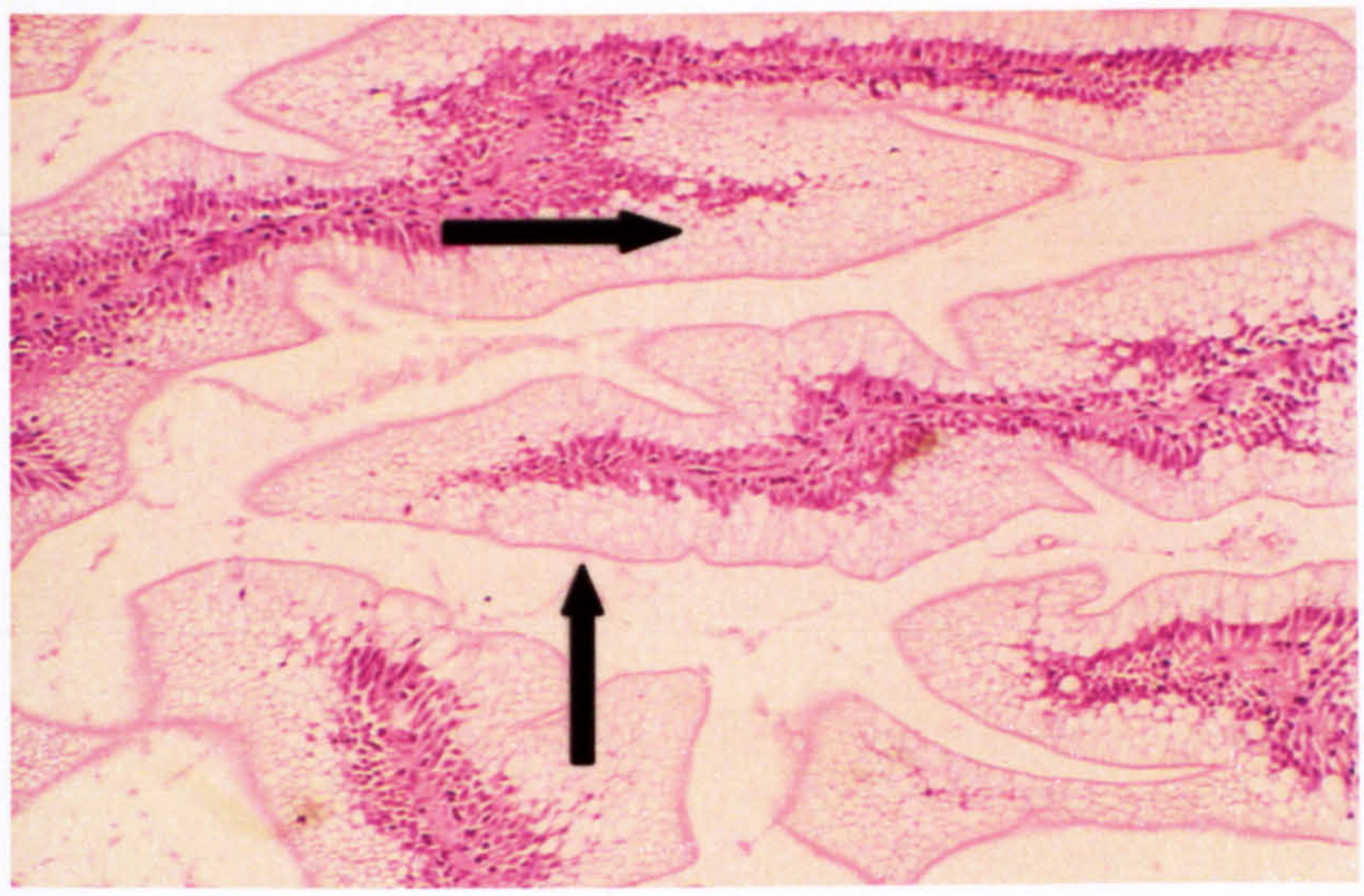
### 5.3.3 *Trial 3 RAFOA Spain – Replacement of FO with LO, RO and OO in European Sea bass*

#### 5.3.3.1 Growth, tissue lipid content and flesh fatty acid composition

There were no significant differences, between dietary treatments, in total length, live mass or specific growth rate (SGR) of the fish at the end of the 34 weeks of feeding the experimental diets according to Mourente *et al.*, (personal communication). The fatty acid composition of flesh total lipid was closely influenced by dietary fatty acid input but specific fatty acids were selectively retained or utilized. There was selective deposition of DHA (flesh DHA > diet DHA). EPA and DHA were significantly reduced and ALA, LA and oleic acids significantly increased in flesh lipids following the inclusion of 60% RO, LO and OO in the diets.

#### 5.3.3.2 Haematology and Macrophage function

Of the blood parameters measured, there were no significant differences in the haematocrit (Figure 5.13a) of fish fed the experimental diets. However, the number of circulating



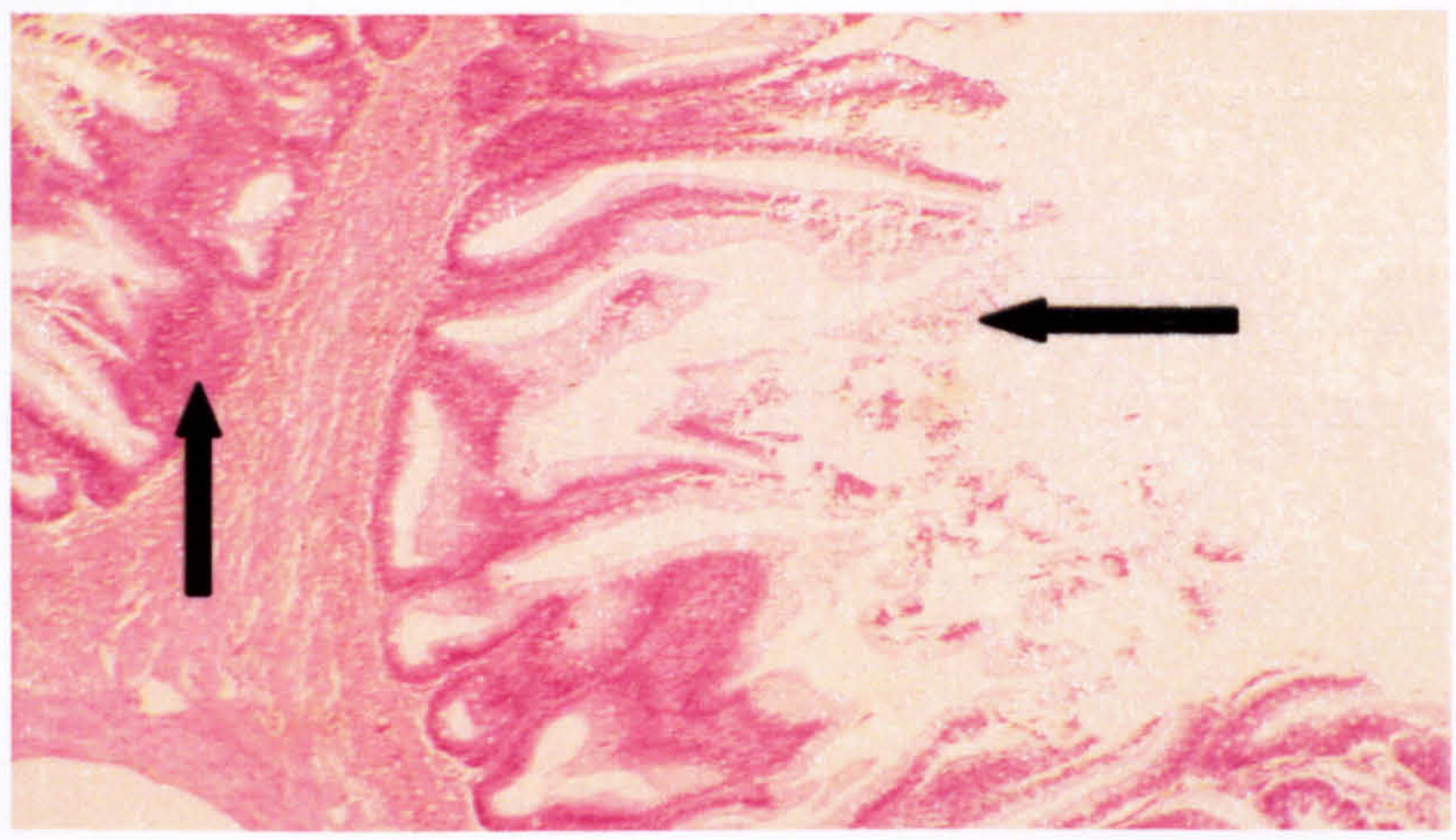




Figure 5.12 Histopathology of Atlantic salmon fed a (a) 25 % RO diet. Distal intestine showing a high degree of vacuolation but no sloughing (mag. x 175) (b) 75 % RO diet. Distal intestine showing cellular infiltration and sloughing of the mucosal membrane (mag. x 70).

leucocytes was significantly affected ($p<0.001$), with samples from fish fed the VO diets being significantly lower than samples from the fish fed the FO diet (FO = 2.1 x $10^8$ cells $ml^{-1}$; LO = 1.5 x $10^8$ cells $ml^{-1}$; OO = 1.3 x $10^8$ cells $ml^{-1}$; RO = 7.5 x $10^7$ cells $ml^{-1}$) (Figure 5.13b). There was no effect of VO on the total number of circulating erythrocytes (Figure 5.13c). Macrophage respiratory burst activity was significantly affected by feeding LO, RO and OO diets as measured by NBT reduction ($p<0.006$) (Figure 5.14). The phagocytic capacity of salmon head kidney macrophages fed the experimental diets is shown in Table 5.9. A reduction in the activity of phagocytosis when macrophages from RO fed fish was observed when compared with fish fed a FO diet.

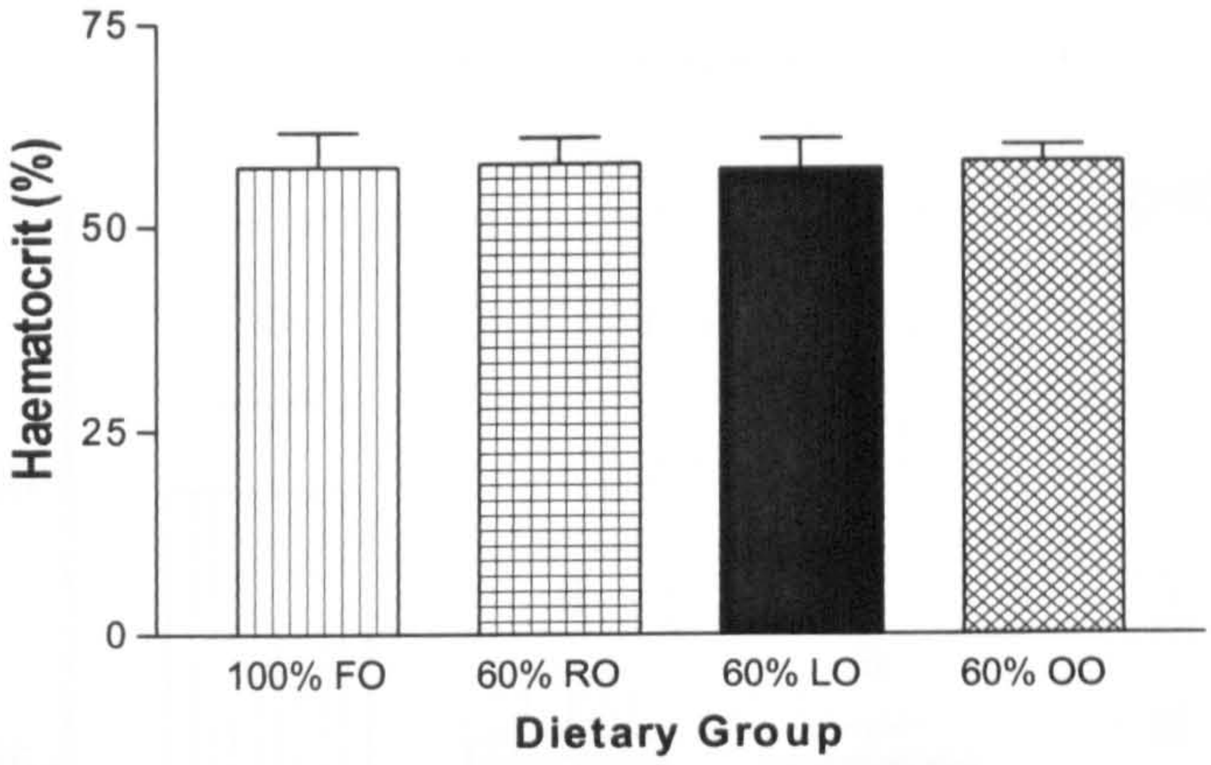
#### 5.3.3.3 Serum lysozyme activity

No significant differences in the serum lysozyme activity between the different dietary groups were found (Figure 5.15).

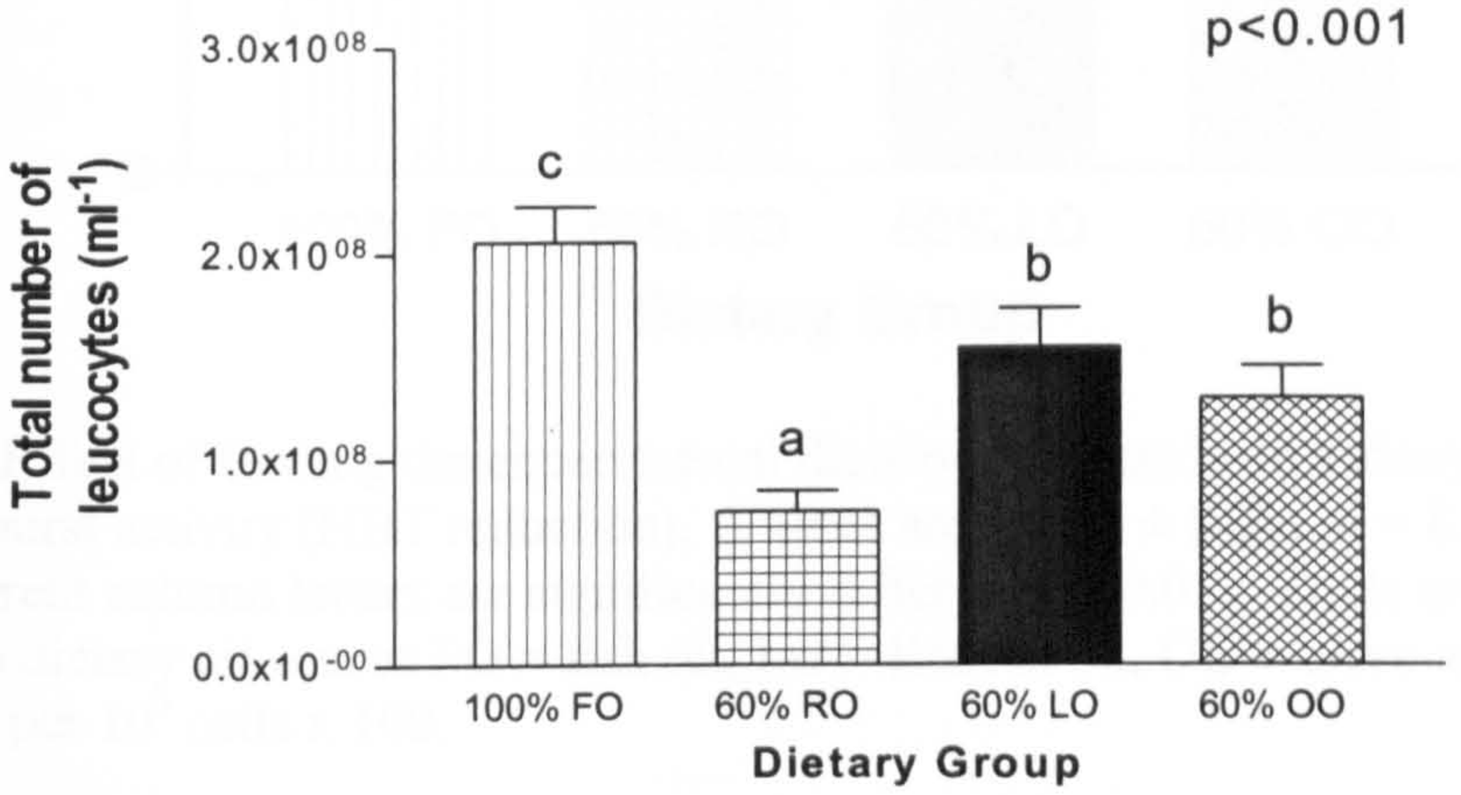
#### 5.3.3.4 Plasma $PGE_2$ concentration

The effect of partial replacement of dietary FO with VO (LO, RO and OO) on $PGE_2$ concentrations in plasma of sea bass during 34 weeks, are shown in Figure 5.15b. No statistical differences were found among different treatment on plasma concentrations of $PGE_2$.

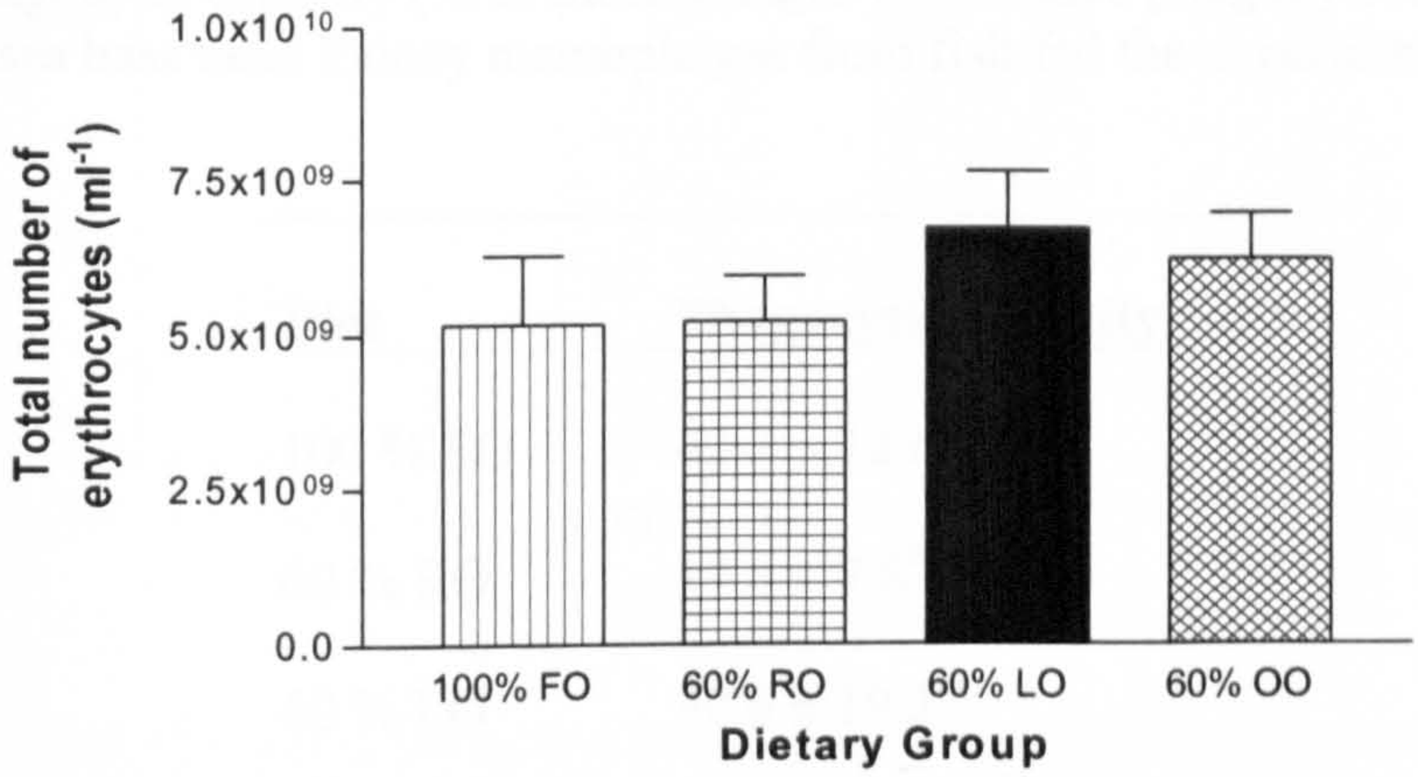
(a)




(b)




(c)




Figures 5.13 Effect of feeding the experimental diets to sea bass on (a) % haematocrit (b) total number of circulating leukocytes (c) total number of circulating erythrocytes. Values are means ± SEM, n = 8. Values having different column letters are significantly different ($p<0.05$). Diets are coded according to dietary oil source FO = fish oil, RO = rapeseed oil, LO = linseed oil, OO = olive oil.

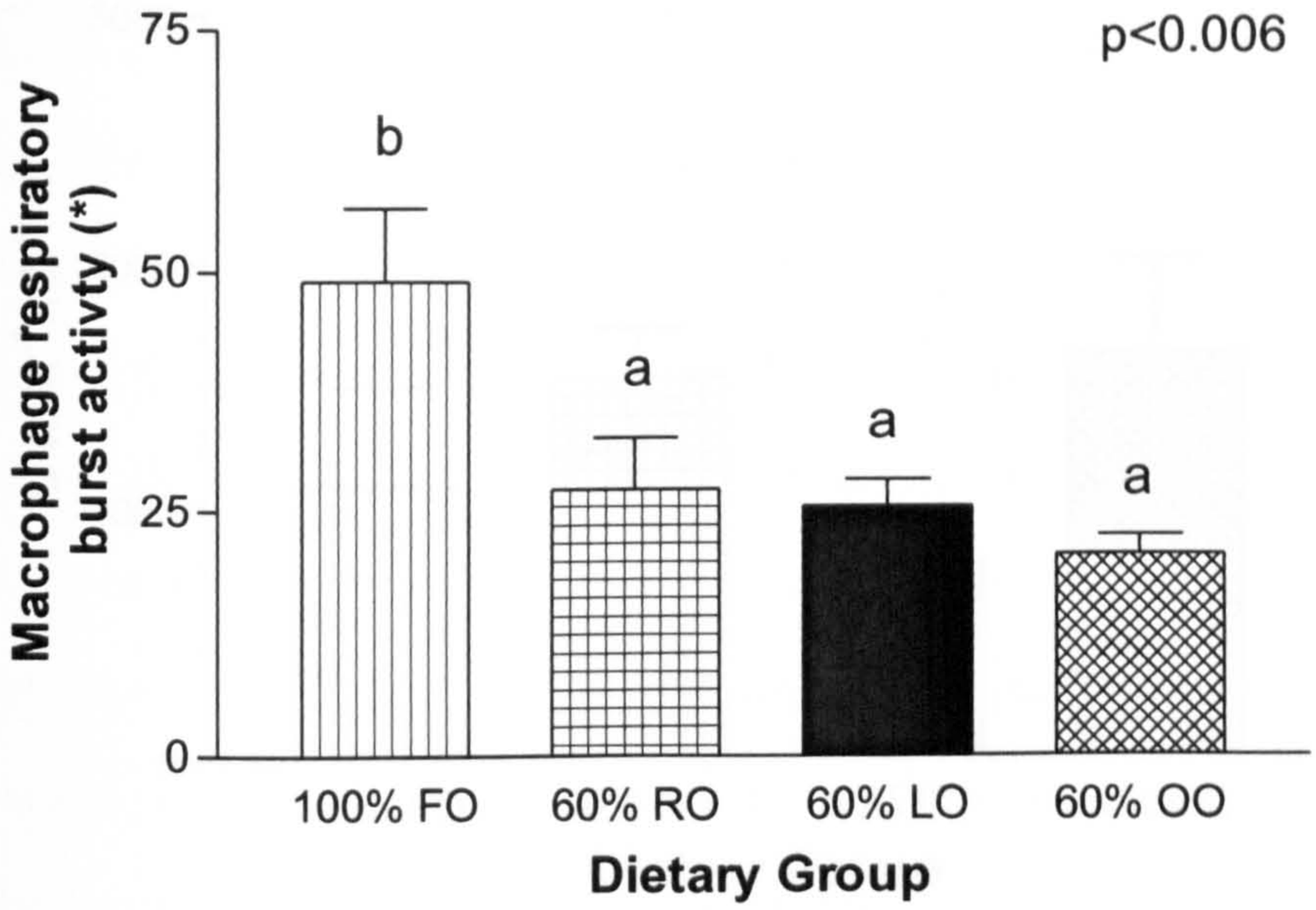
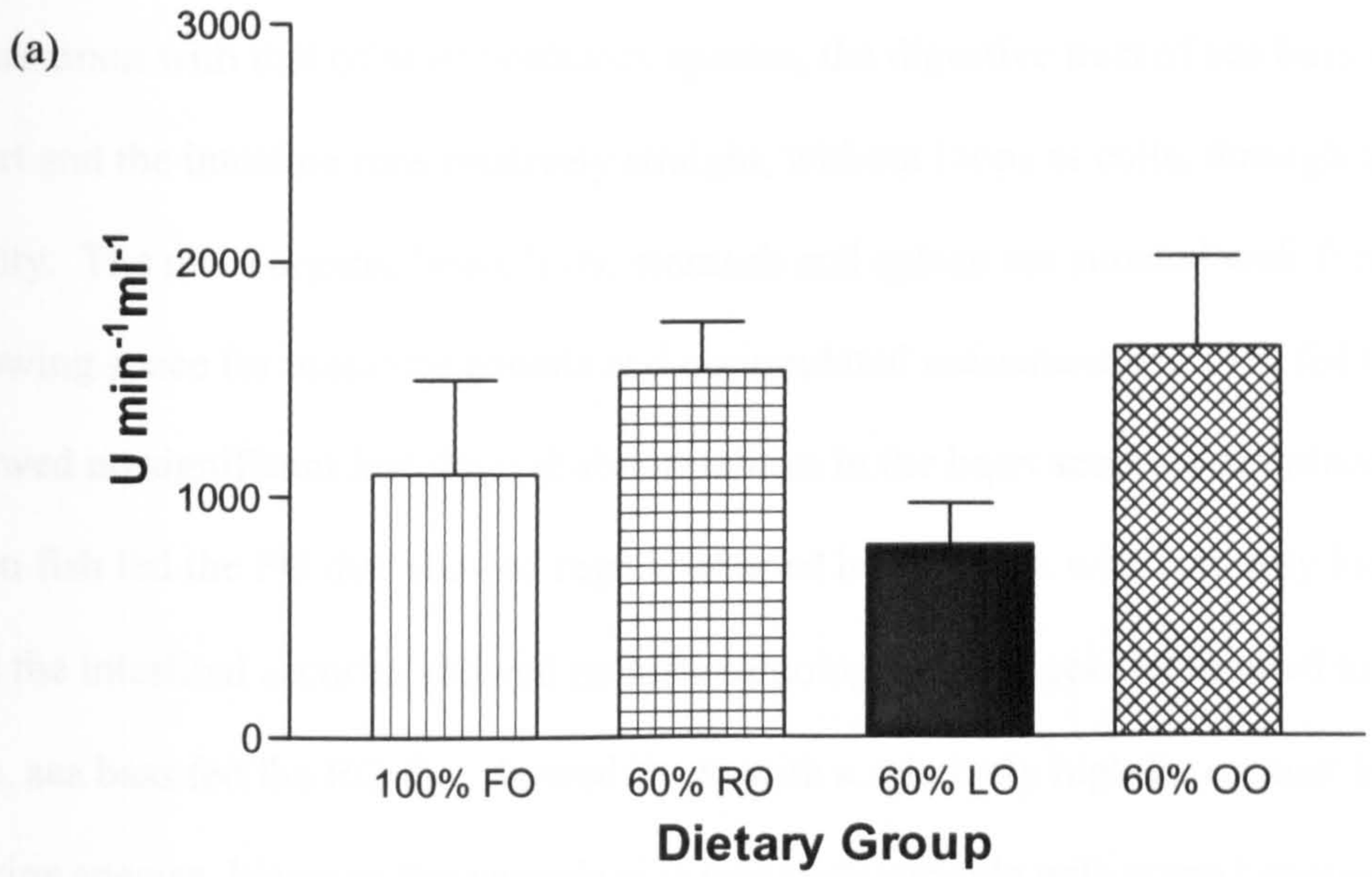



Figure 5.14 Effect of feeding the experimental diets on sea bass head kidney macrophage respiratory burst activity (NBT reduction). Values are means ± SEM, n = 8. Values having different column letters are significantly different ($p<0.05$). Diets are coded according to dietary oil source FO = fish oil, LO = linseed oil, OO = olive oil. * = Absorbance per $10^5$ cells x 100.

Table 5.9 Phagocytic capacity (% of macrophages which have phagocytosed yeast particles) of sea bass head kidney macrophages from fish fed the experimental diets.

| Diet | Phagocytic capacity |
|---|---|
| 100 % FO | $42.2 \pm 12.6^{a}$ |
| 60 % RO | $19.6 \pm 7.8^{b}$ |
| 60 % LO | $30.6 \pm 19.2^{ab}$ |
| 60 % OO | $24.9 \pm 15.3^{ab}$ |

Values are means ± SEM, n = 8. Values in the same column with different superscript letters are significantly different ($p<0.05$). Diets are coded according to dietary oil source FO = fish oil, RO = rapeseed oil, LO = linseed oil, OO = olive oil.



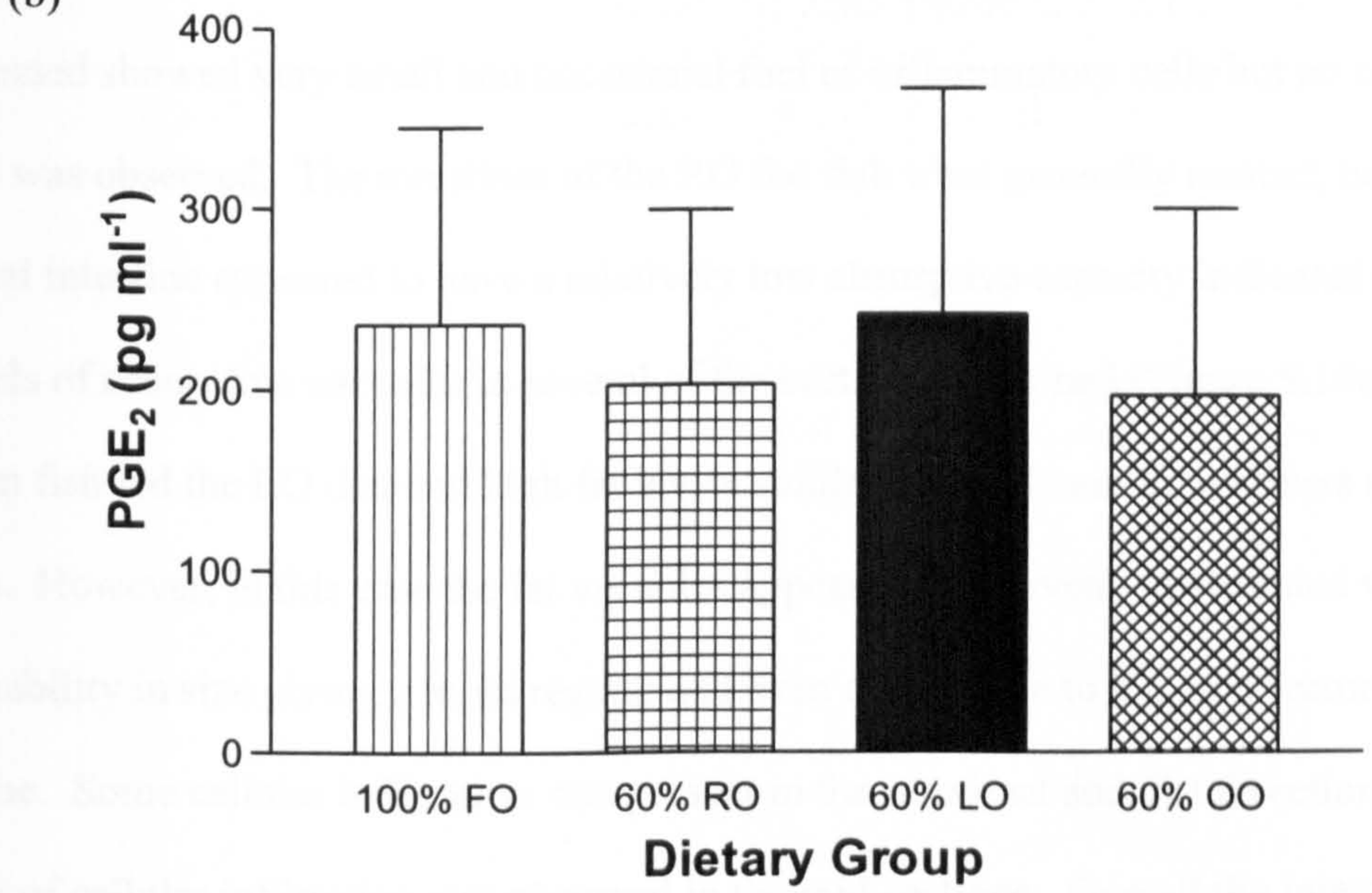
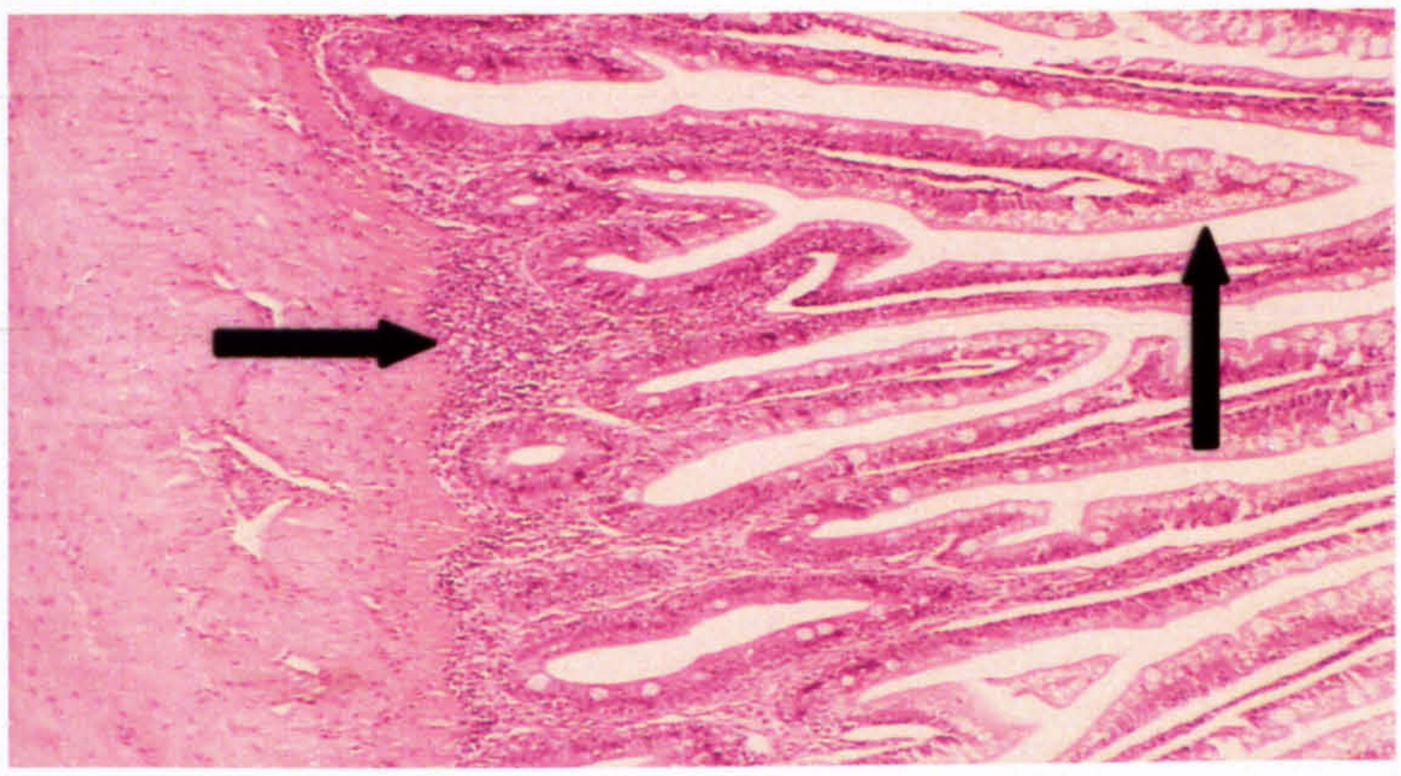




Figure 5.15 Effect of feeding the experimental diets to sea bass on (a) serum lysozyme activity (b) plasma $PGE_2$ concentration. Values are means ± SEM, n = 8. Diets are coded according to dietary oil source FO = fish oil, RO = rapeseed oil, LO = linseed oil, OO = olive oil.

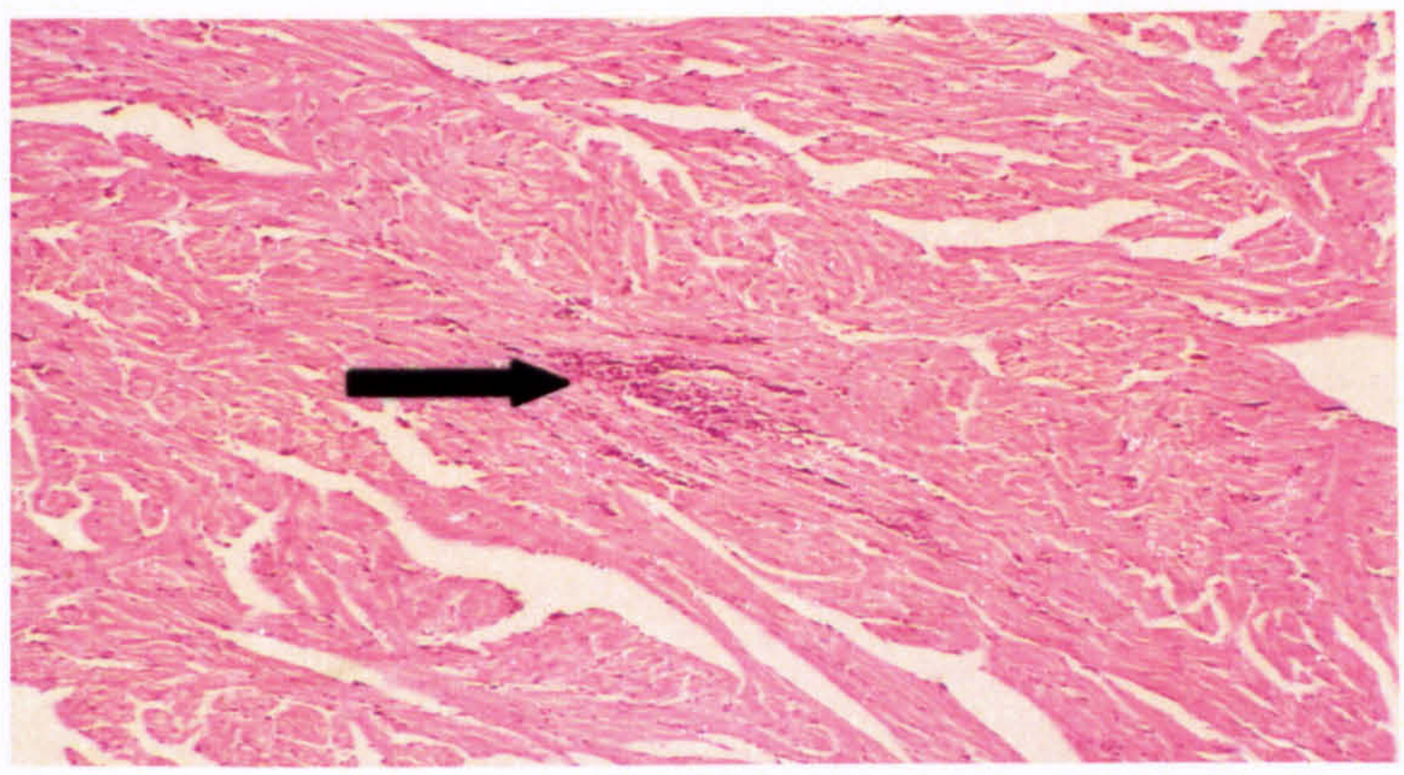
### 5.3.3.5 Histopathology

In common with that of most predatory species, the digestive tract of sea bass is relatively short and the intestine runs relatively straight, without loops or coils, through the body cavity. The main organs, heart, liver, stomach and spleen are situated well forward, allowing space for maturing gonads and accumulated mesenteric fat. Fish fed the FO diet showed no significant histological abnormalities in the heart sections examined. Livers from fish fed the FO diet showed regular-shaped hepatocytes with centrally located nuclei and the intestinal sections showed no histopathological changes. Compared to the FO fed fish, sea bass fed the RO diet showed livers with a relatively high fat content indicative of marine species, however the vacuole size was very variable with some hepatocytes with large individual vacuoles and some with often small multiple vacuoles. All liver sections assessed showed very small and occasional foci of inflammatory cells but no necrosis or pvc was observed. The intestines of the RO fed fish were generally normal, however the distal intestine appeared to have a relatively low absorptive capacity indicated by low levels of absorptive vacuoles in several of the sections examined (Figure 5.16a). Livers from fish fed the LO diet had high fat levels similar to that found in the livers of RO fed fish. However, in this case the fat vacuoles appeared to be evenly distributed with less variability in size giving a more regular and even appearance to the architecture of the tissue. Some cellular infiltration was present in the proximal and distal sections, though no sign of cellular infiltration was observed in the mid sections. Overall the intestines of fish fed the LO diet were normal with a higher absorptive capacity than fish fed the RO diet. A sea bass maintained on a LO diet showing small patches of endocarditis is shown in Figure 5.16b. The majority of livers sampled from the OO dietary group showed a high fat

(a)



(b)



(c)

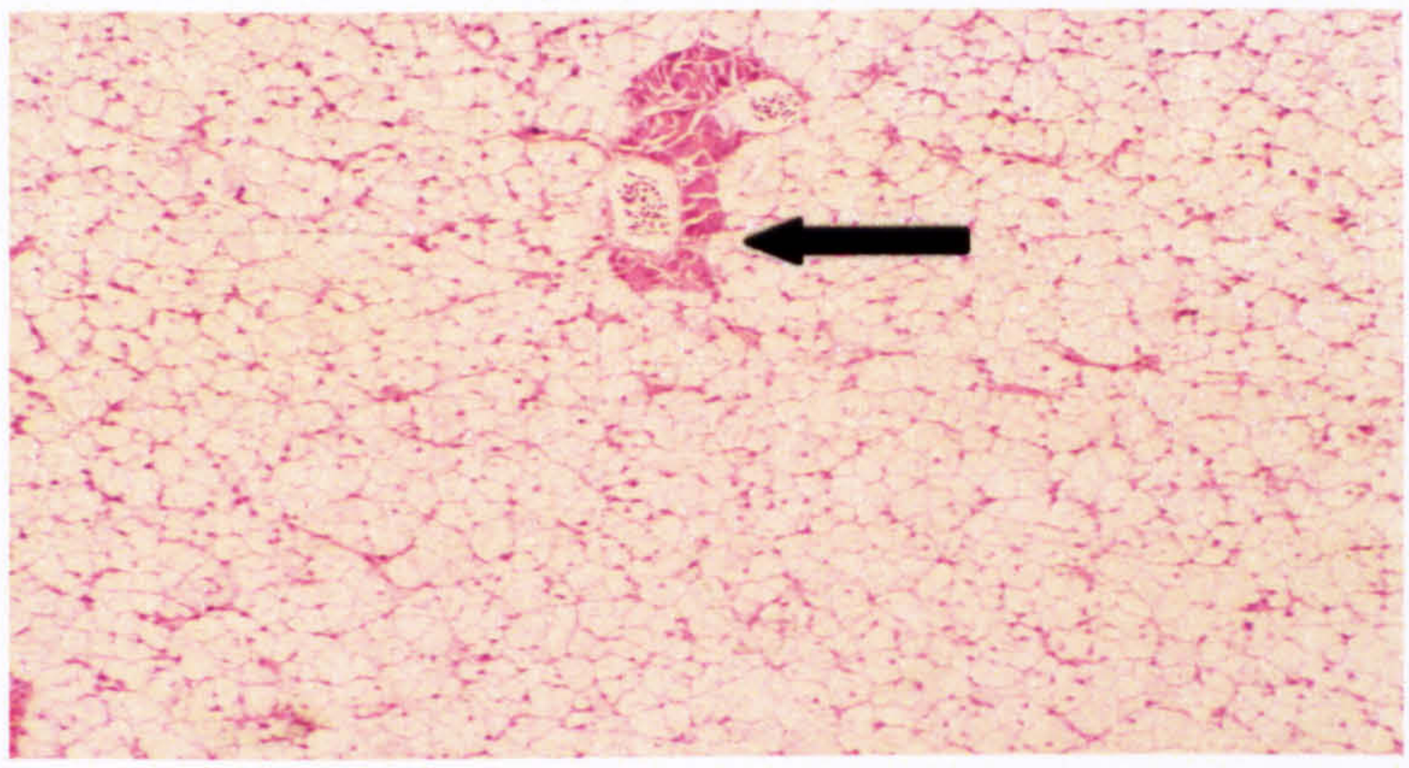


Figure 5.16 Histopathology of sea bass fed a (a) 60 % RO diet. Proximal intestine showing high levels of absorptive vacuoles and some cellular infiltration (mag. x 175) (b) 60 % LO diet. Heart section showing a small foci of endocarditis mag. x 70) (c) a 60 % OO diet. Liver section showing variable sized vacuoles and some cellular breakdown (mag. x 175).

content within the hepatocytes but no other associated pathology. Only one fish showed a variable vacuole appearance with some cellular breakdown (Figure 5.16c). The intestines gave a similar appearance to those fed the FO diet with some increased cellular infiltration in the distal segment only.

## 5.4 Discussion

Several VO have been used as partial substitutes for FO in aquatic feeds for different species without compromising their growth and this was also found to be the case when replacing FO with LO or RO in Atlantic salmon (Bell *et al.*, personal communication; Torstensen *et al.*, 2004). Changes were seen in haematocrit and serum lysozyme activity and more importantly a clear reduction in respiratory burst activity, measured by the reduction of NBT, and plasma $PGE_2$ production in salmon fed LO diets was observed in Trial 1. As mentioned previously (Chapter 4) Bell *et al.*, (1996a) found no statistical differences in haematocrit and serum lysozyme activity in salmon fed diets containing LO compared to those fed FO or sunflower oil. In the same study no significant differences were evident between the dietary groups when measuring head kidney macrophage activity and phagocytosis.

Consistent with the findings reported in Chapters 3 and 4, an assessment of the impact of dietary RO on the immune response of salmon showed a reduction in many of the immune parameters measured. Firstly, feeding salmon with a 100 % RO diet significantly reduced the haematocrit percentage compared to fish fed with lower levels of RO, however no

significant differences were observed between fish fed the RO diets and those fed with the FO diet. Yang *et al.*, (1994) found that Arctic char fed diets containing either 1 % LA or 0.1 % ALA of the total diet, had much lower haematocrit compared to those given diets containing > 0.1 % (n-3) PUFA. Although not always statistically significant, a general reduction in the numbers of circulating leukocytes and erythrocytes was found among fish fed diets containing RO compared to those fed FO diets.

Sea bass fed RO, LO & OO diets showed a significant reduction in the total number of circulating leucocytes and a reduction in macrophage respiratory burst activity (NBT reduction). After feeding sea bass with the RO diet, significant differences were observed in the phagocytic activity of head kidney macrophages compared to fish maintained on a FO diet. Montero *et al.*, (2003) performed a similar trial, feeding gilthead sea bream (*Sparus aurata*) diets containing soybean oil, RO and LO or a blend of these oils compared to a FO control diet. After feeding the experimental diets for 29 weeks some immune parameters were statistically different. While no differences were found in the haematocrit or haemoglobin content, the number of circulating erythrocytes was significantly higher in fish fed the FO diet compared to fish fed the soybean oil and LO diets. Fish fed the soybean oil diet had significantly lower complement activity than fish fed the FO diet but no affect of feeding LO or RO was observed on complement activity. No effect of dietary VO was observed on sea bream lysozyme activity, as was the case in Trial 3 with sea bass.

As mentioned previously, Montero *et al.*, (2003) documented reduced numbers of erythrocytes in seabream fed diets containing either LO or soybean oil compared to those fed FO diets. In contrast, Thompson *et al.*, (1996) found no significant differences in blood

cell numbers between dietary groups of salmon fed either FO or sunflower oil. Despite conflicting effects of n-3 PUFA on the immune response of fish, it appears that n-3 enriched diets consistently have a stabilising effect on cell membranes (Erdal *et al.*, 1991). This is especially important for poikilotherms such as fish where it is essential to have optimal membrane function to maintain biomembranes in a liquid crystalline state in relation to the prevailing water temperature. Findings from Montero and colleagues (2003) are in contrast to an earlier study by Waagbϕ *et al.* (1995) where feeding soybean oil to adult cod (*Gadus morhua*) resulted in significantly increased complement activity compared to fish fed with FO (capelin and sardine). Furthermore, soybean oil feeding significantly reduced haematocrit value when compared to feeding with capelin oil, but had no effect on the number of circulating erythrocytes.

Although a reduction in the activity of salmon head kidney macrophages, as measured by the reduction of NBT, was found in LO fed fish, no significant differences were found when measuring the capacity of macrophages to phagocytose yeast particles from salmon fed the LO diets. However, in Trial 1 feeding with 50 % OO did significantly reduce the phagocytic capacity compared to FO fed fish. Macrophages from salmon maintained on diets containing RO had reduced phagocytic activity when compared to those fed a diet containing FO. This agrees with data from other trials with salmon (Chapter 4) and also other fish species fed RO containing diets (Montero *et al.*, 2003). The macrophage membrane plays a key role in phagocytosis, and any changes in viscosity and permeability can alter their cellular function (Obach *et al.*, 1993). Moreover, the macrophage membrane contains receptors for the Fc fragment of immunoglobulin (Griffin & Mullinax, 1983; Sakai, 1984), and this favours antigen adhesion and engulfment resulting in phagosome

formation. Changes in the membranes may have reduced the receptor sites and the phagocytic capabilities and immunoglobulin levels. In contrast to the results presented in Trial 3, Montero *et al.*, (2003) found no effect of feeding VO on the macrophage respiratory burst activity of sea bream, however, the phagocytic activity of the macrophages was significantly affected in fish fed either RO or soybean oil diets.

In support of the findings presented in Trial 1 on the concentrations of $PGE_2$ in salmon fed LO, Bell *et al.*, (1996a, b) also reported a reduction in the production of $LTB_4$ and $PGE_2$ by stimulated macrophages compared to fish fed FO or sunflower oil. Thus, changes in the phospholipid fatty acid composition of eicosanoid producing cells, as a result of feeding increasing levels of LO (ALA and LA) in turn, can lead to changes in eicosanoid precursors. Bell *et al.*, (1993a, b) found that feeding LO diets to salmon resulted in reduced production of ARA-derived $PGE_2$ and $TXB_2$. The authors reported that feeding LO increased the levels of the Δ 6 desaturation and elongation products, LA and 20:3(n-6) in fish leukocytes but decreased the incorporation of ARA, the product of Δ 5 desaturation. Therefore, it appears the dietary LO, which is rich in LA but even higher in ALA, has an inhibitory effect on the Δ 5 desaturase enzyme responsible for converting 20:3(n-6) to ARA. In LO fed salmon there is competition at the Δ 6 desaturase between LA and ALA and also at the Δ 5 desaturase between 20:3(n-6) and 20:4(n-3) resulting in inhibition of Δ 5 desaturation. In addition, the ability of ALA to inhibit the conversion of LA to ARA has been observed previously in mammals (Brenner, 1981). Bell *et al.*, (1993a, b) found that in LO fed salmon EPA levels were greater or equal to the levels found in FO fed salmon, suggesting that appreciable conversion of ALA to EPA via Δ 6 and Δ 5 desaturation and elongation takes place. VO may therefore affect prostaglandin production, and if these

eicosanoids are produced in high enough concentrations, this could lead to a decrease in macrophage function. Inhibition of respiratory burst activity has also been observed in rainbow trout head kidney macrophages when dime $PGE_2$, a synthetic form of $PGE_2$ was added (Novoa *et al.*, 1996).

Eicosanoid production is associated, very broadly, with stressful situations and is a normal physiological process, with excess eicosanoid production often occurring in pathological conditions (Sargent *et al.*, 2002). Eicosanoids are produced from $C_{20}$ PUFA by the action of cyclooxygenase and lipoxygenase resulting in metabolites that include prostaglandins, leukotrienes and lipoxins that are known to influence a wide range of immune functions (Uhing *et al.*, 1990). Eicosanoids have multiple effects on immune functions, directly by acting on macrophages and lymphocytes or indirectly via cytokines (Stankova & Rola-Pleszczynski, 1992). Prostaglandins, especially ARA-derived $PGE_2$, are produced by monocytes and macrophages, and are associated with modulation of immune cell function (Kinsella *et al.*, 1990), whereas $PGF_{2\alpha}$ is more related to environmental stress adaptation, such as temperature and salinity change (Mustafa & Srivastava 1989). In rainbow trout, $PGE_2$ is known to down-regulate antibody synthesis *in vivo* (Rainger *et al.* 1992), PHA-induced mitogenesis (Secombes *et al.*, 1994c), plasma cell generation *in vitro* (Knight & Rowley, 1995) and respiratory burst (Novoa *et al.*, 1996) and to up-regulate macrophage phagocytosis (Knight *et al.*, 1993). There is good evidence that low concentrations of circulating $PGE_2$ ($<10^{-9}$ M) are required for normal immune function and T-cell differentiation, but that concentrations of $PGE_2 > 10^{-8}$ are immunosuppressive in mammals (Kinsella *et al.*, 1990). In Trial 3, values for $PGE_2$ ranged between $5.6 \times 10^{-10}$ M and $9.7 \times 10^{-10}$ M and no significant differences were observed among treatments. However, these

data should be viewed with caution since important differences in the eicosanoid production pattern have been described among different fish species (Rowley, 1991). The only data available for PGE in European sea bass were in primary testis cell culture incubated with PUFA (ARA, EPA and DHA), where control values were lower than values presented in the present study, but ARA stimulated a significant dose and time dependent increase in PGE production, EPA stimulated PGE production only during the first 6 h (although levels were 26.4 % of those induced with ARA) and DHA reduced PGE production (Asturiano *et al.*, 2000).

Changes in the fatty acid profile of total lipids of PBL broadly reflected the dietary fatty acid composition in Trial 1. Since the fatty acid composition of fish diets influences the fatty acid composition of cell membrane phospholipids this, in turn, can have profound effects on disease resistance and immune function by altering eicosanoid production and because many immune responses are based on leucocyte cell membrane interactions (e.g. phagocytosis and cytokine production). Much has been reported regarding the lipid composition of immunocompetent cells and tissues in various fish species (Pettitt *et al.*, 1989; Pettitt & Rowley, 1990; Thompson *et al.*, 1995; Bell *et al.*, 1996a, b). The ability to modulate immunity depends on the capacity of the fish to selectively incorporate the optimal fatty acids in to membrane structures, in spite of the dietary lipid fatty acid composition and according to temperature. In Trial 1, the levels of oleic acid, ALA and LA were generally increased in fish fed the VO diets compared to FO fed fish. Whereas, EPA and DHA levels were reduced in VO fed fish compared to those maintained on a FO diet. At higher LO inclusion levels (75 % and 100 %), increased amounts of LA, 20:2(n-6) and 20:3(n-6) were found but decreased levels of ARA when compared to FO fed fish. This

supports similar observations found by Bell *et al.*, (1993a, b) where salmon were fed diets containing LO.

The histology of the liver, and to a lesser extent the intestine, was also affected by diet in Trial 1 but the physiological significance of these changes is difficult to interpret. Perhaps accumulation of lipid droplets in the liver of salmon fed high levels of LO may be due to the use of long chain monoenes present in FO as energy sources, in preference to other fatty acids, resulting in storage of particular fatty acids present in the LO diets (e.g. ALA and LA) within the lipid droplets. Caballero *et al.*, (2002) found similar effects when feeding VO diets to rainbow trout. These authors reported the presence of numerous lipid vacuoles in the enterocytes of trout fed soybean oil, RO or a mixture of palm oil (PO) and RO. In addition, the livers sampled from trout maintained on either a RO diet or a RO / PO mixed diet showed swollen hepatocytes with numerous varying-sized lipid vacuoles, similar to the salmon livers assessed in the present study. It is also interesting to note that Olsen *et al.*, (1999, 2000) reported that Arctic char fed LO showed a tendency to accumulate lipid droplets within the cytoplasm of enterocytes, suggesting that this accumulation could be due to insufficient synthesis of phospholipids required for lipoprotein synthesis.

Histological examination in Trial 2 showed few major anomalies between salmon fed the experimental diets. In comparison to other feeding trials using RO as the sole lipid or in a mix with another VO (Chapter 3 and 4) minor pathological changes were observed. As the amount of VO inclusion increased so did the lipid deposition in the liver. However, where in other trials this resulted in a general loss of liver structure and variability in hepatocyte

size and distribution, in this study the livers of RO fed fish appeared normal with only slight changes in their histological appearance. Therefore it is apparent that >50 % replacement of FO with LO, RO and OO may have a detrimental effect on Atlantic salmon immune system and may cause an increase in tissue histopathology. Future studies of dietary oil replacement in Atlantic salmon, should expand on the work carried out here examining their effect on immunological and histological parameters.

The most significant histological difference found among sea bass fed the experimental diets was the occurrence of variable sized vacuoles and large amounts of lipid droplets within the hepatocytes of fish fed diets containing RO and LO compared to fish fed the FO diet. In addition, the distal intestine of the VO fed sea bass showed a higher degree of cellular infiltration in the mucosal folds compared to fish fed the FO diet. The histological changes observed suggest an effect of dietary lipid sources on the transport and metabolism of fat in sea bass, but further studies are required to clarify this. Perhaps, accumulation of lipid droplets in the liver of sea bass fed the RO and LO diets may be due to changes in fatty acids available as energy sources resulting in storage of specific fatty acids (e.g. ALA, LA and oleic acid) within the lipid droplets. Caballero *et al.*, (2003) found that feeding gilthead sea bream (*S. aurata*) either LO, RO or soybean oil diets at 60 % and 80 % resulted in accumulation of supranuclear lipid droplets within intestinal enterocytes. It was noted that the tendency for lipid accumulation was related to a decrease in dietary n-3 HUFA content, which would result in impaired synthesis of lipoproteins (Watanabe, 1982; Sargent *et al.*, 1989; Olsen *et al.*, 1999, 2000). However, accumulation of lipid droplets did not cause pathological damage to the intestinal epithelium and no signs of cellular necrosis were evident. In contrast to this, Olsen *et al.*, (1999, 2000) found a significant impact on

the gastrointestinal tract function and integrity when feeding large amounts of LO to Arctic char (*Salvelinus alpinus* L.). Feeding LO caused high lipid accumulation in the intestines, followed by destruction and loss of gut integrity. These effects are probably caused by impairment of lipoprotein synthesis and lipid transport, and are probably easily alleviated by minor adjustments to the dietary lipid composition. Histological examination of turbot (*Psetta maximus*) fed with diets containing coconut oil showed excessive lipid degeneration of the hepatic parenchyma and significant lesions in the lateral muscle wedge of these fish (Cowey *et al.*, 1976). Histological changes occurring in the fat cells surrounding the lateral lymphatic sinus of turbot given corn oil was less than in fish given the coconut oil diet.

Results from Trial 3 are in broad agreement with earlier findings in other fish species (Caballero *et al.*, 2002, 2003). Rainbow trout fed diets containing either, soybean, palm, OO and RO showed higher accumulation of lipid droplets in the intestinal cells compared to fish fed a FO diet. Similarly, livers from fish fed VO showed large amounts of lipid droplets within the hepatocytes. Caballero *et al.*, (2002) suggested that the accumulation of lipid droplets in the enterocytes might be considered as a temporal storage of lipid due to insufficient lipoprotein synthesis. In addition, hepatocytes with large lipid vacuoles and nuclei located at the periphery of the cell were observed in livers of red drum (*Sciaenops ocellatus*) fed diets containing soybean oil (Tucker *et al.*, 1997). No major histological changes of heart sections from soybean oil fed fish were observed in that study. On the more positive side, Ringϕ *et al.*, (2002) have shown that the intestinal microbiota is significantly influenced by the addition of VO and it is foreseen that it will be possible to manipulate the dietary lipid composition to select for bacteria inhibiting the establishment

of pathogenic bacteria. Ringϕ *et al.*, (2002) showed that mortality dropped from 50% to 20 % in a challenge trial with furunculosis following manipulation of the dietary lipid source.

Several studies have shown that moderate amounts of VO in fish diets do not have a negative impact on fish growth, however, these oils have a fatty acid profile markedly different from that of marine oils and also can contain substances unnatural to fish. These differences may have several implications to the fish in long-term immune studies. The effect of dietary oils on both humoral and cellular immunity could be the result of an imbalance in the fatty acid composition of the membrane phospholipids, affecting the physical properties of the membrane and the activity of membrane-associated receptors, which could explain the decreased haematocrit and macrophage respiratory burst activity. Since the cell membrane contains enzymes that are involved in the production of active forms of oxygen including superoxide anion (Chung & Secombes, 1988), any alterations of membrane properties could affect the production of the reactive oxygen species.

In general, there are a few mechanisms by which dietary fatty acids may affect fish immune function and disease resistance. Firstly, by influencing the cell membrane fatty acid composition and its physical properties which, in turn, can have profound effects on disease resistance because many immune responses are based on leucocyte cell membrane interactions (e.g. activation of cytokine production). Secondly, dietary fatty acids may affect immune function through the production of eicosanoids from ARA and EPA. At high VO substitution levels the production of eicosanoids may be affected resulting in imbalances of both ARA and EPA-derived eicosanoids, which may in turn affect disease resistance. Finally, another possible mechanism by which dietary fatty acids may affect the

immune system involves alteration of signal transductions, possibly due to effects on protein kinase C (Balfry & Higgs 2001).

Examination of the literature shows that changing the concentration of dietary n-3 PUFA in fish feeds can have both beneficial and, in some instances, detrimental effects on disease resistance. Some evidence of an effect of dietary fatty acid imbalances on different mechanisms of the immune system of cultured fish has been reported (Erdal *et al.*, 1991; Sheldon & Blazer 1991; Kiron *et al.*, 1995; Montero *et al.*, 1999, 2003). It has been shown that the respiratory burst activity of phagocytes of European sea bass decreased with increasing dietary lipid level from 9 % to 17 % (Sitja-Bobadilla & Perez-Sanchez 1999). Mortality of channel catfish (*Ictalurus punctatus*) exposed to *E. ictaluri* was significantly higher for fish fed a menhaden oil-supplemented diet than those fed a catfish offal oil-supplemented diet (Li *et al.*, 1994). Fracalossi & Lovell (1994) found that channel catfish fed 7 % menhaden oil or LO had a higher mortality after challenge with *E. ictaluri* than fish fed diets containing corn oil, or beef tallow or a mixture of the three lipid sources at 28 °C. Futhermore, Erdal *et al.*, (1991) found that increasing the amount of dietary n-3 PUFA from 13 to 24 % of the total fatty acids actually had an immunosuppressive effect on Atlantic salmon, and resulted in higher rates of mortality against *Yersinia ruckeri*. The reduction in survival was also associated with degenerative changes in the heart and skeletal muscle as n-3 PUFA was increased.

Other reports show positive effects of n-3 fatty acids on the immune response of fish. The increased activity of head kidney macrophages has been associated with higher levels of dietary n-3 fatty acids in channel catfish (Blazer, 1991; Sheldon & Blazer, 1991) and

rainbow trout (Ashton *et al.*, 1994). One possible mechanism of dietary fat effects on macrophages is altered signal transduction, which, in turn, could alter gene regulation and macrophage function. The biosynthesis of the immunoactive eicosanoids will also be determined by the available HUFA. These factors all contribute to disease resistance but results from different laboratories are often conflicting. A major cause is probably the variation in the type and composition of the dietary lipids and even their susceptibility to oxidation (Fletcher, 1997). Future studies may benefit by the measurement of additional immune parameters (humoral and cellular responses) for documentation of effects on fish health.

From the present study, there is some evidence that immune function, in terms of blood cell counts and macrophage activity may be influenced by VO inclusion. However, the impact of these changes on fish health appear minimal but will be subject to further investigation during the course of the RAFOA II trial presented in Chapter 6.

# Chapter 6 - Researching Alternatives to Fish Oil in Aquaculture RAFOA Part II (Scotland, Norway, Spain)

## 6.1 General Introduction

As a continuation of the RAFOA I study in Chapter 5, the second dietary trial, RAFOA II was a long term study conducted over a whole production cycle, from first feeding up to market size, followed by a period of wash-out with a diet containing FO as the only source of lipid. As in RAFOA I, dietary trials were carried out in Scotland, Norway and Spain, with Atlantic salmon and European Sea bass being the species of choice. The oils substituted in the diets of fish in RAFOA II were blends of three VO. In general, it was concluded from RAFOA I that feeding salmon or sea bass with diets containing a single VO at various inclusion levels ranging from 25 % - 100 % resulted in a reduction of a number of immune parameters. In addition, it was found that at higher inclusion levels, changes to the normal histological appearance of some tissues was evident. The various VO in the present study were blended to achieve a fatty acid composition as similar to FO as possible, in terms of energy content, and saturated, monounsaturated and PUFA content, but without HUFA. The formulated diets for RAFOA II were agreed by the consortium based on all the data collected from RAFOA I and the current available literature. The oils used in all RAFOA II dietary trials were RO, LO and palm oil (PO), but different oil proportions were blended depending on the experimental fish species. Dietary trials involving Atlantic salmon in Scotland and Norway and sea bass in Spain were performed.

### *6.1.1 Palm oil*

This is the first chapter that has dealt with the substitution of FO with palm oil (PO) in aquaculture feeds. From a review of the available literature it has been shown that the use

of PO as a potential substitute for FO has generally shown encouraging results. Ng (2002) reported improvements in growth, protein utilisation, feed efficiency, reproductive performance and higher α-tocopherol concentrations in fillets from PO fed catfish. Research carried out in Atlantic salmon (Torstensen *et al.*, 2000), channel catfish (Legendre *et al.*, 1995) and climbing perch (*Anabas testudineus*) (Varghese & Oommen, 2000) resulted in growth and feed utilisation efficiency comparable to fish fed with equivalent levels of dietary FO.

Palm belongs to the species *Elaeis* and after soybean oil is the largest VO produced in the world (Gascon *et al.*, 1989). PO currently accounts for 13 % of the total world production of oils and production is still rising. Malaysia is the world's largest supplier of palm oil with production rising from 8.3 million mt in 1998 to 10.6 million mt the following year. Crude palm oil is orange in colour due to the high content of carotenoids, mainly α and β-carotene (Tan & Ng, 1987) and is rich in vitamin E, which helps prevent the oil from being oxidised (Arroyo, 1974; Higuera *et al.*, 1977). From the current literature no trial has focused on the effect of PO diets on fish health and immune function. The principal aim of this chapter was to investigate how blends of dietary VO would impact on selected aspects of the innate immune response and histopathology in salmon and sea bass from first feeding to harvest. Similar biochemical and immune analyses were conducted to those carried out in RAFOA I to establish if blended VO diets had a detrimental effect on the immune response of experimental fish as was seen in RAFOA I and earlier studies.

## 6.2 Materials and Methods

The effects of replacing dietary FO with a 75 % blended VO diet on the immune function and tissue histology of Scottish farmed Atlantic salmon are presented in Trial 1. The effects of substituting FO with a 100 % blended VO diet on the serum lysozyme activity of farmed Atlantic salmon in Norway was examined in Trial 2. The effects of replacing FO with different blends of LO, RO and PO in sea bass farmed in Spain was investigated in Trial 3.

### *6.2.1 Trial 1 RAFOA II Scotland – Replacement of FO with a blend of LO, RO and PO in Atlantic salmon*

#### 6.2.1.1 Dietary Groups

The diets prepared for salmon contained 45 % protein (fish meal), and 30 % oil. The VO component comprised RO, LO and PO in the ratio 41.25/11.25/22.5 with the remaining 25 % being capelin oil. Atlantic salmon fed either a control FO diet (capelin oil) (in duplicate, n = 3000 fry tank$^{-1}$) or the test VO diet (25 % FO + 75 % VO (LO/RO/PO mix)) (in triplicate, n = 3000 fry tank$^{-1}$) were initially held at the Marine Harvest hatchery at Invergarry, Scotland (Figure 6.1). Feeding commenced on the 15$^{th}$ March 2002 and ended for my concern on the 18$^{th}$ November 2003. In April 2003 fish were transported to Marine Harvest FTU at Loch Duich, Lochalsh, Scotland for the continuation of the study in sea water. Feeds were produced at Nutreco Technology Centre (part of Nutreco ARC).

Figure 6.1 Tank facilities at Marine Harvest Invergarry hatchery site.

The dietary formulations and fatty acid compositions used for salmon are shown in Tables 6.1 and 6.2. The major protein content of all diets was provided by fish meal. However, since fish meal itself contains some FO, the replacement of FO with VO was maximised by reducing the level of fish meal in the diets by using maize gluten and extracted soyabean meal as alternative protein sources.

### 6.2.1.2 Collection of samples for immunological studies

Twelve fish were sampled per dietary treatment at five different time points; at 0 weeks (prior to vaccination, 34 weeks from onset of feeding), 3, 6 and 9 weeks post-vaccination (47, 50 and 53 weeks from the onset of feeding) and after transfer to sea water (33 weeks post-sea water transfer) a further sample was taken.

### 6.2.1.3 Haematology and Macrophage function

Blood was collected according to Section 2.3.2 and head kidneys according to Section 2.3.4. Heparinised blood samples were used to determine haematocrit (Section 2.3.2.3) and the total numbers of leukocytes and erythrocytes (Section 2.3.2.1 and 2.3.2.2). Head kidney macrophages were prepared following the methods outlined in Sections 2.3.4 and 2.3.7.2 and used in determining macrophage respiratory burst activity according to Section

Table 6.1 Composition (g $kg^{-1}$ feed) of diets used in Trials 1 and 2 for Atlantic salmon

| | Pellet Size (mm) | | |
|---|---|---|---|
| Ingredient | 4 | 6 | 9 |
| Fish meal, Scandinavian | | | 385.8 |
| Fish meal, S-American | 506.1 | 472.5 | |
| Corn gluten | 100.0 | 100.0 | 100.0 |
| Soybean meal, extracted | 100.0 | 100.0 | 100.0 |
| Wheat | 46.4 | 79.7 | 98.7 |
| Oil (FO, RO, LO, PO) | 222.5 | 222.8 | 290.5 |
| Premix | 25.0 | 25.0 | 25.0 |

Scandinavian LT-fish meal (Nordsildmel, Norway)

S-American fish meal (Consortio Malla, Pisco, Peru)

FO (capelin oil) (Nordsildmel, Norway)

RO (Oelmühle Hamburg, Germany)

LO (crude E.C.C. from N.V. Oliefabriek Lictervelde, Belgium)

PO (Migase, Spain)

FO was supplemented with 200 ppm BHT, LO with 500 ppm Ronoxan A (Roche, Switzerland) as antioxidants. No antioxidants were added to the RO.

Table 6.2 Fatty acid compositions (% of total fatty acids by weight) of the experimental diets given in Trial 1 and 2.

| | 6mm | | | 9mm | | |
|---|---|---|---|---|---|---|
| | FO Diet | 75%VO Diet | 100% VO Diet | FO Diet | 75%VO Diet | 100% VO Diet |
| % lipid | 27.9 | 27.5 | | 30.8 | 32.5 | |
| 14:0 | 6.1 | 2.4 | 1.0 | 6.3 | 2.1 | 0.6 |
| 15:0 | 0.5 | 0.2 | | 0.5 | 0.2 | |
| 16:0 | 14.7 | 16.0 | 15.9 | 14.2 | 16.3 | 15.3 |
| 18:0 | 2.8 | 3.3 | 3.4 | 2.0 | 2.6 | 2.7 |
| Total saturated | 24.3 | 21.9 | 21.9 | 23.0 | 21.2 | 19.4 |
| 16:1n-9 | 0.2 | 0.1 | | 0.2 | 0.1 | |
| 16:1n-7 | 4.8 | 1.9 | 0.8 | 4.6 | 1.5 | 0.5 |
| 18:1n-9 | 13.5 | 35.2 | 42.7 | 13.0 | 35.2 | 43.0 |
| 18:1n-7 | 2.5 | 2.3 | 2.2 | 2.3 | 2.6 | 2.4 |
| 20:1n-11 | 0.6 | 0.2 | 0.0 | 0.5 | 0.2 | 0.0 |
| 20:1n-9 | 9.6 | 3.3 | 1.3 | 11.0 | 3.7 | 1.3 |
| 20:1n-7 | 0.2 | 0.1 | 0.0 | 0.2 | 0.1 | 0.0 |
| 22:1n-11 | 14.0 | 4.2 | 0.7 | 16.6 | 4.7 | 0.8 |
| 22:1n-9 | 0.9 | 0.6 | 0.2 | 1.4 | 0.6 | 0.2 |
| 24:1n-9 | 0.7 | 0.3 | 0.0 | 0.7 | 0.2 | 0.0 |
| Total monounsaturated | 47.0 | 48.2 | 47.9 | 50.5 | 49.1 | 48.2 |
| 18:2n-6 | 4.0 | 11.8 | 14.6 | 3.2 | 13.5 | 17.1 |
| 18:3n-6 | 0.1 | 0.0 | | 0.1 | 0.0 | |
| 20:2n-6 | 0.2 | 0.1 | 0.0 | 0.3 | 0.1 | 0.0 |
| 20:3n-6 | 0.1 | 0.0 | | 0.0 | 0.1 | |
| 20:4n-6 | 0.5 | 0.2 | 0.1 | 0.4 | 0.1 | 0.0 |
| 22:4n-6 | 0.0 | 0.0 | | 0.0 | 0.0 | |
| 22:5n-6 | 0.2 | 0.1 | | 0.2 | 0.0 | |
| Total n-6 | 5.1 | 12.2 | 14.7 | 4.1 | 13.8 | 17.1 |
| 18:3n-3 | 1.1 | 8.5 | 11.4 | 1.3 | 9.5 | 13.4 |
| 18:4n-3 | 2.4 | 0.8 | 0.2 | 2.6 | 0.8 | 0.2 |
| 20:3n-3 | 0.1 | 0.1 | | 0.2 | 0.0 | |
| 20:4n-3 | 0.7 | 0.2 | 0.0 | 0.7 | 0.2 | 0.0 |
| 20:5n-3 | 6.7 | 2.8 | 1.2 | 6.4 | 2.0 | 0.6 |
| 22:5n-3 | 1.1 | 0.4 | 0.2 | 0.8 | 0.2 | 0.0 |
| 22:6n-3 | 10.4 | 4.5 | 2.1 | 9.5 | 2.9 | 1.0 |
| Total n-3 | 22.4 | 17.3 | 15.2 | 21.3 | 15.7 | 15.2 |

FO = fish oil diet, VO = blended vegetable oil diet. Data are presented as mean (n = 2).

2.4.2. Phagocytic activity of head kidney macrophages was determined according to Section 2.4.3.

### 6.2.1.4 Serum lysozyme activity

Serum was collected from blood samples according to Section 2.3.3 to measure serum lysozyme activity (Section 2.4.5) and in Trial 1 to determine antibody titres against *A. salmonicida* (i.e. these fish were vaccinated against *A. salmonicida*) (Section 2.5.3).

### 6.2.1.5 Fatty acid analysis of peripheral blood leukocytes

Samples of PBL from 4 fish dietary treatment$^{-1}$ were isolated from whole blood by density gradient centrifugation according to Section 2.8.1. Total lipid from the isolated PBL was extracted following the procedure outlined in Section 2.8.2 and by Folch *et al.*, (1957). Gas chromatography was used to identify the fatty acid methyl esters following the method outlined in Section 2.8.2.

### 6.2.1.6 Histopathology

Heart, liver and intestinal sections were sampled for histopathological assessment from 6 fish dietary treatment$^{-1}$ according to the methods outlined in Sections 2.3.5, 2.7.1 and 2.7.2. Random slides were assessed "blind" externally by a fish veterinarian of the Fish Vet. Group, Inverness according to Section 2.7.3., to eliminate any bias in interpretation.

### 6.2.1.7 Determination of antibody titres to *Aeromonas salmonicida* in Trial 1

In the freshwater stage Atlantic salmon were vaccinated against furunculosis with an inactivated culture of *A. salmonicida* subspecies s*almonicida* using an Alphaject 1200 vaccine in January 2003 at Marine Harvest hatchery, Invergarry, Scotland. Twelve fish from both dietary treatments were bled and serum separated and stored at –20 °C until used in the ELISA. Serum from 12 individual fish, from both dietary treatments, were sampled prior to vaccination (0 weeks) and at 3, 6 and 9 weeks after vaccination (34, 47, 50 and 53 weeks from onset of feeding), and tested for antibodies against *A. salmonicida* by ELISA (Section 2.5.3).

### 6.2.1.8 *Vibrio anguillarum* bacterial challenge

To measure the natural resistance of Atlantic salmon to a bacterial challenge, a sub group of Atlantic salmon fed either with the control FO diet or the VO diet were moved to Marine Harvest's Lochailort Trials Unit, Lochailort, Scotland. Sixty fish from each dietary treatment were pan-jet marked with an aqueous solution of alcian blue dye (1 % w/v) to distinguish between dietary groups. Ten fish from both dietary treatments were grouped into six replicate tanks. Fish were allowed 9-12 weeks to acclimate after transport and marking. Although initially fish were to be challenged by co-habitation this was later ruled out due to insufficient stocking densities achieved using this method. This was because of the low numbers of available fish for the bacterial challenge, as the remaining fish were required for continuance onto the seawater stage of the trial. Fish were challenged using an i.p injection of *V. anguillarum* (*Listonella anguillarum*) (0.1 ml fish$^{-1}$, > 0.4 x $10^8$ cells) originally obtained from BioMar (ref. DK91) and the disease challenge ran for 21 days.

The antibody response is normally at a maximum around 6 weeks post-vaccination although this is temperature dependent. It should be noted that on the advise of the staff at the challenge facility there was no cross protection from the vaccination with *A. salmonicida*, so ultimately the fish used in the challenge would all be classed as unvaccinated with regard to *V. anguillarum*.

#### 6.2.1.9 Statistical analysis

Significance of difference between dietary treatments within each sample time point was determined by Student's t-test using $p<0.05$ as a cut off for significance. Differences between means were determined by Newman-Keuls post-test.

### *6.2.2 Trial 2 RAFOA II Norway – Replacement of FO with a blend of LO, RO and PO in Atlantic salmon*

#### 6.2.2.1 Dietary Groups

The feeding experiment started on the 5th April 2002 at Lerang Research Station, Nutreco ARC, Stavanger, Norway. Juvenile Atlantic salmon, ready for exogenous feeding (mean weight 0.187 g) were distributed into six 1 $m^2$ tanks (n = 2000 fish $tank^{-1}$). The two experimental diets with either 100 % FO (capelin oil) or 100 % VO (made up of 55 % RO, 15 % LO and 30 % PO) were fed in excess to triplicate tanks. These VO blends were chosen to give a similar ratio of saturated, monounsaturated and polyunsaturated fatty acids to that found in Northern hemisphere FO. The diets were produced by Nutreco ARC, Stavanger and the dietary formulation and fatty acid compositions are presented in Tables

6.1 and 6.2. Average temperature was 12.6 °C from start to June, and 9.7 °C from June to October and the tanks were exposed to continuous light.

### 6.2.2.2 Collection of samples for immunological studies

Trial 2 was sampled by Dr Bente Torstensen of the National Institute of Nutrition and Seafood Research (NIFES) in Norway. Fifteen fish dietary treatment$^{-1}$ were bled and serum collected according to Section 2.3.3 and frozen immediately. Frozen serum samples were sent to the Institute of Aquaculture for determination of serum lysozyme activity (Section 2.4.5). Fish were bled at weeks 0, 3, 6, and 9 weeks post vaccination (28, 32, 35 and 38 weeks from the onset of feeding (December 2002), respectively). The salmon parr were vaccinated in the fresh water stage by i.p injection of NORVAX® MINOVA 6 Vet (Intervet Norbio) containing *A. salmonicida* susp. *salmonicida*, *V. salmonicida*, *V. anguillarum* serotype 01 and 02, *V. viscosus* and a surface protein from infectious pancreatic necrosis virus (IPNV) serotype Sp.

### 6.2.2.3 Statistical analysis

Significance of difference between dietary treatments was determined by Student's t-test using $p<0.05$ as a cut off for significance. Differences between means were determined by Newman-Keuls post-test.

### *6.2.3 Trial 3 RAFOA II Spain – Replacement of FO with a blend of LO, RO and PO in Sea bass*

#### 6.2.3.1 Dietary Groups

On the 10th of July 2002, approximately 1800 sea bass (average total length = 7.9 ± 0.5 cm; average total body mass = 5.2 ± 1.0 g) were purchased from MARESA in Huelva, Spain, and transported to the laboratory facilities at the University of Cádiz in the Faculty of Marine and Environmental Sciences in Puerto Real (Cádiz). Fish were transported at a density of approx. 1.8 kg/m$^3$, salinity 39 %, temperature 24 °C and sea water in the transport tank was saturated with oxygen. On arrival at the laboratory facilities, fish were stocked in 5000 l rectangular tanks at 600 fish tank$^{-1}$ (approximately 0.6 kg / m$^3$) (Figure 6.2). On the 12th of July 2002, fish were started on the experimental diets, provided by NUTRECO, and fed *ad libitum* to satiation with mechanic belt automatic feeders. Diets were provided in 1, 2 and 3mm of pellet sizes. The dietary formulation and fatty acid composition of the experimental diets are presented in Tables 6.3 and 6.4. The diets tested for sea bass contained approximately 20 - 23 % oil. The control diet contained anchovy oil and the added oil combinations for the experimental diets were as follows: 100 % anchovy oil (FO); 40 % anchovy oil, 35 % LO, 15 % PO and 10 % RO (VO1); 40 % anchovy oil, 24 % LO, 12 % PO and 24 % RO (VO2). The blends of VO used for inclusion in the sea bass diets had a maximum level of 60 % VO due to the limited ability of marine fish to convert ALA, EPA and DHA.

Figure 6.2 Tank facilities at the University of Cádiz in the Faculty of Marine and Environmental Sciences.

Table 6.3 Composition of experimental diets used in Trial 3 (g $kg^{-1}$).

| **Diets** | FO | VO1 | VO2 |
|---|---|---|---|
| Protein | 47.8 | 46.2 | 47.8 |
| Lipids | 20.2 | 21.3 | 21.1 |
| Carbohydrate | 15.1 | 15.3 | 15.5 |
| Ash | 6.7 | 6.3 | 6.2 |
| Moisture | 10.2 | 10.9 | 9.4 |
| **Components** | | | |
| Fish meal [1] | 400.0 | 400.0 | 400.0 |
| Maize gluten [2] | 262.7 | 262.7 | 262.7 |
| Wheat [3] | 152.3 | 152.3 | 152.3 |
| Oil | 160.0 | 160.0 | 160.0 |
| Premixes [4] | 25.0 | 25.0 | 25.0 |
| **Composition (%) of added oil** | | | |
| Anchovy oil (FO) [5] | 100 | 40 | 40 |
| RO [6] | 0 | 10 | 24 |
| LO [7] | 0 | 35 | 24 |
| PO [8] | 0 | 15 | 12 |

[1] Scandinavian LT-fish meal (Nordsildmel, Norway)

[2] Cargill, Staley, USA

[3] Statkorn, Oslo, Norway

[4] Vitamin and mineral premix added min. to NRC recommendations

[5] Anchovy oil (Denofa, Fredrikstad, Norway) supplemented with 200 ppm BHT

[6] Crude rapeseed oil (Oelmunhle Hamburg, Germany) no antioxidant added

[7] Crude E.C.C. linseed oil (N.V. Oliefabriek Lictervelde, Belgium) supplemented with 500 ppm

[8] Crude palm oil (Migase, Spain)

FO = fish oil, VO1 = anchovy oil, linseed oil, palm oil and rapeseed oil (40:35:15:10)

VO2 = anchovy oil, linseed oil, palm oil and rapeseed oil (40:24:12:24)

Table 6.4 Fatty acid compositions (% of total fatty acids by weight) of the experimental diets given in Trial 3.

| Dietary Treatments | FO | VO1 | VO2 |
|---|---|---|---|
| Fatty Acid (%) | | | |
| 14:0 | 5.3 ± 0.1 | 3.2 ± 0.1 | 3.1 ± 0.0 |
| 15:0 ISO | 0.5 ± 0.1 | 0.5 ± 0.2 | 0.4 ± 0.1 |
| 15:0 | 0.5 ± 0.0 | 0.4 ± 0.0 | 0.3 ±0.0 |
| 16:0 | 16.4 ± 0.2 | 14.9 ± 0.2 | 14.2 ± 0.2 |
| 18:0 | 3.4 ± 0.0 | 3.2 ± 0.0 | 3.0 ± 0.0 |
| Total saturates | 26.4 ± 0.3 | 22.3 ± 0.3 | 21.3 ± 0.2 |
| 16:1n-9 | 5.2 ± 0.1 | 3.1 ± 0.0 | 3.0 ± 0.1 |
| 16:1n-7 | 2.3 ± 0.4 | 1.8 ± 0.1 | 1.8 ± 0.0 |
| 18:1n-9 | 11.2 ± 0.1 | 17.7 ± 0.2 | 20.4 ± 0.3 |
| 18:1n-7 | 2.1 ± 0.0 | 1.7 ± 0.0 | 1.8 ± 0.0 |
| 20:1n-9 | 2.7 ± 0.0 | - | - |
| 22:1n-11 | 2.8 ± 0.0 | 2.4 ± 0.0 | 2.6 ± 0.1 |
| 24:1n-9 | 0.6 ± 0.0 | 0.5 ± 0.0 | 0.5 ± 0.0 |
| Total monoenes | 26.9 ± 0.6 | 27.2 ± 0.2 | 30.2 ± 0.2 |
| 16:2 | 0.7 ± 0.0 | 0.4 ± 0.0 | 0.5 ± 0.1 |
| 16:3 | 0.8 ± 0.0 | 0.4 ± 0.0 | 0.3 ± 0.1 |
| 16:4 | 1.1 ± 0.0 | 0.6 ± 0.0 | 0.6 ± 0.0 |
| 18:2n-6 | 7.1 ± 0.0 | 10.9 ± 0.1 | 11.3 ± 0.2 |
| 18:3n-3 | 2.8 ± 0.1 | 13.1 ± 0.1 | 10.6 ± 0.1 |
| 18:4n-3 | 2.2 ± 0.0 | 1.4 ± 0.0 | 1.3 ± 0.0 |
| 20:4n-6 | 0.7 ± 0.0 | 0.4 ± 0.0 | 0.4 ± 0.0 |
| 20:4n-3 | 0.6 ± 0.0 | 0.3 ± 0.0 | 0.3 ± 0.0 |
| 20:5n-3 | 11.3 ± 0.1 | 6.8 ±0.1 | 6.7 ± 0.1 |
| 22:5n-6 | 0.2 ± 0.0 | 0.1 ± 0.0 | 0.2 ± 0.0 |
| 22:5n-3 | 1.3 ± 0.0 | 0.8 ± 0.0 | 0.7 ± 0.0 |
| 22:6n-3 | 11.0 ± 0.1 | 6.9 ± 0.1 | 6.5 ± 0.1 |
| Total n-6 | 9.2 ± 0.1 | 12.4 ± 0.3 | 13.4 ± 0.4 |
| Total n-3 | 31.6 ± 0.4 | 30.5 ± 0.3 | 27.2 ± 0.3 |
| HUFA n-6 | 1.2 ± 0.1 | 0.7 ± 0.1 | 0.9 ± 0.5 |
| HUFA n-3 | 24.6 ± 0.3 | 14.9 ± 0.2 | 14.4 ± 0.0 |

Results are means ± SD, n=3. Values having different superscript letters are significantly different ($p<0.05$). HUFA, highly unsaturated fatty acid, (-) represents not detected. FO = fish oil diet, VO = blended vegetable oil diet.

#### 6.2.3.2 Collection of samples for immunological and histological studies

The methodology described in Sections 6.2.1.3 – 6.2.1.6 were followed in dietary Trial 3. Nine fish dietary treatment$^{-1}$ were sampled after 64 weeks (October 2003) from the onset of feeding the experimental diets.

#### 6.2.3.3 Measurement of $PGE_2$

Plasma was isolated from heparinised blood samples from Trial 3 and used in an EIA to determine the concentration of $PGE_2$ in plasma according to Section 2.6.

#### 6.2.3.4 Statistical analysis

Significance of difference between dietary treatments was determined by ANOVA using $p<0.05$ as a cut off for significance. Differences between means were determined by Newman-Keuls post-test.

## 6.3 Results

### *6.3.1 Trial 1 RAFOA II Scotland –Replacement of FO with a blend of LO, RO and PO in Atlantic salmon*

#### 6.3.1.1 Growth, tissue lipid content and flesh fatty acid compositions

According to Bell *et al.*, (personal communication) there was no difference in growth between fish fed the FO-based or the 75 % VO-based diets prior to transferring them to sea

water or when measured 33 weeks post-sea water transfer. Fish fed the FO diet had an average weight of 1.25 kg and those maintained on the VO diet had an average weight of 1.28 kg. Fish sampled 16 weeks post-sea water transfer showed no differences in flesh and liver lipid content between treatments. However, the fatty acid composition of these tissues was correlated with the dietary fatty acids, a significant increase in the flesh levels of 16:0, 18:0, oleic acid, LA, 20:2(n-6), 20:3(n-6), ALA, 20:4(n-3) and total n-6 PUFA were found in salmon fed the 75 % VO diet compared to fish fed the FO diet. Conversely, a significant decrease in 14:0, 16:1(n-7), 18:1(n-7), 20:1(n-9), 22:1(n-11), 24:1, 18:4(n-3), EPA, DHA and the ratio of n-3/n-6 was found in VO fed salmon. It is worth noting that fish fed the 75 % VO diet had a higher incidence of cataracts compared to FO fed fish (Bell *et al.*, personal communication).

### 6.3.1.2 Haematology and Immune function

Salmon parr were assayed before vaccination against Furunculosis in January 2003 (after 34 weeks from the onset of feeding) and some significant differences were found in non-specific immune activity between dietary groups (Figure 6.3). Significant differences were found in % haematocrit values, and the total number of circulating leukocytes and erythrocytes. Salmon fed the VO diet for 34 weeks had a haematocrit value of 31.9 % compared to 20.1 % in fish fed the FO diet ($p=0.0003$) (Figure 6.3a). Fish fed the VO diet after 47 weeks had significantly higher haematocrit values (40.6 %) compared to fish fed the FO diet (34.3 %, $p = 0.0008$) as shown in Figure 6.3a. No differences in % haematocrit were reported after 50 weeks, however after 53 weeks, fish fed the VO diet had a

significantly higher haematocrit value (37.5 %) compared to fish fed the FO diet (29.2 %) ($p=0.0217$) (Figure 6.3a). After post-sea water transfer, no differences in % haematocrit were reported between dietary groups.

The only statistical difference found when measuring the total number of circulating leukocytes was found after 34 weeks. Fish fed the VO diet had an average count of 1.9 x $10^7$ cells $ml^{-1}$ compared to 1.2 x$10^7$ cells $ml^{-1}$ found in fish fed the FO diet as shown in Figure 6.3b ($p=0.0146$). The effect of feeding the experimental diets on the total number of circulating erythocytes at the different sampling points are shown in Figure 6.4. After feeding the diets for 34 weeks, the fish fed the VO diet had significantly higher erythrocyte numbers (7.0 x $10^8$ cells $ml^{-1}$) than fish fed the FO diet (3.4 x $10^8$ cells $ml^{-1}$) ($p<0.0001$). The opposite was found when fish were sampled after 47 weeks (3 weeks post-vaccination). Fish fed the VO diet had significantly reduced numbers of erythrocytes (5.2 x $10^8$ cells $ml^{-1}$) compared to those fish maintained on the FO diet (7.6 x $10^8$ cells $ml^{-1}$) ($p=0.0078$)(Figure 6.4). No other differences between dietary treatments were found at any of the other sample points.

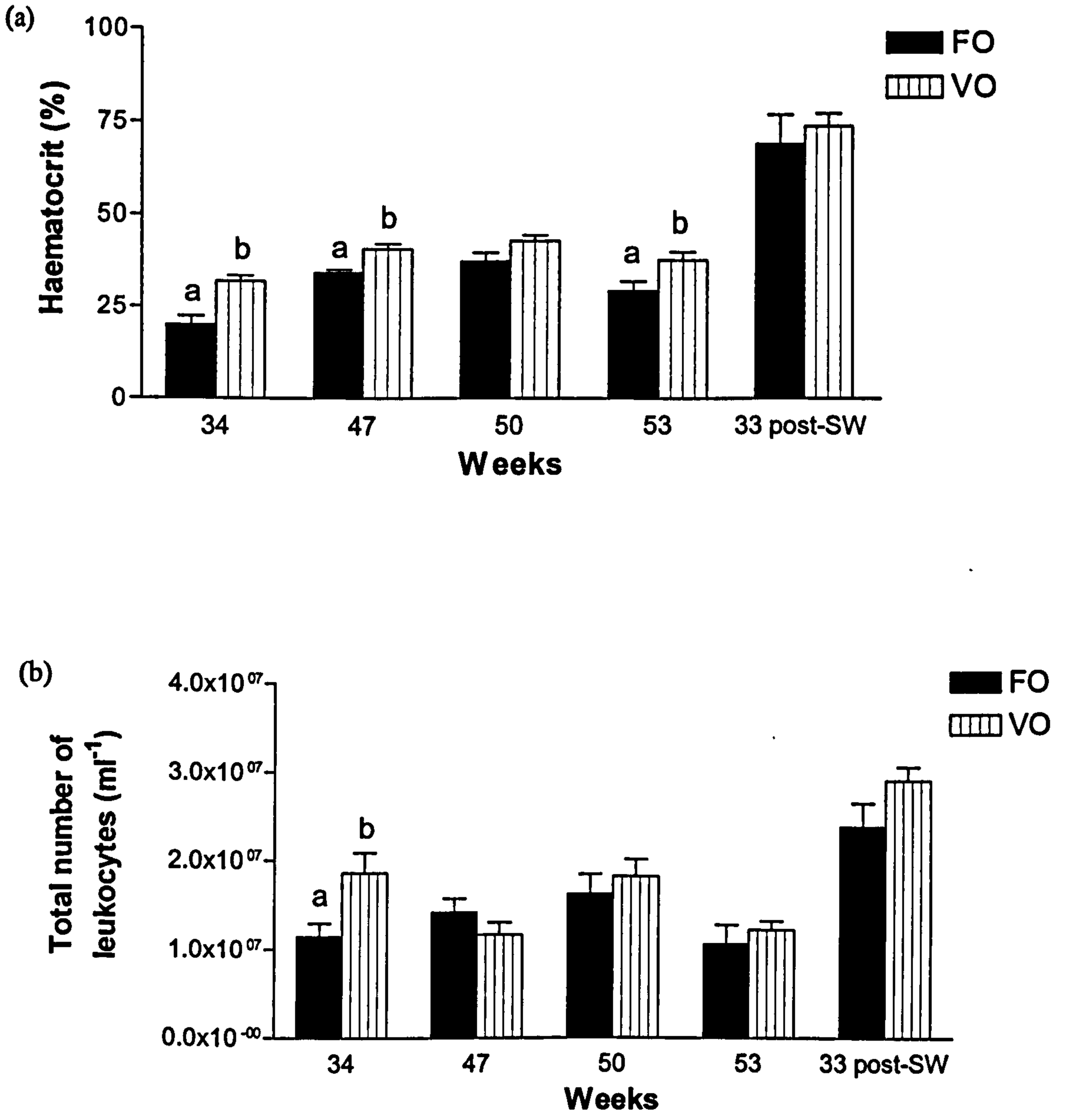



Figure 6.3 Effect of feeding the experimental diets to salmon on (a) % haematocrit (b) the total number of circulating leukocytes measured at each sample time point. Values are means ± SEM, n=12. FO = fish oil diet, VO = blended vegetable oil diet, SW = sea water transfer. Significant differences ($p<0.05$) within a time point (weeks) are represented by different column letters.

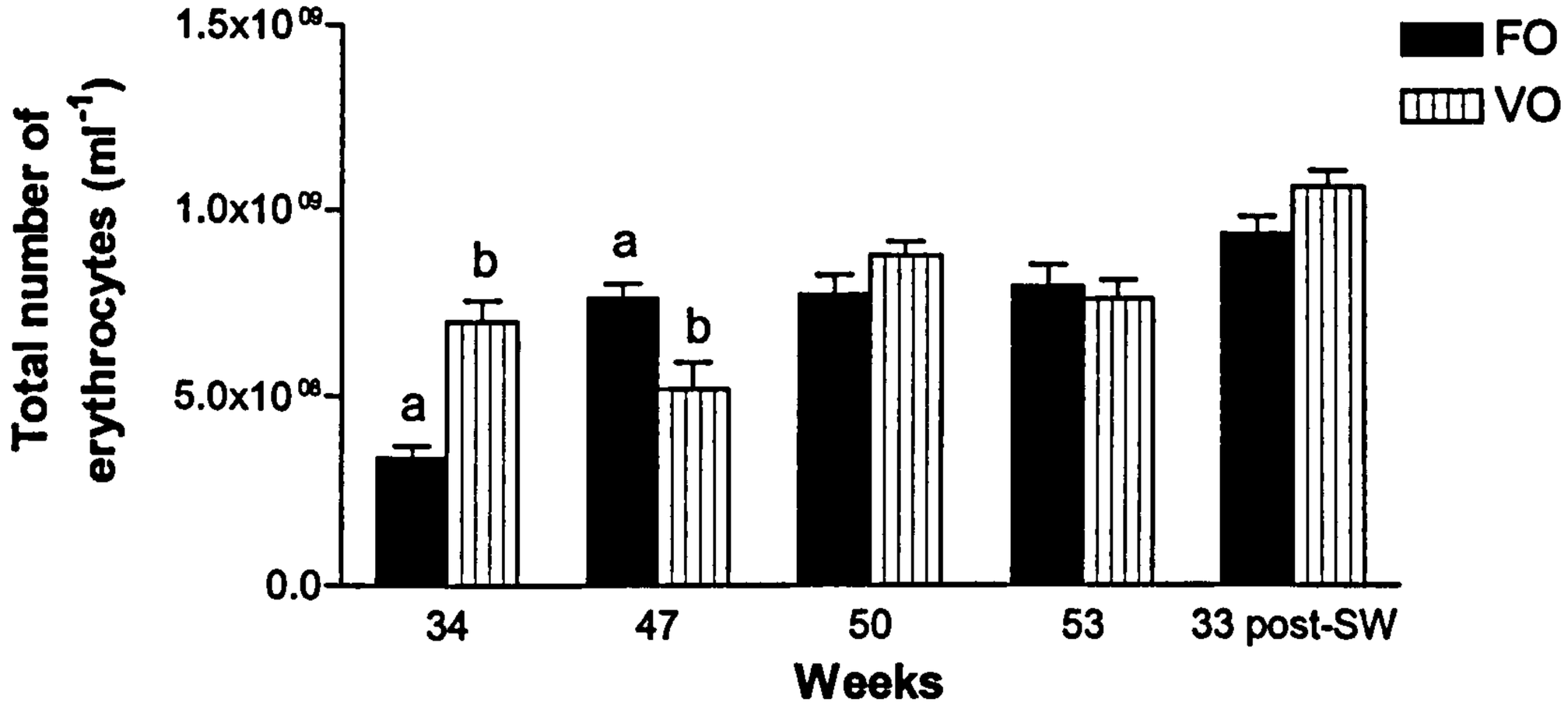



Figure 6.4 Effect of feeding the experimental diets to salmon on the total number of circulating erythrocytes measured at each sample time point. Values are means ± SEM, n=12. FO = fish oil diet, VO = blended vegetable oil diet, SW = sea water transfer. Significant differences within a time point (weeks) are represented by different column letters.

### 6.3.1.3 Macrophage function

Macrophage function as measured by the reduction of NBT by salmon head kidney macrophages was not altered by feeding either the 100 % FO or 75 % VO diet. No significant differences were apparent between dietary treatments measured at any of the time points sampled as shown in Figure 6.5. The only noticeable feature was that after feeding the experimental diets for 50 weeks, the head kidney macrophage respiratory burst activity was higher in both dietary groups compared to fish sampled at any of the other time points. The ability of salmon head kidney macrophages to phagocytose yeast particles at the various sample time points is shown in Table 6.5. No significant change in the phagocytic capacity was observed between the FO and VO dietary treatments.

### 6.3.1.4 Serum lysozyme activity

Salmon sampled pre- and post-vaccination in fresh water and after 33 weeks post-sea water transfer, showed no significant differences in serum lysozyme activity between fish fed the FO or VO diet as shown in Figure 6.6.

### 6.3.1.5 Fatty acid composition of peripheral blood leucocytes

After sampling the fish post-sea water transfer, the fatty acid composition of PBL was determined (Table 6.6). Overall there were no significant differences between the lipids of PBL from fish fed either the 100 % FO diet or the 75 % VO diet. The only major difference found was in the total amount of saturated fatty acids present. Fish fed the FO diet showed a higher value of total saturates (24.0 %) compared to 21.5 % found in fish fed the 75 %

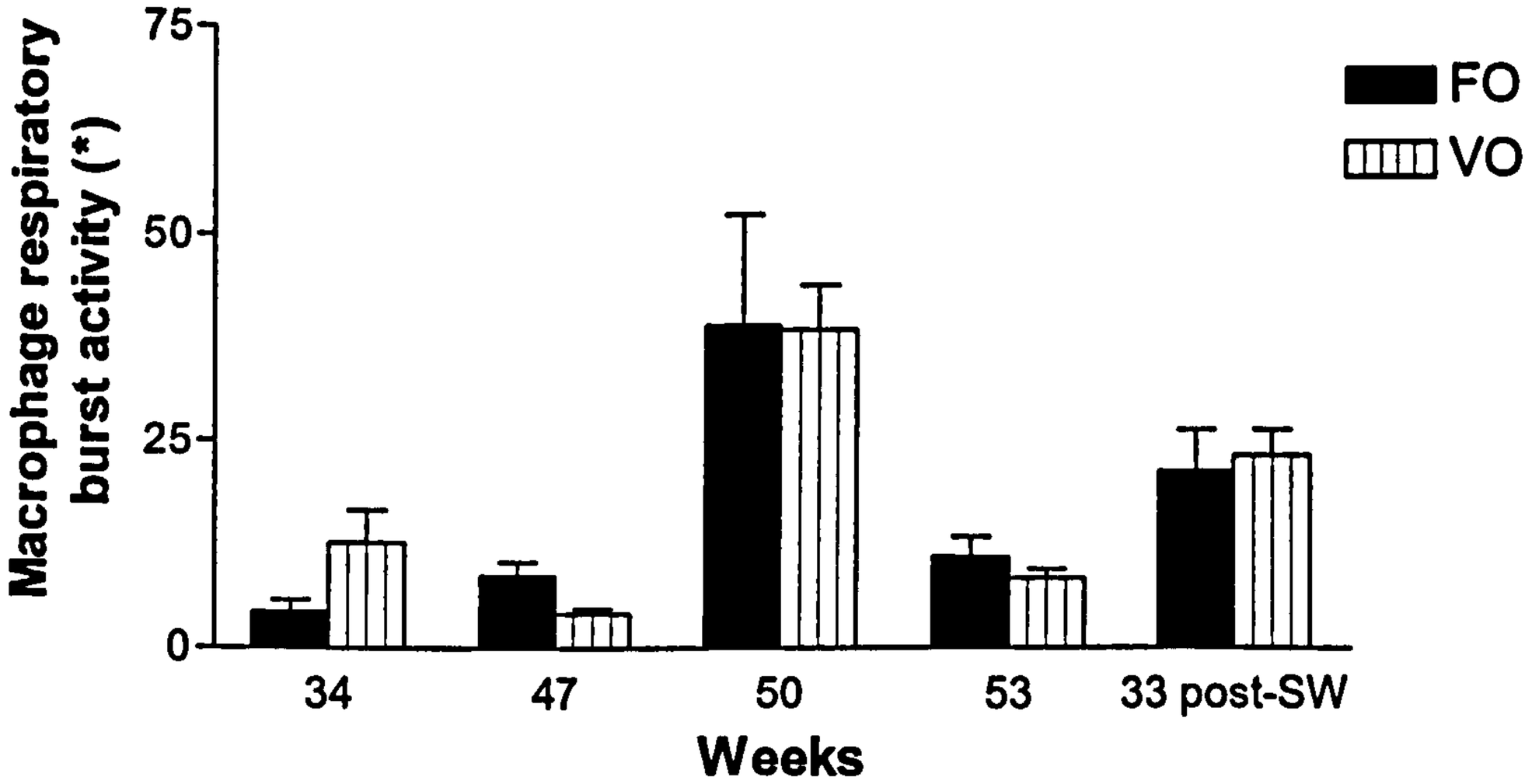



Figure 6.5 Effect of feeding the experimental diets on salmon head kidney macrophage respiratory burst activity (NBT reduction) sampled at each time point. Values are means ± SEM, n = 12. FO = fish oil diet, VO = blended vegetable oil diet, SW = sea water transfer. * = Absorbance per $10^5$cells x 100.

Table 6.5 Phagocytic capacity (% of macrophages which have phagocytosed yeast particles) of salmon head kidney macrophages fed the experimental diets at the various sampling time points. Values are means ± SEM, n = 12.

| Sample time point | FO Diet | VO Diet |
|---|---|---|
| 34 weeks feeding | 40.9 ± 17.4 | 44.7 ± 23.5 |
| 47 weeks feeding | 64.2 ± 26.3 | 52.0 ± 15.5 |
| 50 weeks feeding | 74.2 ± 11.6 | 59.6 ± 8.7 |
| 53 weeks feeding | 53.9 ± 14.7 | 40.2 ± 9.6 |
| 33 weeks post-sea water transfer | 46.3 ± 18.6 | 43.7 ± 16.9 |

FO = fish oil diet. VO = blended vegetable oil diet (25 % fish oil+ 75 % vegetable oil (linseed oil/rapeseed oil/palm oil mix).

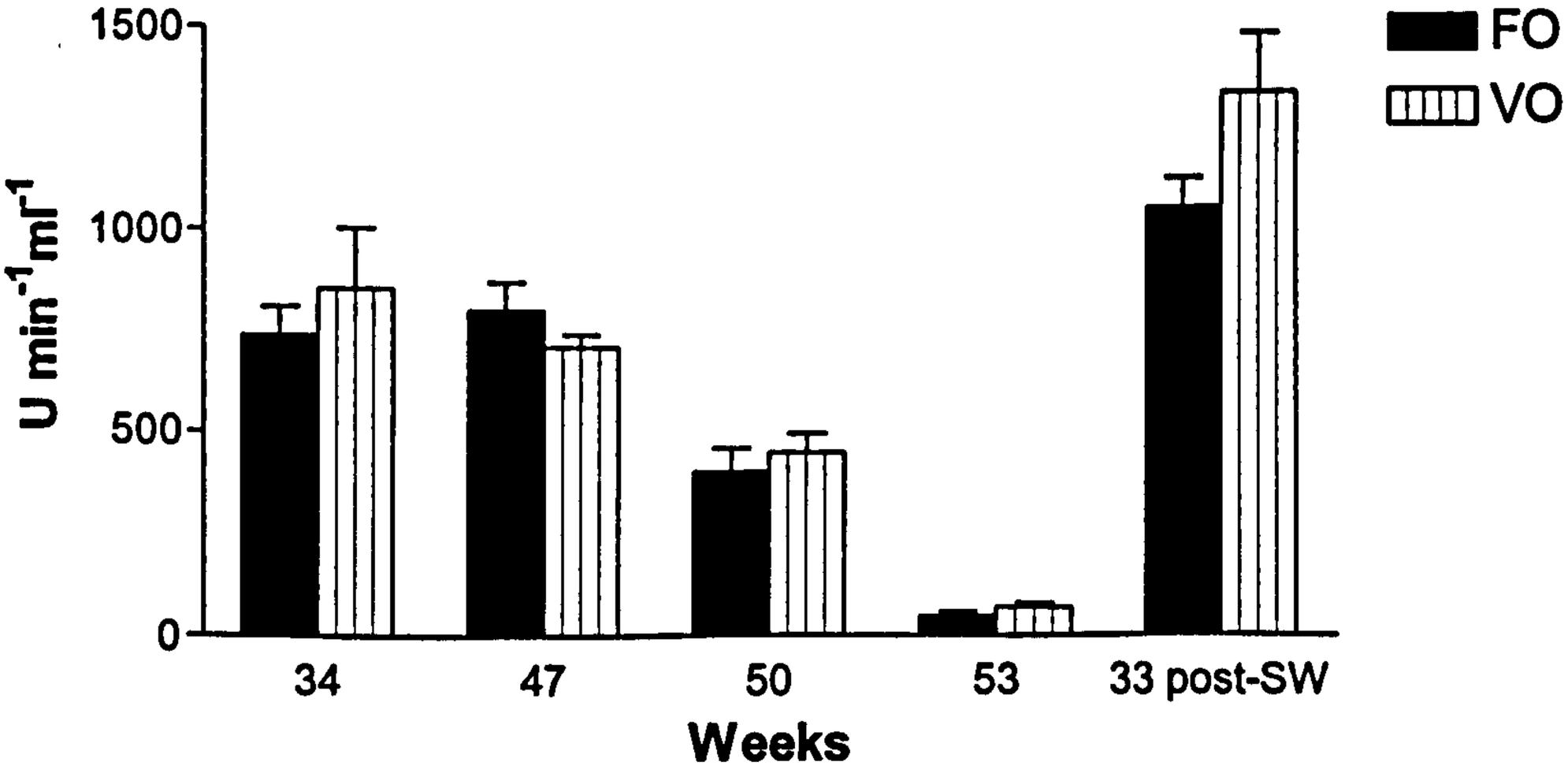



Figure 6.6 Effects of feeding the experimental diets on serum lysozyme activity sampled at each time point. Values are means ± SEM, n = 12. FO = fish oil diet, VO = blended vegetable oil diet, SW = sea water transfer.

Table 6.6 Fatty acid composition (wt %) of Atlantic salmon PBL isolated post-sea water transfer. Values are expressed as the mean per cent of total fatty acids for four fish ± SD. Values with different superscript letters within a row are significantly different ($p<0.05$).

| Fatty Acids | FO diet | VO diet |
|---|---|---|
| 14:0 | 1.6 ± 0.8 | 1.1 ± 0.7 |
| 15:0 | 0.4 ± 0.1 | 0.4 ± 0.2 |
| 16:0 | 17.2 ± 0.8 | 16.1 ± 1.4 |
| 18:0 | 4.4 ± 0.5 | 3.6 ± 0.5 |
| 20:0 | 0.2 ± 0.1 | 0.1 ± 0.0 |
| 22:0 | 0.3 ± 0.1 | 0.3 ± 0.2 |
| Total saturates | 24.0 ± 0.8[a] | 21.5 ± 1.8[b] |
| 16:1 (n-7) | 2.4 ± 0.9 | 2.5 ± 0.2 |
| 18:1 (n-9) | 12.9 ± 4.3 | 15.2 ± 4.1 |
| 18:1 (n-7) | 2.0 ± 0.2 | 1.6 ± 0.5 |
| 20:1 (n-9) | 3.3 ± 0.8 | 2.7 ± 1.2 |
| 20:1 (n-7) | 0.3 ± 0.1 | 0.4 ± 0.2 |
| 22:1 (n-11) | 2.7 ± 1.7 | 0.7 ± 0.1 |
| 22:1 (n-9) | 0.4 ± 0.2 | 0.4 ± 0.2 |
| 24:1 | 2.8 ± 1.0 | 2.6 ± 0.3 |
| Total monoenes | 26.8 ±1.9 | 24.6 ± 1.6 |
| 18:2 (n-6) | 2.9± 1.7 | 4.1 ± 1.8 |
| 18:3 (n-6) | 0.2 ± 0.0 | 0.2 ± 0.1 |
| 20:2 (n-6) | 0.6 ± 0.4 | 0.8 ± 0.3 |
| 20:3 (n-6) | 0.3 ± 0.2 | 0.3 ± 0.1 |
| 20:4 (n-6) | 1.8 ± 0.4 | 1.4 ± 0.5 |
| 22:4 (n-6) | 0.1 ± 0.0 | 0.1 ± 0.0 |
| 22:5 (n-6) | 0.4 ± 0.0 | 0.4 ± 0.1 |
| Total (n-6) | 6.2 ± 2.1 | 7.3 ± 1.7 |
| 18:3 (n-3) | 1.1 ± 0.8 | 2.0 ± 1.1 |
| 18:4 (n-3) | 0.3 ± 0.0 | 0.3± 0.2 |
| 20:3 (n-3) | 0.3 ± 0.2 | 0.3 ± 0.1 |
| 20:4 (n-3) | 0.7 ± 0.2 | 0.6 ± 0.1 |
| 20:5 (n-3) | 9.5 ± 2.0 | 8.0 ± 2.2 |
| 22:5 (n-3) | 2.1 ± 0.3 | 1.7 ± 0.4 |
| 22:6 (n-3) | 23.8 ± 2.0 | 23.8 ± 4.8 |
| Total (n-3) | 37.8 ± 2.5 | 36.7 ± 6.1 |
| Total PUFA | 44.0 ± 2.6 | 44.0 ± 6.7 |
| (n-3):(n-6) | 6.7 ± 2.5 | 5.2 ± 1.6 |

VO diet. Although the levels of oleic acid, LA and ALA were higher in fish fed with the VO diet, no significant differences were found.

### 6.3.1.6 Antibody Titres

Antibody titres from the present study are shown in Figure 6.7, where values are expressed as antibody titre (-Log 2 +1) ± SE, measured by ELISA. Antibody levels from vaccinated fish were monitored at 3 week intervals after vaccination. No significant differences were observed between Atlantic salmon fed 100 % FO and 75 % VO regarding vaccination efficacy. In conclusion, no effect on vaccination efficacy of dietary lipid source was observed.

### 6.3.1.7 Histopathology

No pathology was observed in any of the heart sections taken from fish fed for 34 weeks (pre-vaccination) on the FO diet (Figure 6.8a), apart from a very small area of apical pericarditis in one fish. Salmon maintained on the VO diet exhibited no cardiomyopathy in any of the heart sections examined. One fish had a small degree of pericarditis affecting the apex of the ventricle as shown in Figure 6.8b, but no other heart pathology was found. Another fish had some small focal areas of endocarditis with associated small mural thrombi but no other associated pathology was found.

When comparing the histological findings from fish sampled after 47, 50 and 53 weeks of feeding (3, 6, and 9 weeks post vaccination), no significant pathology was seen in any heart

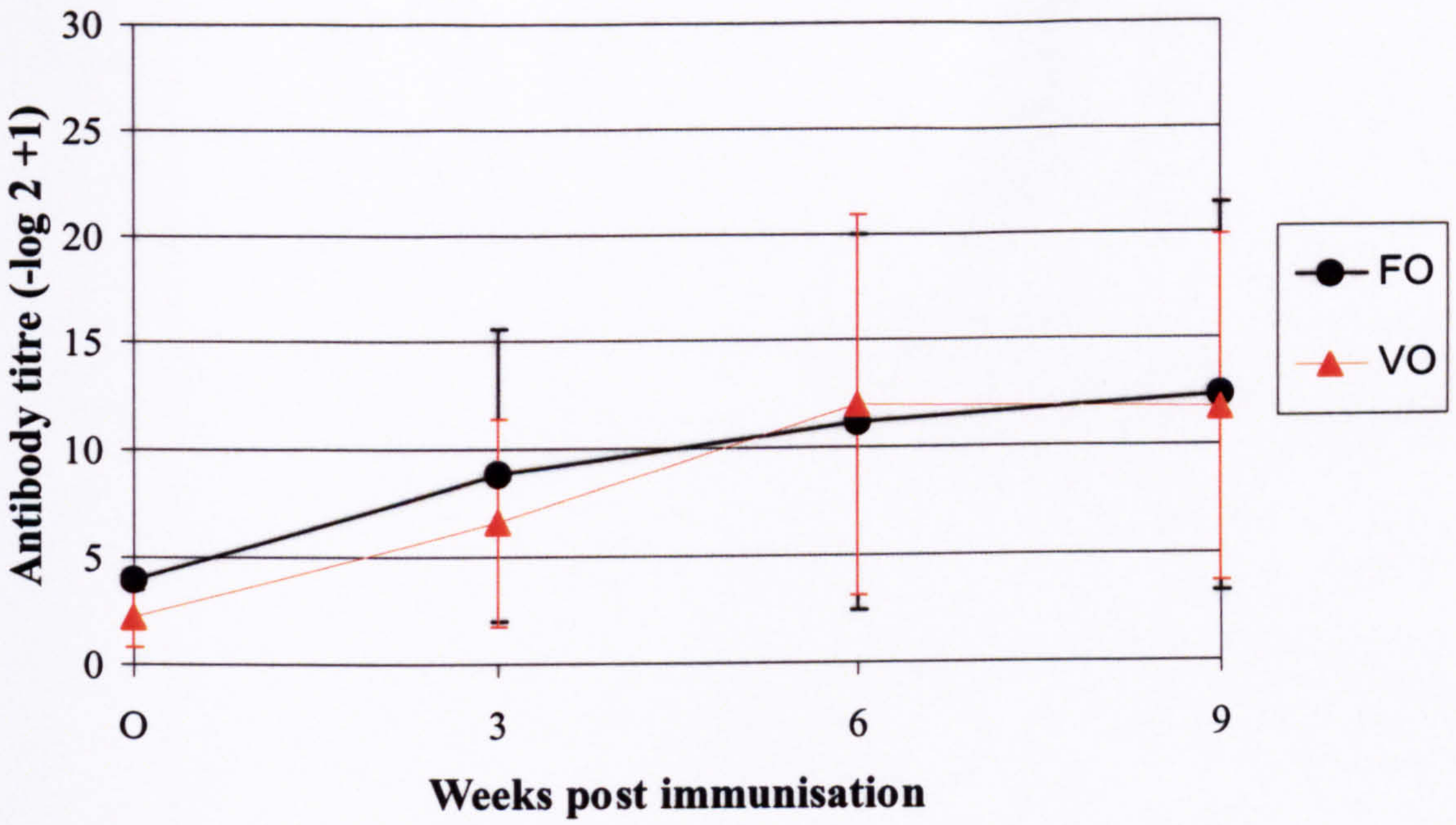



Figure 6.7 Serum antibody titres of Atlantic salmon in Trial 1 vaccinated with *Aeromonas salmonicida* vaccine measured by ELISA. Values are means ± SEM, n = 12.

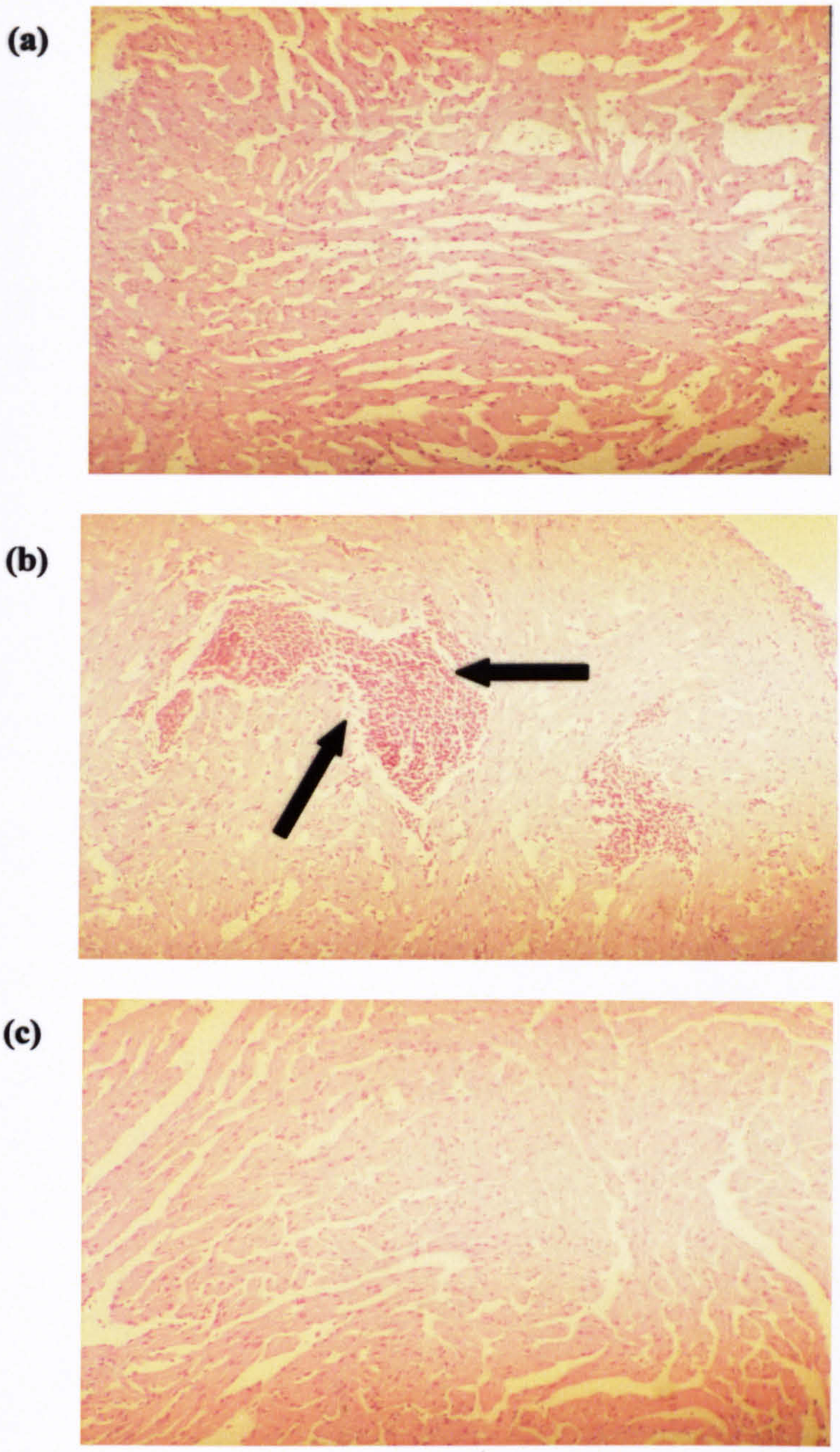



Figure 6.8 Histopathology of salmon hearts (a) sampled after 34 weeks feeding 100 % FO diet (pre-vaccination) showing a normal structure (mag.x 175) (b) sampled 34 weeks feeding 75 % VO diet (pre-vaccination) showing slight pericarditis at the apex of the ventricle (mag.x 175) (c) sampled after 47 weeks feeding 100 % FO diet (3 weeks post vaccination) showing a normal histological appearance (mag.x 175).

section from fish fed the FO diet or the VO diet. A normal histological appearance was evident in heart from a fish fed the FO diet sampled after 47 weeks of feeding (Figure 6.8c). Occasional minor pericarditis on the apex of the ventricle was seen, but this is often a frequent finding and can sometimes be associated with vaccination, but was at a very low level so can be dismissed. After seawater transfer very few pathological changes were identified in any of the heart sections examined. Only very minor levels of endocarditis were seen in sections from fish fed the FO diet and in one section on the VO diet. Minimal pericarditis was present in one section from a fish on the FO diet. One section from both dietary treatments had a small focus of necrosis and inflammation within the muscle. In all cases these were very minor changes and very unlikely to have been clinically significant or had any effect on overall health of the fish.

A moderate fat content was found in the livers of most fish, sampled after 34 weeks feeding (pre-vaccination) with single fat vacuoles within the hepatocytes, which were variable in size and distribution. Inflammation was confined to the occasional inflammatory cell, apart from in three fish where patches of inflammation were seen as illustrated in Figure 6.9a, which shows a section of liver from a fish fed the FO diet. In general however, there appeared to be less liver pathology in the FO fish compared with the VO diet. All fish had moderate or low to moderate fat levels within the liver (Figure 6.9b). Fat vacuolation within the hepatocytes was mostly in single vacuoles but very variable in size and distribution throughout the tissue. Two of the fish from the VO dietary group tended to have smaller vacuoles. Necrosis of the hepatocytes was seen to a greater or lesser extent in all fish. This varied from occasional single cell necrosis in one of the fish to a greater degree of patchy necrosis (Figure 6.9c) and cellular breakdown resulting in loss

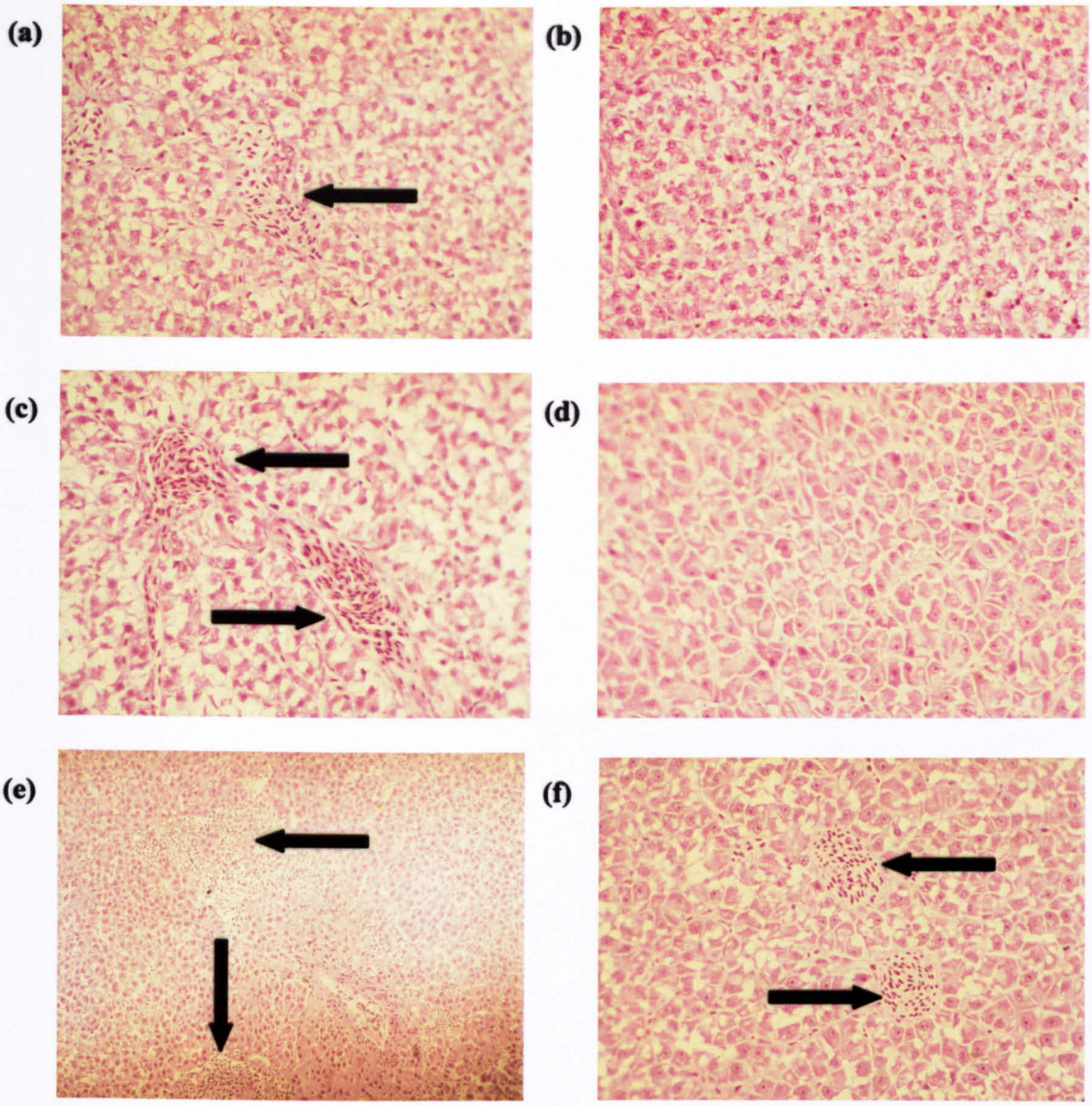



Figure 6.9 Histology of salmon livers (a) sampled after 34 weeks feeding 100% FO diet (pre- vaccination) showing a small area of inflammation (mag.x 430) (b) sampled after 34 weeks feeding 75% VO diet (pre-vaccination) showing a normal appearance with moderate fat content with variable fat vacuoles (mag.x 430) (c) sampled after 34 weeks feeding 75% VO diet (pre-vaccination) showing patchy necrosis and the presence of cellular infiltrate (mag.x 430) (d) sampled after 47 weeks feeding 100 % FO diet (3 weeks post vaccination) showing a normal appearance with moderate fat content with single vacuoles (mag.x 430) (e) sampled after 47 weeks feeding 75% VO diet (3 weeks post vaccination) showing a small degree of single cell necrosis (mag.x 175) (f) sampled after 50 weeks feeding 100 % FO diet (6 weeks post vaccination) showing more signs of single cell necrosis than after 47 weeks feeding (mag. X 430).

of areas of normal architecture in all of the other five fish examined. Levels of inflammatory cell infiltration varied from occasional inflammatory cells in three of the fish to areas of inflammatory cell accumulation in the other three fish liver sections. Moderate pvc was seen in all but one of the fish examined. In the liver of salmon fed the VO diet, fat was moderate in most sections assessed (scored 2-3) as shown in Figure 6.9d. Fat vacuoles were mostly single and normal within the hepatocytes. There was a little necrosis in all sections (scored mostly 1, but had increased a little to mostly score 2 after 50 weeks from the onset of feeding, this had reduced again after 53 weeks feeding). The necrosis was invariably single cell necrosis scattered throughout the tissue as shown in Figure 6.9e from a fish fed the VO diet, but no areas or extensive necrosis was seen. Score 1-2 is very low and was likely to have been of low significance, as it would appear that some single cell necrosis is a fairly common finding, although it can indicate some osmoregulatory stress or more general chronic stress. Inflammatory cell infiltration and pvc was also seen at very low levels in a small number of fish at each sampling point, but again would have been of very little clinical significance. The level of necrosis in VO fed fish was similar to fish fed the FO diet at all time points. There appeared to be slightly higher levels of inflammatory cells, single cell necrosis and pvc in the VO fed fish, particularly after 50 weeks from the onset of feeding (Figure 6.9f), although this seems to have reduced after 53 weeks feeding. In general there appeared to be more liver pathology in the VO group compared to the FO group.

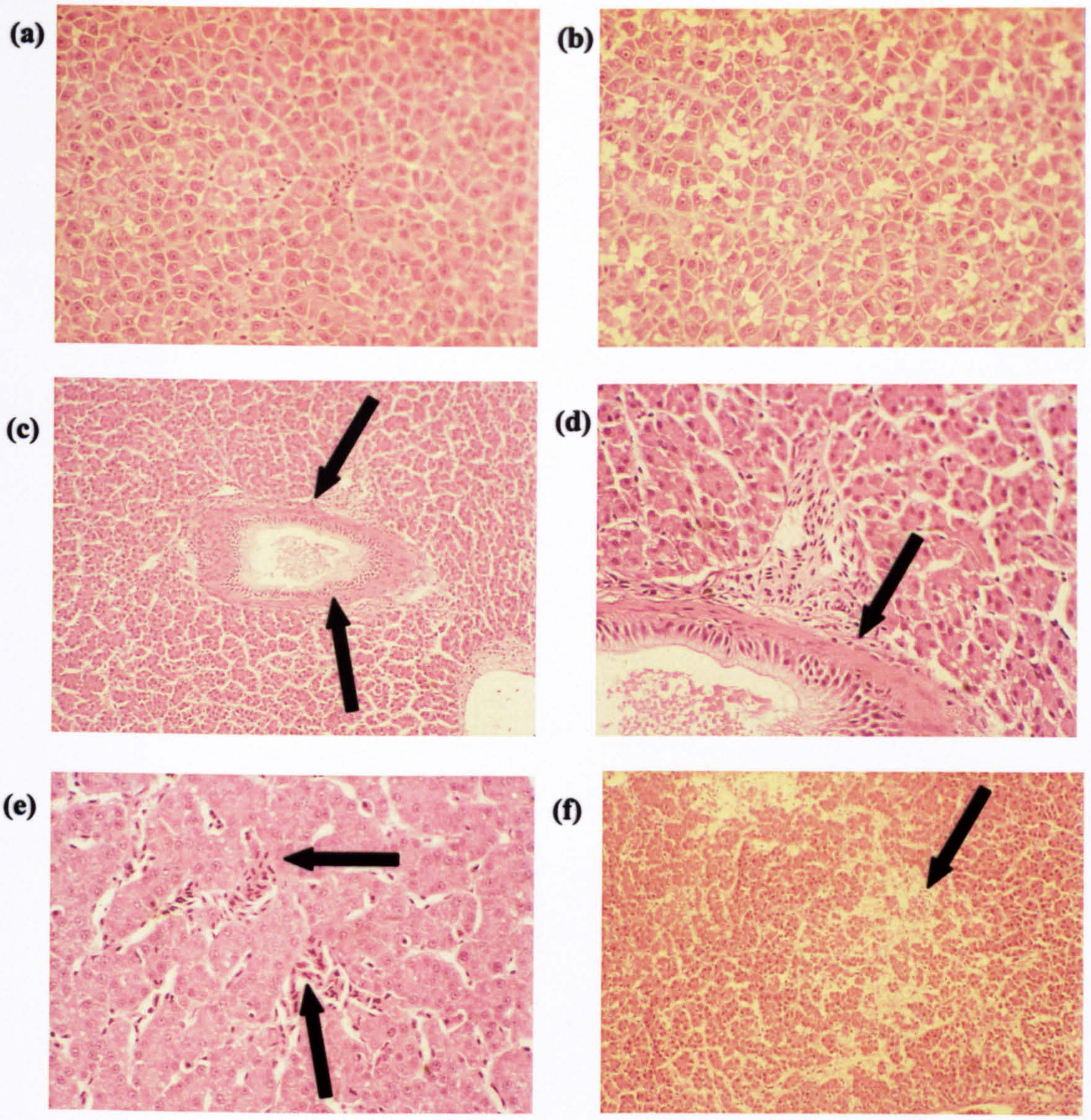



Figure 6.10 Histopathology of salmon livers (a) sampled after 50 weeks feeding 75% VO (6 weeks post vaccination) showing less fat within the vacuoles compared to those sampled after 47 weeks and some single cell necrosis (mag.x 430) (b) sampled after 53 weeks feeding 75% VO diet (9 weeks post vaccination) showing lower fat levels, single cell necrosis and inflammation compared those sampled after 50 weeks feeding (mag.x 430) (c) sampled 33 weeks post-sea water transfer feeding a 100 % FO diet showing signs of pvc (mag. x 175) (d) sampled 33 weeks post-sea water transfer feeding a 100 % FO diet showing signs of necrosis and pvc (mag.x 430) (e) sampled 33 weeks post-sea water transfer feeding a 75 % VO diet showing signs of necrosis and inflammatory cell accumulation (mag.x 430) (f) sampled 33 weeks post-sea water transfer feeding a 75 % VO diet showing an irregular structure and necrosis (mag.x 175).

Fat levels in the liver appeared to be slightly lower in fish fed the VO diet particularly after 47 and 50 weeks feeding as shown in Figures 6.10a and 6.10b, after 47 weeks feeding there was little difference between the FO group and the VO group. Fat levels within the liver were a little variable in both dietary groups and vacuoles were single within each hepatocyte but were quite variable in size throughout the tissue (i.e. some hepatocytes having small vacuoles and some having large vacuoles, this gave an appearance of variable fat deposition within each section). None of this would be considered abnormal and there were no apparent differences between the two diets. Small levels of necrosis were seen in some sections on both diets, again minimal in most cases and represented minor single cell necrosis, which again is frequently seen in normal fish. One fish in both groups had a slightly higher degree of necrosis. Inflammatory cells were also seen in three fish on the FO diet and one fish on the VO diet and this was similar to the levels of pvc seen in both groups (Figures 6.10c, d, e, and f). All the liver pathology was relatively minor and there appeared to be very little overall difference between the two groups.

No significant pathology was seen in any gut section examined from fish sampled after 34 weeks feeding (pre-vaccination). The proximal section of one of the fish fed the FO diet had some inflammatory cell infiltration up the lamina propria. Vacuolation and mucus activity appeared normal. The appearance of the absorptive vacuoles appeared to be multiple and variable in all sections i.e. multiple vacuoles were present in the enterocytes and these were of variable size, but there appeared to be no abnormality associated with this. Mucosal sloughing was pronounced in many fish, but this did not appear to be due to any ante-mortem degeneration and most likely due to post-mortem change and processing artefact. The distal intestine from one fish maintained on the FO diet showing a normal

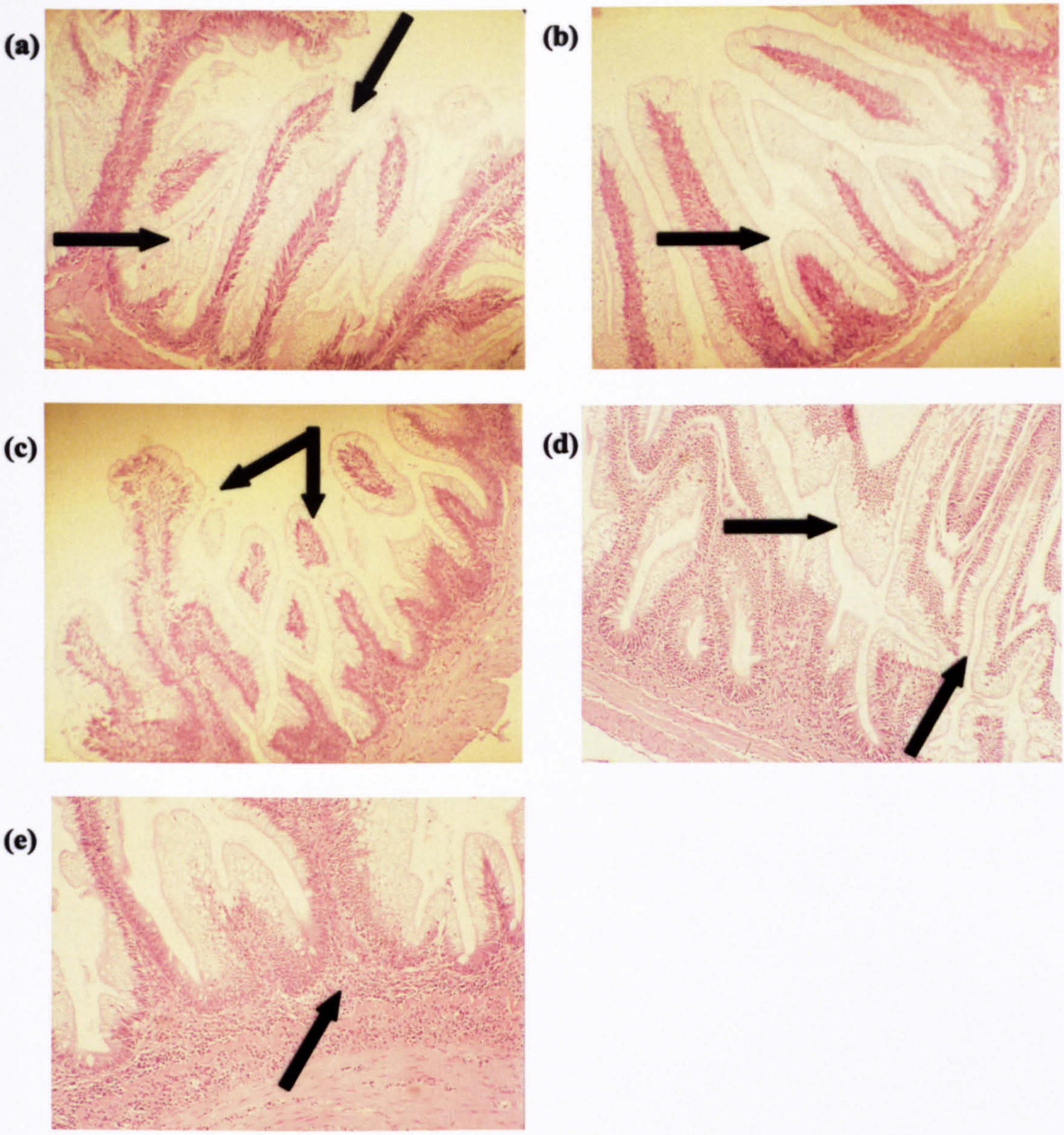
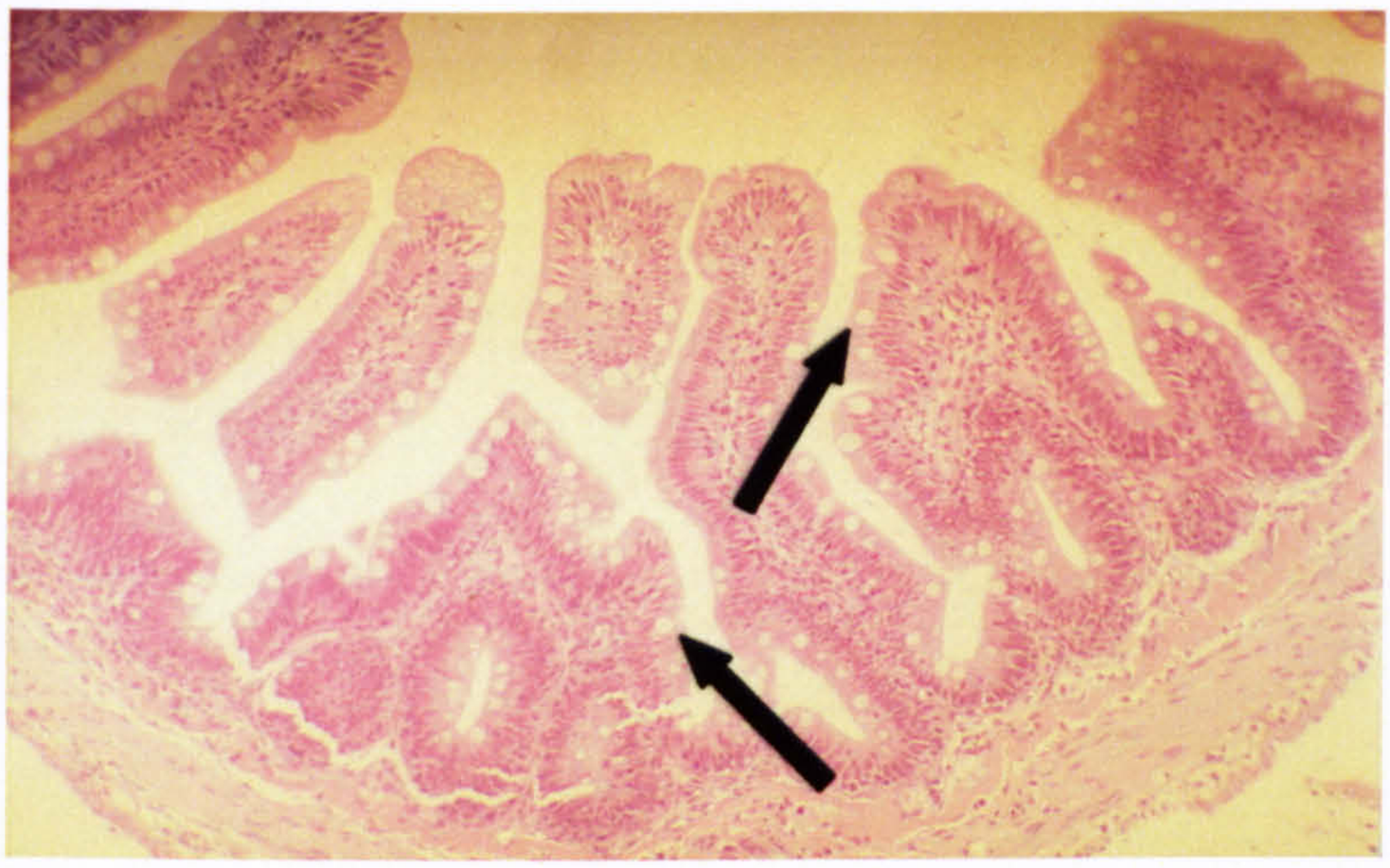



Figure 6.11 Histopathology of salmon distal intestine (a) sampled after 34 weeks feeding 100 % FO diet (pre-vaccination) showing a normal appearance with multiple and variable absorptive vacuoles (mag.x 175) (b) sampled after 34 weeks feeding 75 % VO diet (pre-vaccination) showing a normal appearance: no sloughing or cellular infiltration (mag.x 175) (c) sampled after 53 weeks feeding 75 % VO diet (9 weeks post vaccination) showing high vacuolation of the mucosal folds (mag.x 175) (d) sampled 33 weeks post-sea water transfer fed 100 % FO diet showing a highly vacuolated mucosal membrane (mag. X 430) (e) sampled 33 weeks post-sea water transfer fed 75 % VO diet showing cellular infiltration up the lamina propria (mag.x 175).

appearance with multiple vacuoles is shown in Figure 6.11a. A normal structural appearance of the distal intestine sampled from one fish maintained on the VO diet is shown in Figure 6.11b. Vacuolation and mucus activity appeared normal and any mucosal sloughing was thought to be most likely due to post-mortem change and processing artefacts. Overall, after feeding the experimental diets for 47, 50 and 53 weeks, all the intestinal sections appeared normal with very little significant pathology seen. Mucus activity and absorptive vacuoles appeared to be at normal levels for these sections in both dietary groups at all time points sampled. After 53 weeks feeding the VO diet, increased vacuolation was evident in some distal sections as shown in Figure 6.11c. Any breakdown and sloughing of the mucosa appeared to be at the apex of the mucosal folds. Absorptive vacuoles were mostly small and multiple and appeared normal. There was occasional cellular infiltration into the lamina propria but this was again at a very low level and there did not seem to be any difference between the dietary groups or time points. After fish had been sampled in sea water the results of the examination of the intestinal segments showed a great deal of similarity between the two groups. Mucus activity and vacuolation were similar, although vacuolation appeared a little more prominent in the distal segment of fish fed the VO diet. Vacuoles were mostly multiple within the enterocytes and variable in size and again there was no difference between the two groups. The degree of vacuolation evident in the distal intestine of a fish fed the FO diet is shown in Figure 6.11d. Cellular infiltration into the lamina propria was seen at grade 2 in one distal section from both groups. Figure 6.11e shows a section of distal intestine from a fish fed the VO diet showing signs of cellular infiltration. Sloughing of the mucosa was similar between the two groups and did not appear to be due to any significant pathological degeneration. Figure

**(a)**



**(b)**

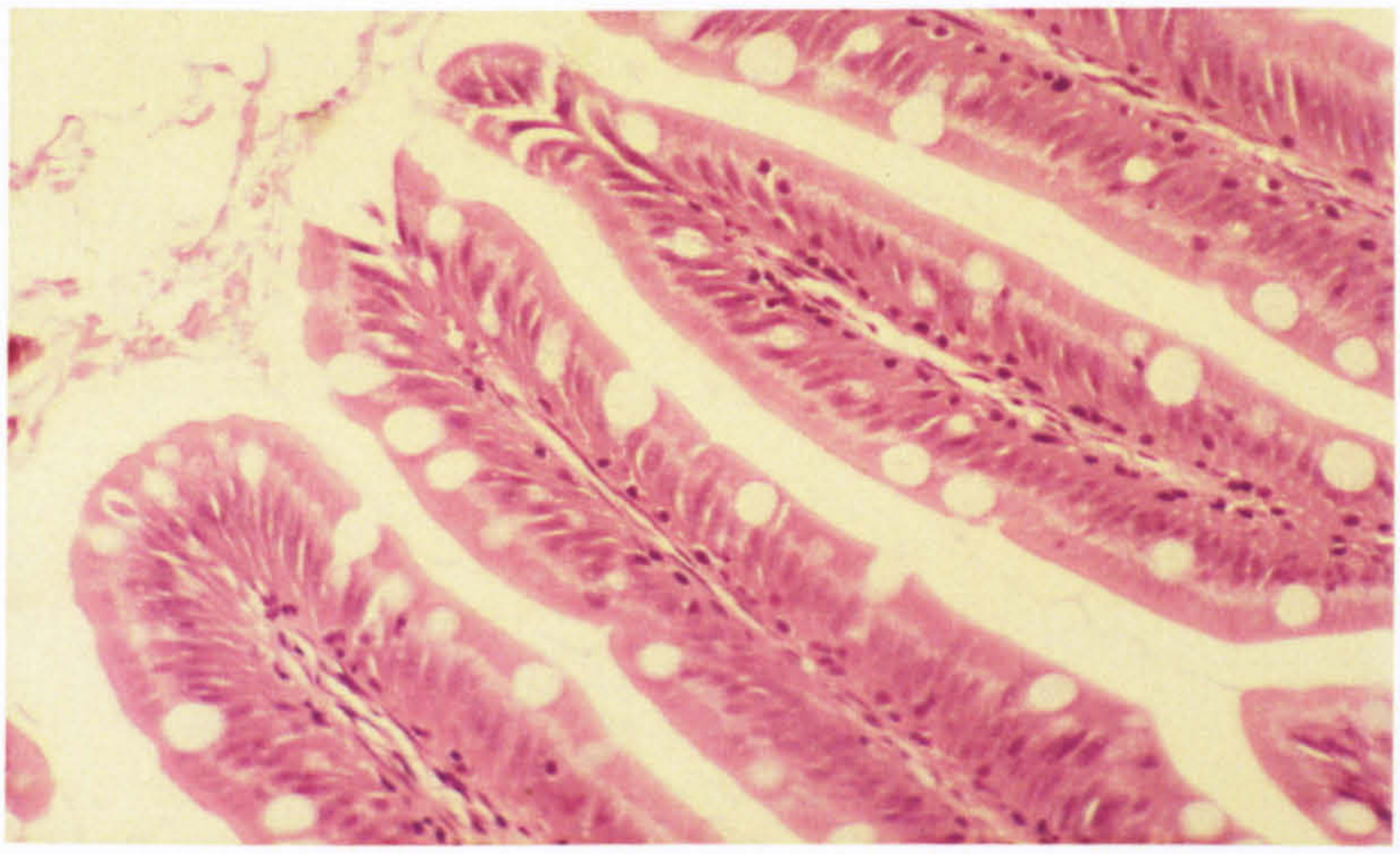


**Figure 6.12 Histopathology of salmon proximal intestine (a) sampled after 47 weeks feeding 75 % VO diet (3 weeks post vaccination) showing a normal appearance with regular absorptive vacuoles and no sloughing of the mucosa (mag. X 175) (b) sampled after 53 weeks feeding 75 % VO diet (9 weeks post vaccination) showing a normal appearance with regular absorptive vacuoles and no sloughing of the mucosa (mag.x 175).**

6.12a shows a section of proximal intestine from a fish fed the VO diet for 47 weeks, with regular vacuoles and no evidence of sloughing. A few proximal sections sampled after 53 weeks feeding the VO diet showed increased mucus activity (Figure 6.12b) but no significant pathological change.

### 6.3.1.8 Bacterial Challenge

Vaccination and challenge were used as *in vivo* indicators of the influence of different dietary oils on innate and adaptive immunity in Atlantic salmon. Atlantic salmon were challenged with *V. anguillarum* to determine the natural resistance of the fish to this bacterial pathogen. While it is more meaningful to assess vaccine efficacy by a challenge system resembling natural exposure, water bourne challenges are often difficult to accomplish. It was recommended that the fish were injected i.p. with *V. anguillarum* as it would have been difficult to achieve the correct stocking densities for a co-habitation challenge with the numbers of fish available for experimentation. The clinical signs in fish challenged with *V. anguillarum* were haemorrhaging around the mouth, on the gills and body which in some cases turned into necrotic lesions. Cumulative mortalities of fish fed with the experimental diets after bacterial challenge with *V. anguillarum* are shown in Figure 6.13. Fish maintained on the FO diet had an overall mortality rate of 12 %, whereas the fish fed the VO diet had a mortality rate of 30 %. Statistical survival analysis (Kaplan-Meir) was carried out on both dietary groups and the difference in mortality was found to be significantly different ($p=0.0425$).

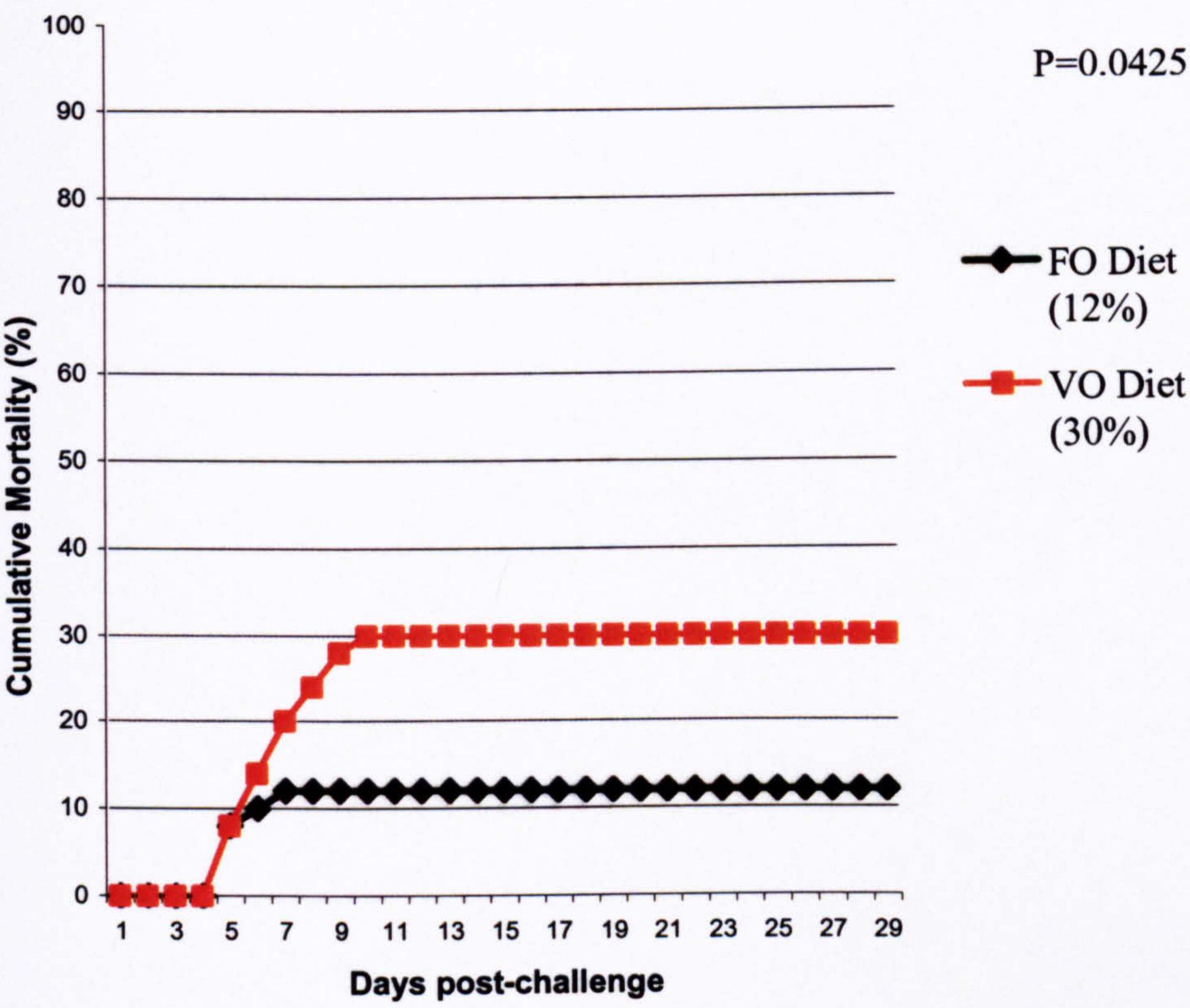



Figure 6.13 Cumulative mortalities of fish fed the experimental diets after challenge with *Vibrio anguillarum* (n = 120).

### *6.3.2 Trial 2 RAFOA II Norway – Replacement of FO with a blend of LO, RO and PO in Atlantic salmon*

#### 6.3.2.1 Growth, tissue lipid content and flesh fatty acid compositions

No differences in growth were seen from April 2002 to August 2003 between dietary treatments, however there was a tendency for increased growth in the 100 % FO group in October 2002, but this was not found to be significant (Torstensen *et al.*, personal communication). The fatty acid compositions of fish flesh reflected that of the dietary fatty acid compositions of the two experimental diets with regards to saturated, monounsaturated and n-6 fatty acids. The findings from this trial also suggest a connection between dietary oil source and cataract development in Atlantic salmon. A significantly higher incidence of visible cataracts were found in the 100 % VO group compared with the 100 % FO group. Previously cataracts have been related to growth but no differences in growth were observed in this study.

#### 6.3.2.2 Serum lysozyme activity before and after vaccination with NORVAX® MINOVA 6 Vet

No significant differences were observed between salmon fed 100 % FO and 100 % VO with regard to serum lysozyme activity either before of after vaccination with NORVAX® MINOVA 6 Vet. The effect of feeding the experimental diets on lysozyme activity is shown in Figure 6.14.

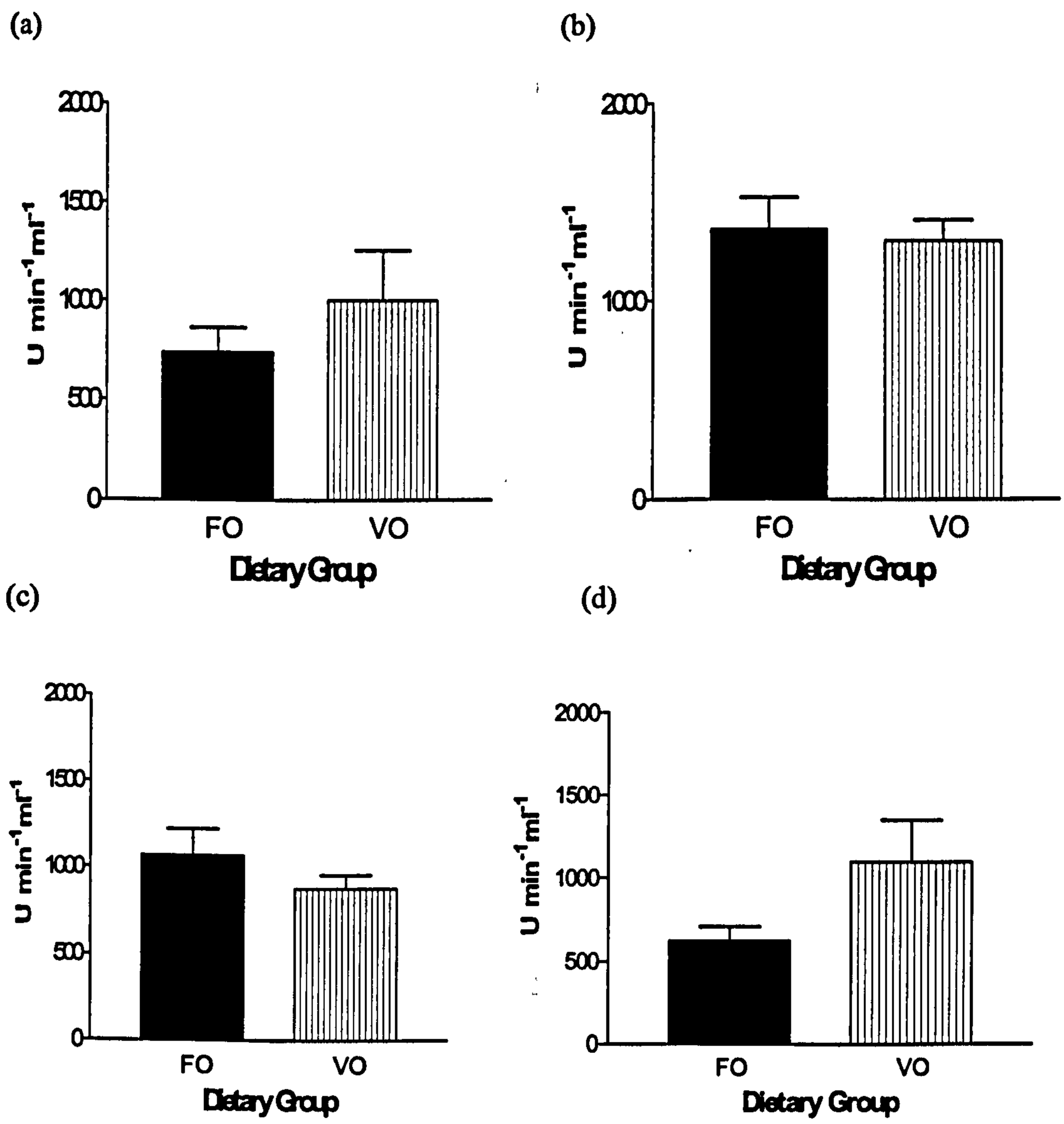



Figure 6.14 Serum lysozyme of Atlantic salmon fed the experimental diets sampled (a) prior to vaccination (b) 3 weeks post vaccination (c) 6 weeks post vaccination (d) 9 weeks post vaccination (n = 15)

### *6.3.3 Trial 3 RAFOA II Spain – Replacement of FO with a blend of LO, RO and PO in sea bass*

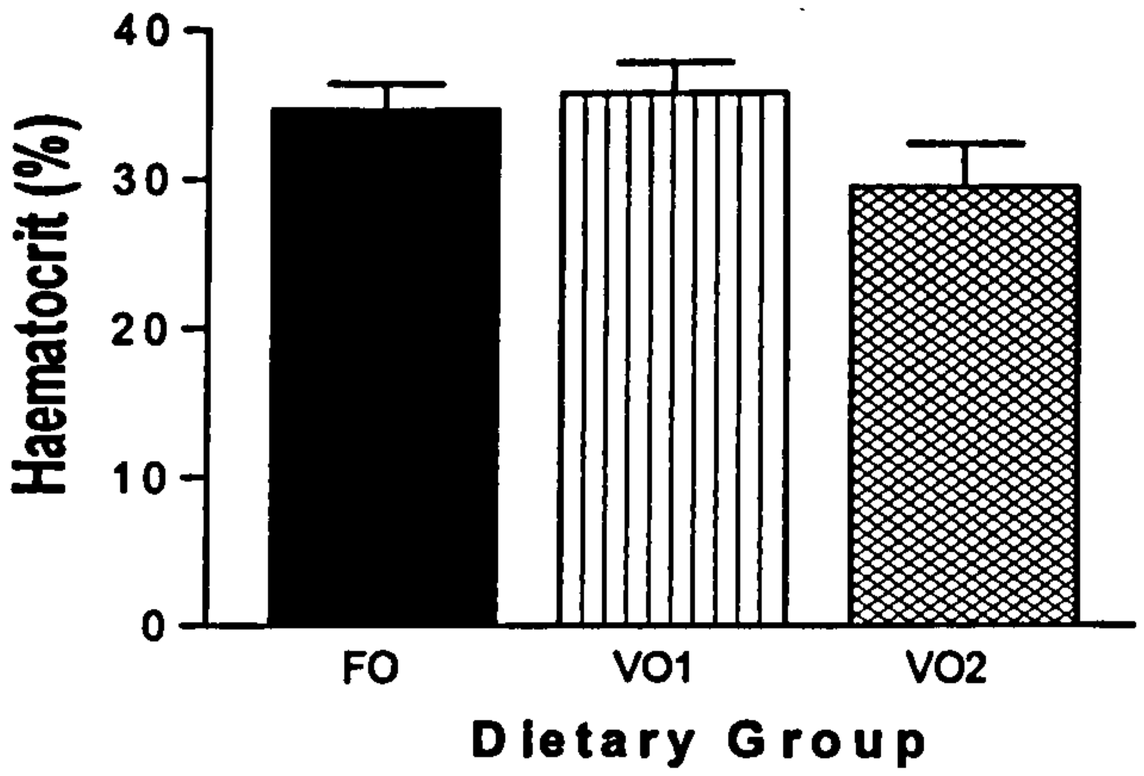
#### 6.3.3.1 Growth, tissue lipid content and flesh fatty acid compositions

Sea bass grew well on all three experimental diets with no significant differences in growth being recorded (Mourente *et al.*, personal communication). Fish fed on the FO diet had an average weight of 176.2 g compared to 143.2 g for fish fed the VO1 diet and 159.8 g for fish fed VO2 diet. The fatty acid composition of fish flesh varied with the dietary source. Total saturated fatty acids (primarily 16:0) were highest in the FO diet and VO1 diet and lowest in the VO2 diet. Total monoenes (primarily oleic acid) were identical in all of the dietary treatments, as were total polyenes. However, fish fed the 100 % FO diet showed the lowest values of LA and ALA and highest in ARA, EPA and DHA. The fatty acid composition of the liver showed no significant differences in the total number of saturated fatty acids (primarily 16:0) from different treatments, whereas total monoenes (primarily oleic acid) were highest in lipids from livers from the fish fed the VO2 diet and lowest in total lipids from those fed the FO diet and VO1 diet. The proportion of total polyenes was identical for all dietary treatments but fish fed the FO diet were richest in EPA and DHA and fish fed the VO1 diet and the VO2 diet were richest in LA and ALA (Mourente *et al.*, personal communication).

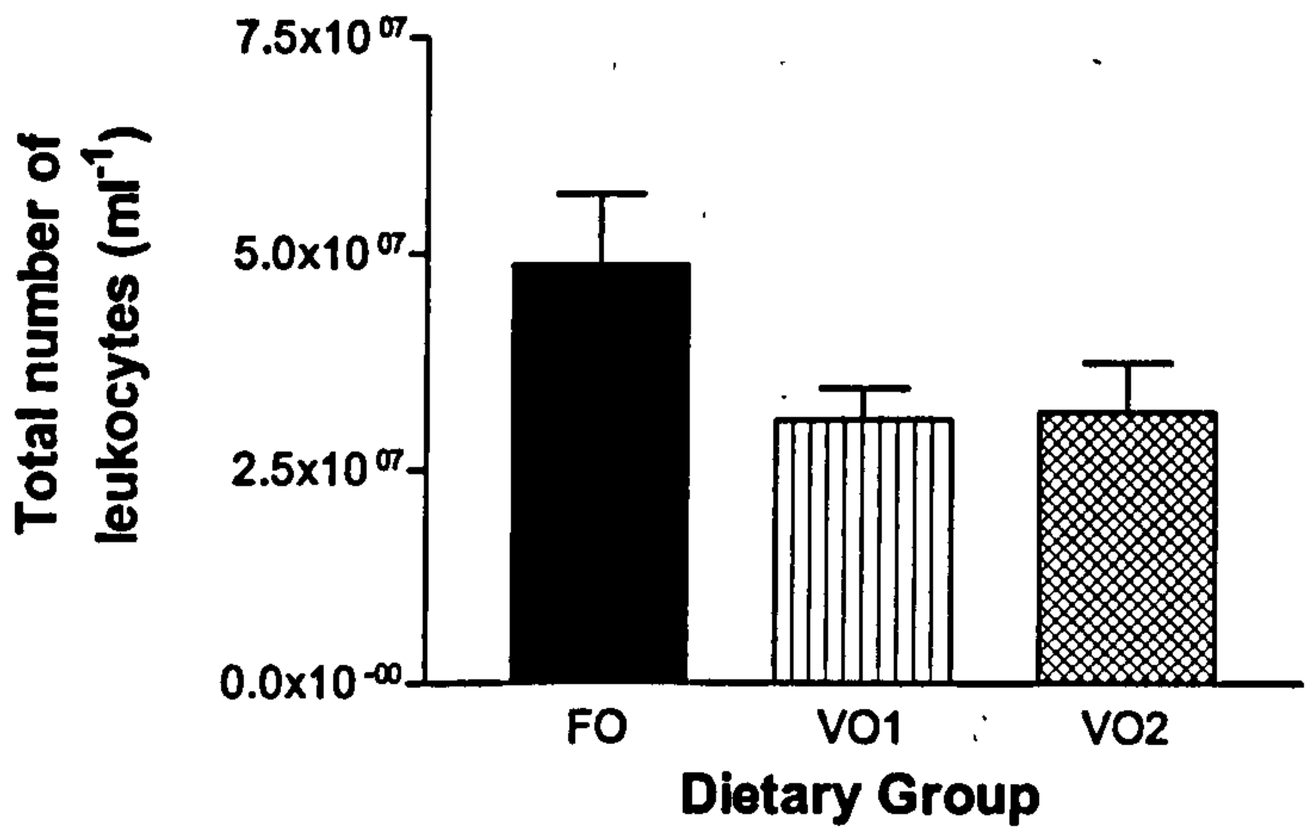
#### 6.3.3.2 Haematology and Immune function

Dietary lipid sources did not affect any of the haematological parameters measured. No significant differences were found when measuring haematocrit (Figure 6.15a), the total

(a)




(b)




(c)

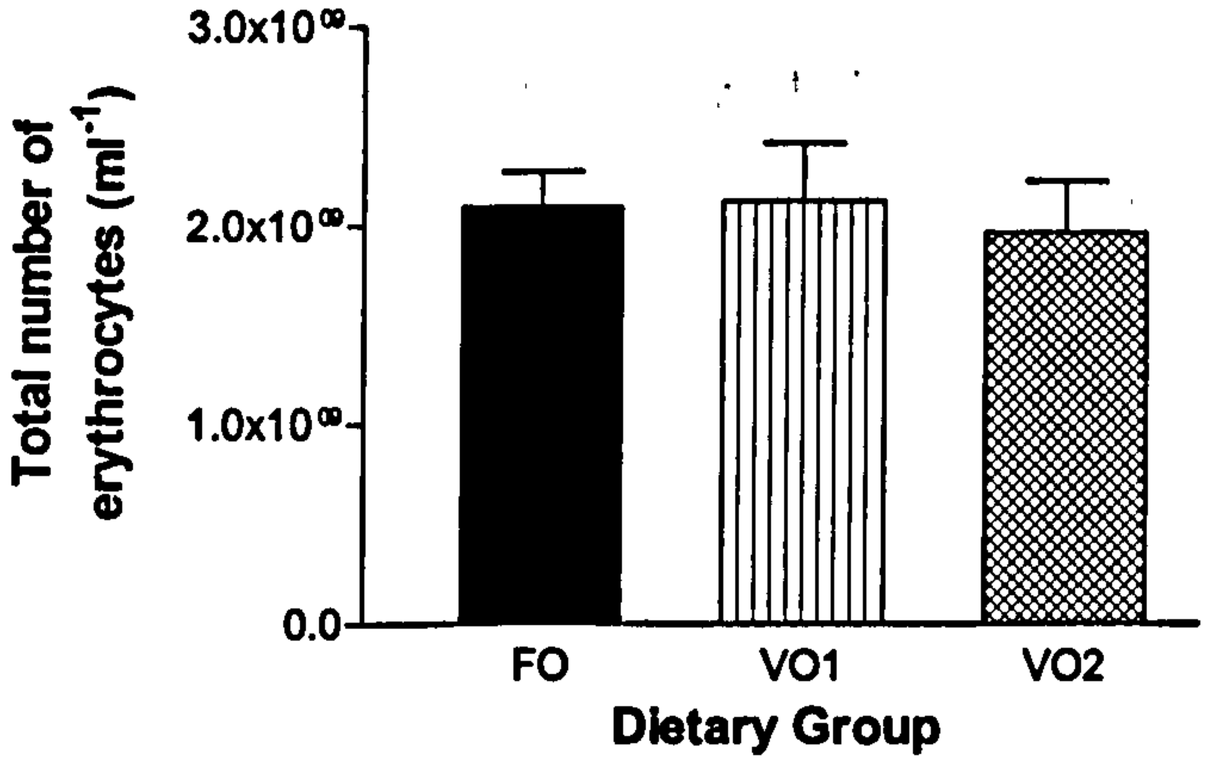



Figure 6.15 Effect of feeding the experimental diets to sea bass on (a) % haematocrit (b) total number of circulating leukocytes (c) total number of circulating erythrocytes. Values are means ± SEM, n=9.

number of leukocytes (Figure 6.15b) and erythrocytes (Figure 6.15c). Although fish fed the two VO mixed diets showed lower numbers of circulating leukocytes than fish fed the FO diet, no statistical differences were found, perhaps due to wide variations in individual responses.

### 6.3.3.3 Macrophage function

The production of superoxide anion by head kidney macrophages was measured by the NBT method and results are presented in Figure 6.18. The results show that following PMA triggering, the respiratory burst activity was significantly reduced in fish fed the VO based diets. However, there was no effect on the cellular immunity of VO fed fish as measured by the phagocytic activity of head kidney macrophages. The phagocytic capacity of head kidney macrophages isolated from fish fed the experimental treatments are presented in Table 6.7.

### 6.3.3.4 Serum lysozyme activity

No effect of dietary VO was observed on sea bass serum lysozyme activity (Figure 6.17a). Fish fed the FO diet showed the highest (1452.5 U $ml^{-1}$ $min^{-1}$) (but not significant) value of lysozyme activity in serum compared to 1351.1 U $ml^{-1}$ $min^{-1}$ found for the VO1 diet and 1171.1 U $ml^{-1}$ $min^{-1}$ for the VO2 diet.

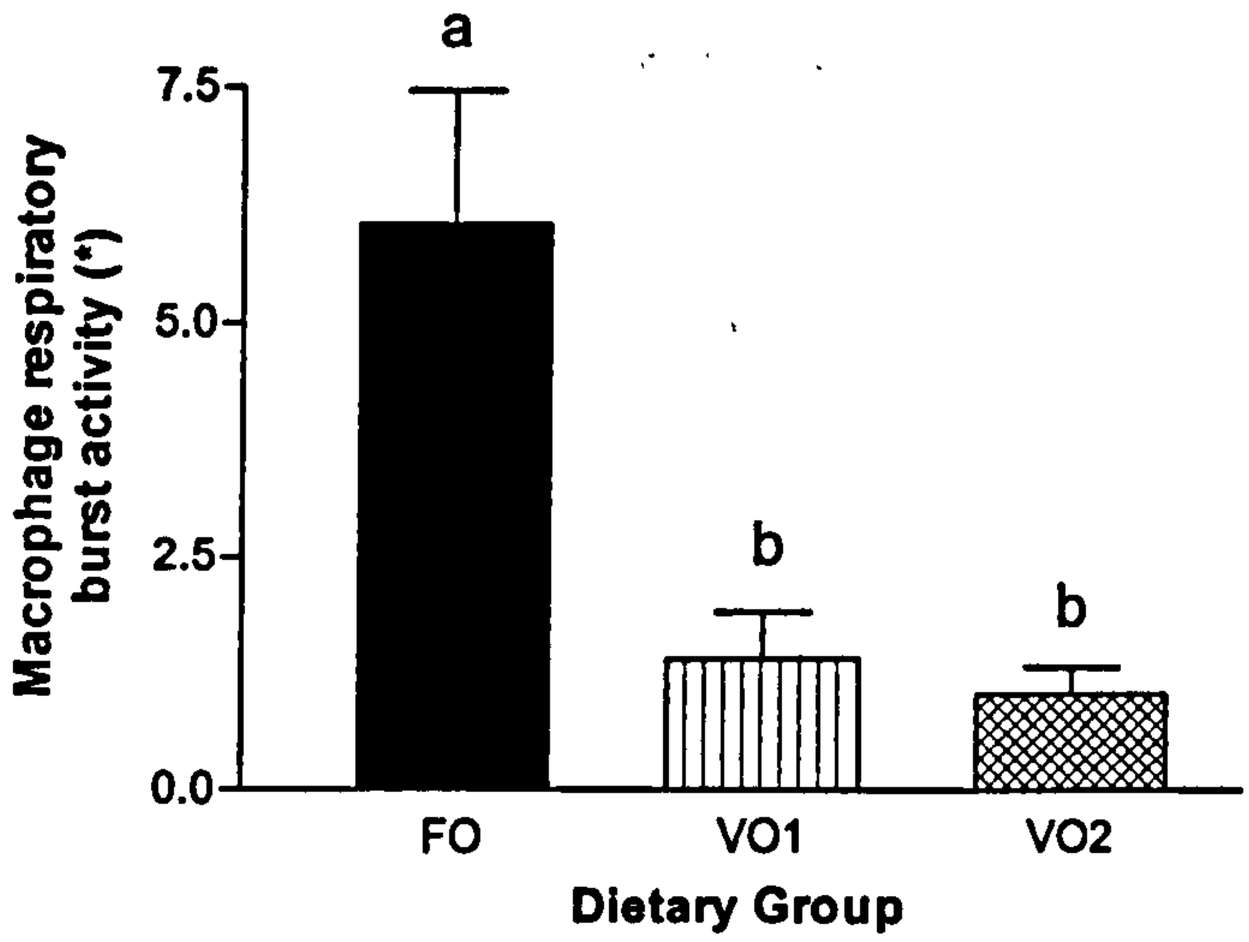


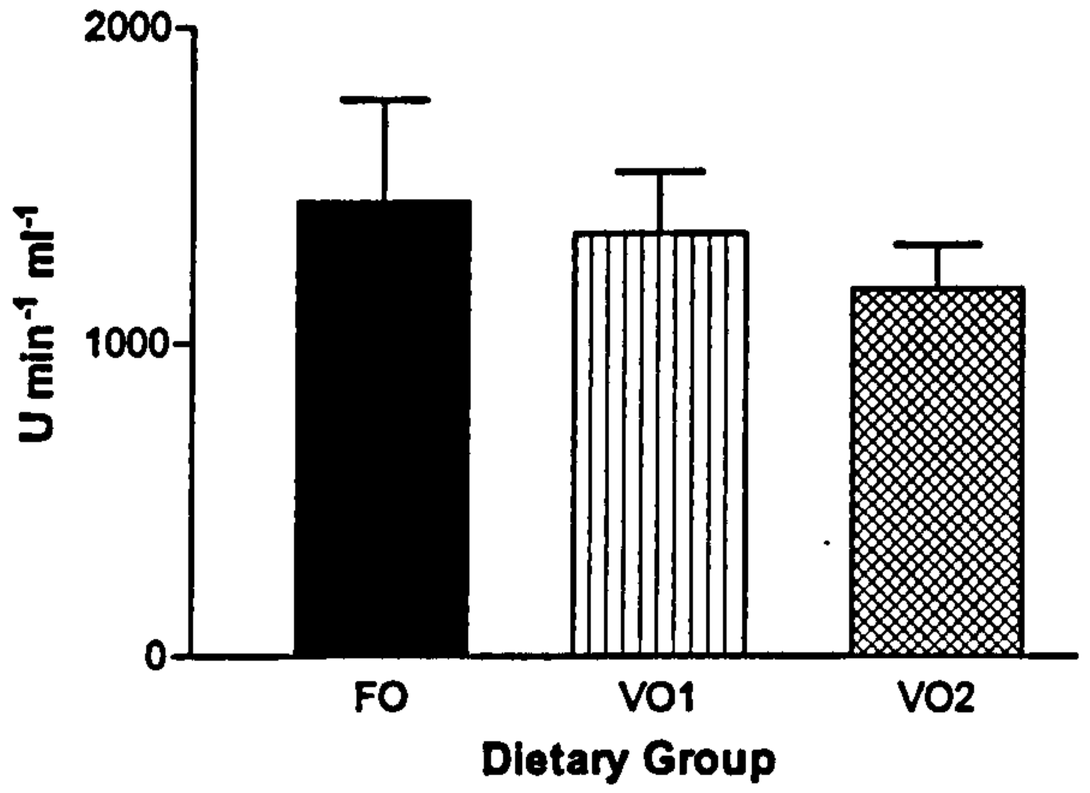

Figure 6.16 Effect of feeding the experimental diets on sea bass head kidney macrophage respiratory burst activity (NBT reduction). Values are means ± SEM, n = 9. Values having different column letters are significantly different. * = Absorbance per $10^5$ cells x100.

Table 6.7 Phagocytic capacity (% of macrophages which have phagocytosed yeast particles) of sea bass head kidney macrophages from fish fed the experimental diets

| Dietary treatment | Phagocytic capacity |
|---|---|
| FO | 39.6 ± 13.8 |
| VO1 | 31.6 ± 18.5 |
| VO2 | 30.4 ± 15.4 |

Values are means ± SEM, n = 9.

(a)




(b)

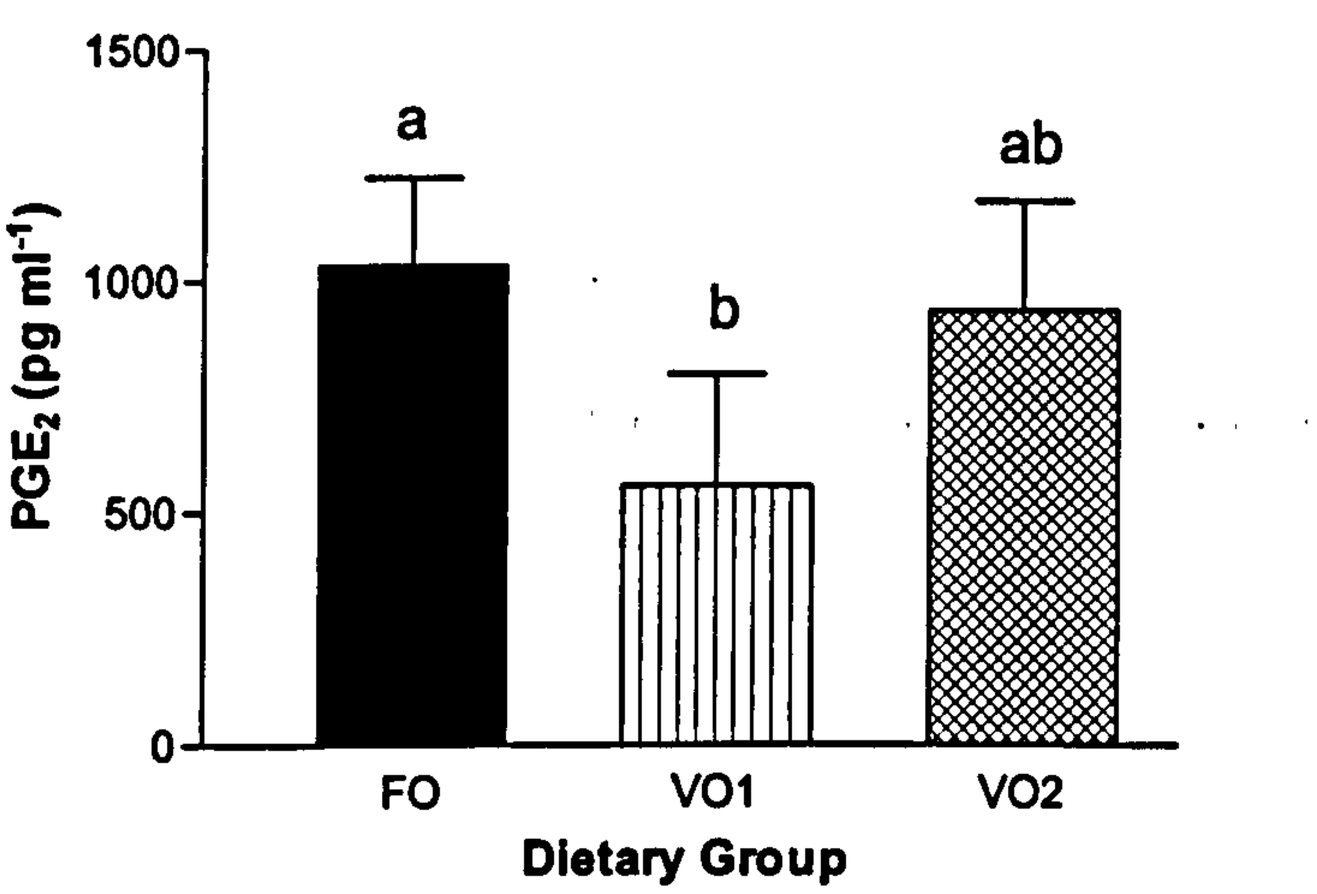



Figure 6.17 Effect of feeding the experimental diets to sea bass on (a) serum lysozyme activity (b) $PGE_2$ concentration. Values are means ± SEM, n=9. Values having different column letters are significantly different ($p<0.05$).

### 6.3.3.5 Plasma $PGE_2$ concentration

The concentrations of $PGE_2$ in sea bass plasma are shown in Figure 6.17b. In plasma from fish fed with the VO1 diet the concentration of $PGE_2$ was significantly lower than fish fed the FO diet and the VO2 diet. Sea bass fed the VO1 diet had a $PGE_2$ concentration of 556.7 pg $ml^{-1}$ compared to 1034.3 pg $ml^{-1}$ for the FO diet and 935.4 pg $ml^{-1}$ for the VO2 diet.

### 6.3.3.6 Fatty acid composition of peripheral blood leucocytes

Inclusion of VO in the diets for sea bass modified the fatty acid composition of isolated peripheral blood leukocytes. The fatty acid compositions of PBL from FO fed fish were somewhat different from the corresponding PBL from fish fed VO, with the latter showing greater monoene fatty acids and higher n-6 PUFA. Sea bass maintained on the VO1 diet and the VO2 diet had significantly increased levels of 18:0, oleic acid, LA, 20:2(n-6) and ALA and significantly reduced amounts of n-3 PUFA, ARA, EPA, DHA and others as shown in Table 6.8. In addition, the overall ratio of n-3/n-6 was reduced significantly in sea bass fed the VO diets.

### 6.3.3.7 Histopathology

Hearts examined from all three dietary groups showed no signs of pathological change. In livers, fat vacuoles were variable in size in many sections with some very large vacuoles present within some hepatocytes and relatively smaller vacuoles in some other hepatocytes. Due to the level of vacuolation in some hepatocytes there was some distortion of the cellular architecture and occasional breakdown of cells. Again, there were no differences

Table 6.8 Fatty acid composition (wt %) of sea bass peripheral blood leukocytes. Values are expressed as the mean per cent of total fatty acids for four fish ± SD. Values with different superscript letters within a row are significantly different ($p<0.05$).

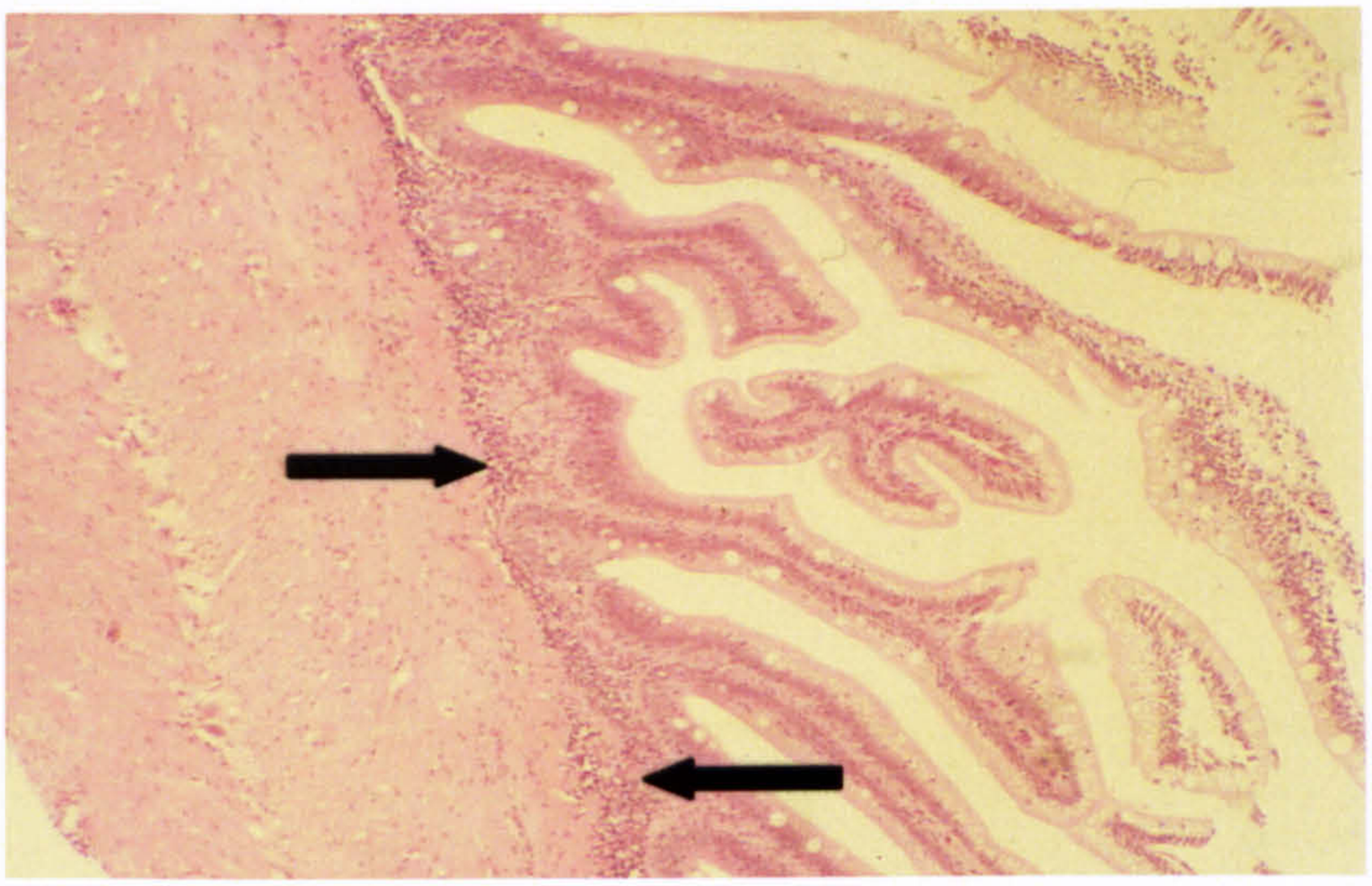
| Fatty Acids | FO | VO1 | VO2 |
|---|---|---|---|
| 14:0 | 1.6±0.0 [a] | 1.0±0.2 [b] | 1.1±0.1 [b] |
| 15:0 | 0.5±0.1 [a] | 0.3±0.0 [b] | 0.3±0.1 [b] |
| 16:0 | 17.2±1.4 | 16.8±0.6 | 16.7±0.8 |
| 18:0 | 3.9±0.2 [a] | 5.6±0.5 [b] | 4.7±0.3 [c] |
| 20:0 | 0.2±0.1 | 0.2±0.0 | 0.2±0.0 |
| 22:0 | 0.2±0.0 | 0.2±0.1 | 0.2±0.1 |
| Total saturates | 23.5±1.8 | 24.0±0.7 | 23.1±0.7 |
| | | | |
| 16:1 (n-7) | 3.4±0.4 [a] | 2.5±0.5 [b] | 2.6±0.2 [b] |
| 18:1 (n-9) | 10.5±1.7 [a] | 18.8±0.4 [b] | 20.4±1.8 [b] |
| 18:1 (n-7) | 2.0±0.2 | 1.9±0.2 | 1.9±0.1 |
| 20:1 (n-9) | 1.7±0.2 | 1.8±0.2 | 1.8±0.3 |
| 20:1 (n-7) | 0.2±0.1 [a] | 0.0±0.0 [b] | 0.0±0.0 [b] |
| 22:1 (n-11) | 0.9±0.2 | 1.0±0.3 | 1.0±0.34 |
| 22:1 (n-9) | 0.2±0.0 | 0.2±0.1 | 0.2±0.0 |
| 24:1 | 1.4±0.5 | 1.4±0.5 | 1.0±0.2 |
| Total monoenes | 20.1±2.7 [a] | 27.6±1.4 [b] | 28.9±2.6 [b] |
| | | | |
| 18:2 (n-6) | 2.6±0.2 [a] | 5.8±0.4 [b] | 6.1±0.7 [b] |
| 18:3 (n-6) | 0.2±0.1 | 0.2±0.0 | 0.2±0.0 |
| 20:2 (n-6) | 0.3±0.1 [a] | 0.5±0.0 [b] | 0.4±0.0 [b] |
| 20:3 (n-6) | 0.3±0.1 [a] | 0.0±0.0 [b] | 0.1±0.0 [c] |
| 20:4 (n-6) | 1.2±0.1 [a] | 0.7±0.1 [b] | 0.7±0.1 [b] |
| 22:4 (n-6) | 0.3±0.1 [a] | 0.2±0.0 [a] | 0.0±0.0 [b] |
| 22:5 (n-6) | 0.4±0.0 [a] | 0.3±0.0 [b] | 0.3±0.0 [c] |
| Total (n-6) | 4.9±0.4 [a] | 7.5±0.5 [b] | 7.7±0.7 [b] |
| | | | |
| 18:3 (n-3) | 0.7±0.2 [a] | 5.9±0.5 [b] | 4.8±0.6 [c] |
| 18:4 (n-3) | 0.9±0.2 [a] | 0.7±0.1 [ab] | 0.6±0.1 [b] |
| 20:4 (n-3) | 0.3±0.1 [a] | 0.2±0.2 [b] | 0.2±0.0 [b] |
| 20:5 (n-3) | 13.4±0.5 [a] | 8.9±0.2 [b] | 8.3±0.4 [b] |
| 22:5 (n-3) | 1.2±0.1 [a] | 0.9±0.2 [b] | 0.8±0.0 [b] |
| 22:6 (n-3) | 27.6±2.6 [a] | 19.7±1.0 [b] | 20.6±2.9 [b] |
| Total (n-3) | 44.1±2.6 [a] | 36.4±1.5 [b] | 35.3±2.6 [b] |
| | | | |
| Total PUFA | 49.1±2.4 [a] | 43.9±1.9 [b] | 43.1±2.2 [b] |
| | | | |
| n-3/n-6 | 9.0±1.2 [a] | 4.8±0.2 [b] | 4.6±0.7 [b] |

between the three dietary groups. Small foci of inflammation were seen in some sections in all three groups, with a slightly higher incidence in the VO2 diet. PVC was not a feature in any of the dietary groups examined. With regard to the intestinal sections, mucus levels appeared very similar in all segments and in all dietary groups. Absorptive vacuoles were small and multiple in all sections. In the FO diet these were at relatively low levels in the proximal and mid segments and higher in the distal segments. In fish fed the VO1 diet and the VO2 diet vacuolation in the proximal segments appeared to be much more pronounced, interestingly less so than in the mid sections. Some cellular infiltration was seen in the lamina propria of one fish in the FO diet (Figure 6.18a) and two fish on the VO2 diet (Figure 6.18b). Sloughing of the mucosal membrane was not a feature in any of the sections examined. The major difference seen between these groups was the level of absorptive vacuolation in the proximal segment of the fish fed the VO1 diet and the VO2 diet compared with the FO diet.

## 6.4 Discussion

The results of immune activity in the present trial suggest an improvement in the overall immune response of fish compared to RAFOA I, where a number of the parameters measured were reduced in salmon fed diets containing RO and LO compared to fish fed FO diets. From data presented in Trial 1, it would seem that feeding diets to Atlantic salmon with a 75 % blended VO diet resulted in only minor changes in haematocrit values and levels of circulating erythrocytes and leukocytes in the freshwater stage of production.

(a)



(b)

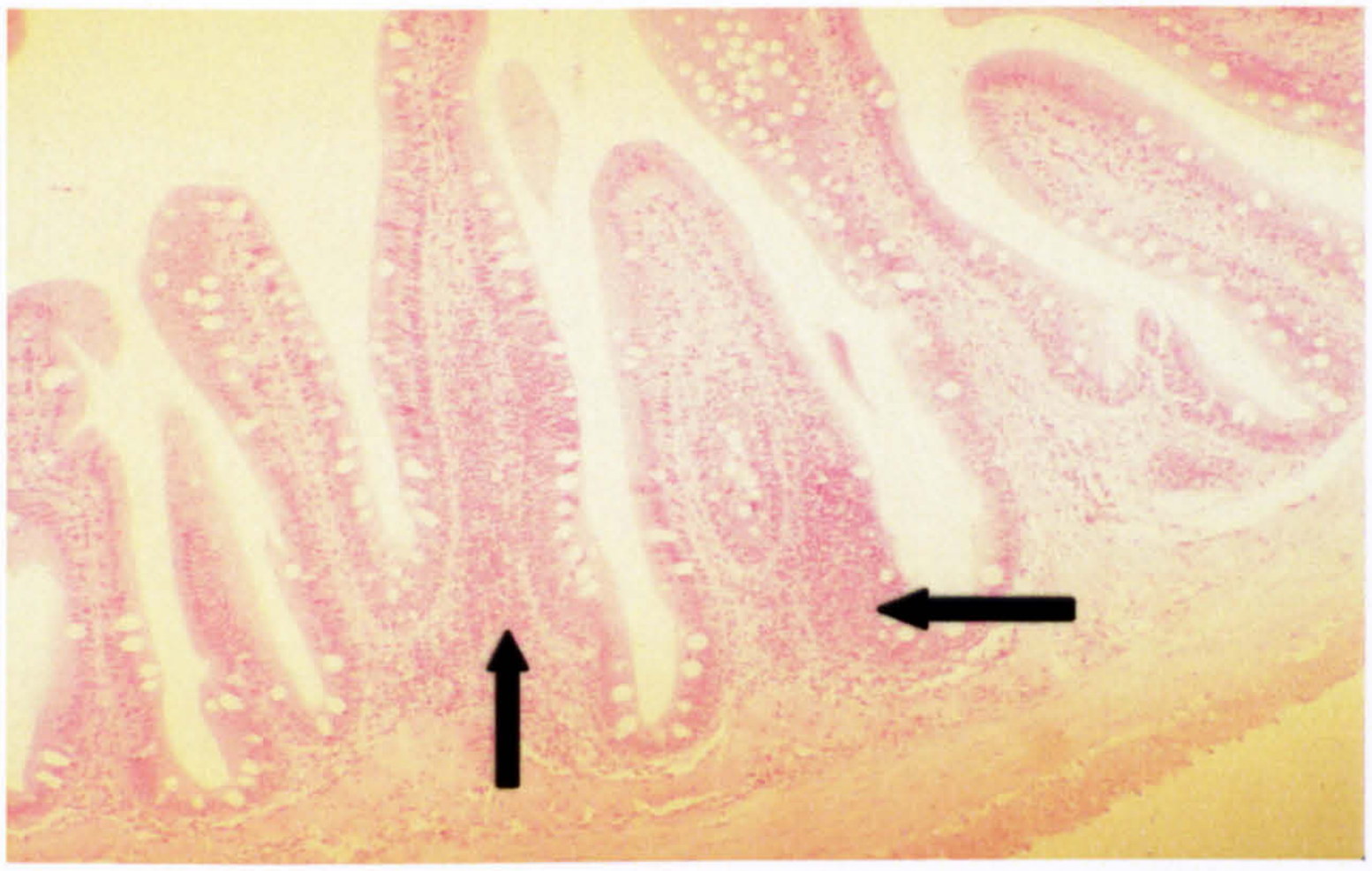


Figure 6.18 Histopathology of sea bass fed a (a) FO diet, distal intestine showing slight cellular infiltration in the lamina propria and high levels of absorptive vacuoles (mag.x 175) (b) VO2 diet, distal intestine showing cellular infiltration but no sloughing of the mucosal folds (mag.x 430).

After sea water transfer, no significant differences in humoral or cellular immune responses were found after feeding 75 % VO. Interestingly, the impact of dietary VO on disease resistance against *V. anguillarum*, showed significant differences in cumulative mortalities compared to fish fed a FO diet. This is in agreement to a study by Bransden *et al.*, (2003) in which the authors reported significant differences in mortalities of salmon fed sunflower oil after challenge with *V. anguillarum*. Erdal *et al.*, (1991) found decreased antibody titres and survival in salmon fed diets with high n-3 PUFA levels. The authors reported that increasing the dietary concentration of n-3 HUFA, (EPA and DHA) from 12.7 % to 24.2 % of the total dietary fatty acids caused a reduction in antibody production and survival when challenged with *Y. ruckeri*. An increased incidence of degenerative changes was also observed, however, on the other hand, increased levels of omega-3 fatty acids increased erythrocyte cell wall strength.

Findings from previous chapters (Chapters 3-5), reported that feeding VO diets to salmon resulted in haematocrit values significantly lower than those found in fish fed FO diets. In this present study, however, it was found that fish fed a blend of dietary VO resulted in an elevated haematocrit value. Although there is often a high degree of variation in haematocrit values among fish within the same group, high values may be indicative of an increased oxygen demand (Casillas & Smith 1977). Sirbopoulos (2003) studied the effects of feeding PO only diets on the immune system of rainbow trout and found no significant differences in haematocrit values in fish fed PO diets compared to those maintained on a FO diet.

Sirbopoulous (2003) also reported significant differences in the total number of circulating leukocytes 4 weeks after feeding. Fish fed either 25 % or 50 % PO diets having a significant reduction in numbers compared to fish fed a 100 % FO diet. However, no significant differences were recorded after 8 weeks of feeding. Trout fed with a 100 % PO diet showed lower numbers of erythrocytes compared to FO fed fish although not statistically different. Sirbopoulous, found that feeding trout for four weeks on either 25 %, 50 % or 100 % PO only diets, resulted in a significant difference in the phagocytic capacity of head kidney macrophages compared with those from FO fed fish.

In previous chapters (Chapters 3-5) a significant reduction in macrophage function was reported when VO diets were fed to salmon and sea bass. In the current trial, however, this was not the case. No differences were found in either respiratory burst activity or phagocytic capacity between the two dietary groups. It may be that feeding a blended VO diet does not dramatically alter the macrophage membrane fluidity or composition, as seen when feeding a single VO, and so no effect was observed on macrophage function. Respiratory burst activity is generally reported to increase following vaccination, with a peak activity being observed 30 days post-injection (Secombes, 1994b). Similarly, in the current trial, the maximum amount of respiratory burst activity was found 6 weeks post vaccination. In addition, a study by Kitlen *et al.*, (1997) also reported heightened respiratory burst activity 4 weeks post-vaccination.

Lysozyme plays an important bactericidal role, primarily through lytic actions on the pathogen cell wall. High lysozyme activity may be desirable in cultured fish because it may aid against infection in situations in which fish are kept at high densities and

consequently are exposed to high bacterial loads (Grinde *et al.*, 1988). In RAFOA II no differences in serum lysozyme activity were observed at any of the sample time points. These results are in agreement with findings from Sirbopoulos (2003) in which PO diets had no effect on serum lysozyme activity when compared to fish fed FO diets.

Prior to vaccination fish were examined for histopathologies in heart, liver and intestinal sections. Heart and gut sections appeared normal with no apparent cellular pathologies present in fish from either treatment. In livers, there was evidence of increased inflammatory activity in fish fed 75 % VO compared to those fed FO. Necrosis of the hepatocytes was seen to a greater or lesser extent in all fish from the 75 % VO group. Very few pathological changes were identified in any of the tissues sampled post-sea water transfer. Futhermore, any changes were very minor and extremely unlikely to have had any effect on performance of the fish. The pathological effects observed in many of the dietary trials presented in previous chapters (Chapters 3-5) poses the question is there a level at which the dietary ratio of n-6/n-3 PUFA becomes high enough that it causes pathological change in fish. In the present study, less apparent histological changes were observed in fish fed a blended VO diet, which was produced to give a similar ratio of saturated, monounsaturated and PUFA to that found in the FO diet.

The concept of vaccinating fish on a commercial scale has been realised especially with respect to bacterial diseases such as *V. anguillarum* and *A. salmonicida* species. The techniques available for mass immunisation of fish include i.p., bath immersion, oral or even via spray application. Each method however has its own disadvantages and advantages in terms of stress on the fish, level and duration of protection achieved, labour

costs and time, and accessibility of the fish. Research has shown that age, temperature, season, antigen dose, the route of administration, antigenic competition and adjuvant effects may affect the immune response in fish.

In the present study an ELISA was used to determine the levels of antibodies in fish vaccinated against *A. salmonicida*. The ELISA technique is simple, rapid, sensitive and inexpensive, and has numerous major applications in fish pathology including, the detection of pathogens in diseased fish, identification of pathogens isolated in culture and the detection of fish antibodies produced in response to a pathogen (Huston, 1979; Roberson, 1981). The over-riding prerequisite for the ELISA technique is for antiserum of suitable titre and avidity. If this is met then it is likely that most bacterial and viral fish pathogens could be rapidly detected by this method. High antibody titres does not always mean conferment of protection against a particular pathogen and in some cases it is the action of macrophages which may be equally, if not more important than the antibody response in conferring protection against BKD (Evenden *et al.*, 1993). In the present study, there were no significant differences in antibody titre between groups of fish fed the experimental diets therefore, no effect on vaccination efficacy of dietary lipid source was observed. It is worth noting however, that large variations between individual fish within each dietary group was observed resulting in large standard errors.

Antibody titres are used to assess the state of the non-specific immune system, although they are not always indicative of the protective value of a vaccine or correlated with the survival of the fish. It has been demonstrated that the outcome of a disease challenge may be independent of the agglutinating antibody titre in the serum of fish (Thuvander *et al.*,

1993), and it is specificity rather than the quantity of the antibodies that are produced that is important (Olivier *et al.*, 1985). Therefore antibodies must act in conjunction with other factors of the immune system to combat pathogenic infection. Erdal and Reitan (1992) have reported that antibody titres in fish normally increase considerably between 3 and 8 weeks after vaccination and peak between 3 and 6 months (Cisar & Fryer, 1974) with the actual timing of the response being dependent on the temperature of the water (Manning & Mughal, 1985).

Protection against fish pathogens is probably due to many elements and is possibly more dependent on non-specific and cell-mediated protection, than on antibody-mediated protection. Fracalossi and Lovell (1994) observed low disease resistance and immune function in channel catfish fed diets high in n-3 PUFA. The survival of channel catfish after challenge with *E. ictaluri* was the lowest in fish fed menhaden oil and LO compared to fish fed corn oil (high LA), beef tallow or one containing an equal mixture of corn oil, beef tallow and menhaden oil. After feeding the different lipid sources to catfish at 28 °C and subsequent challenge, fish fed the menhaden oil and LO had significantly lower survival rates than fish fed, the beef tallow, corn oil or mixed oil diets. At the lower temperature of 17 °C, there was no difference in survival among dietary groups.

Antibody production was highest in fish fed the menhaden oil diet, which indicates that circulating antibody was not correlated to survival. The highest antibody production was evident at week 2 in fish fed the menhaden oil and beef tallow diets. These two diets contained markedly lower concentrations of LA than the other diets, which may suggest the diets high in LA or n-6 fatty acids suppress antibody production in channel catfish. Erdal *et*

*al.*, (1991) found decreased antibody titres and survival in salmon fed diets with high n-3 PUFA levels. They reported that increasing the dietary concentration of n-3 HUFA, EPA and DHA from 12.7 % to 24.2 % of the total fatty acids caused a reduction in antibody production and likelihood of survival in salmon challenged with *Y. ruckeri*.

The results from Trial 2 suggest that neither the dietary VO nor the vaccination procedure had a significant effect on the levels of serum lysozyme activity in Atlantic salmon. As in Trial 1, the findings from the Trial 2 suggest that feeding blended VO diets to salmon does not alter serum lysozyme activity when compared to salmon maintained on FO diets.

Ackerman *et al.*, (2000) studied the effect of vaccination on kidney lysozyme activity of rainbow trout. The authors reported significant differences in lysozyme activity when sampled 5, 12 and 24 weeks post vaccination with *A. salmonicida*. However they found no consistent pattern in the differences found. Levels fell from 2381 $\mu g\ ml^{-1}$ after 5 weeks post vaccination to 685.4 $\mu g\ ml^{-1}$ after 12 weeks, then rose again to 902.5$\mu g\ ml^{-1}$ after 24 weeks. Fletcher & White (1976) observed that lysozyme activity may decrease by up to 70 % during the coldest time of year. Temperature variations between sampling time points may account for the small differences, although not significant, between dietary groups when measuring lysozyme activity in the Trial 2.

Increased lysozyme activity in fish after activation of the immune system has been recorded by various authors (Fletcher & White, 1973; Studnicka *et al.*, 1986), and increased lysozyme activity in Atlantic salmon has been detected 30 days after challenging the fish with the pathogenic bacterium *A. salmonicida* (Mϕyner *et al.*, 1993).

In Trial 3 feeding diets with a 60 % FO replacement with combinations of LO, RO and PO did not significantly depress the humoral immune response in sea bass in comparison to diets containing 100 % FO. However, the inclusion of different VO in the diet did affect the ability of head kidney macrophages to phagocytose yeast particles. During respiratory burst, superoxide and hydrogen peroxide are produced and released into the surrounding tissue (Jang *et al.*, 1995; Secombes, 1990). Inhibition of this process by immunotoxic substances can lead to a prolonged survival of invading bacteria or fungi followed by an increased growth and subsequently leading to increased influence of the pathogenic organism on normal physiology, resulting in increased disease with possible fatal effects (Kollner *et al.*, 2002). Corresponding to the reduced respiratory burst activity of sea bass macrophages from VO fed fish, the concentration of $PGE_2$ of plasma from fish fed the VO1 diet and the VO2 diet were reduced compared to the control FO diet. Only the VO1 diet, however, showed significantly reduced levels of plasma $PGE_2$.

Inclusion of VO increased LA, ALA and 20:2(n-6) fatty acids in PBL of fish fed the VO diets. Oleic acid was increased in PBL of fish containing the VO diets, whereas fish fed the FO diet showed the highest n-3 HUFA concentrations in PBL. There were no significant differences in the DHA (product of Δ6-desaturation and elongation of EPA) content of lymphocytes of fish fed the two VO diets, although PBL from fish fed the FO diet had a significantly higher DHA content. Fish fed the FO diet had significantly more ARA, the product of Δ5-desaturation, in PBL than the fish fed the VO diets. It therefore appears that feeding diets containing VO has an inhibitory effect on Δ5 and Δ6 desaturase, which is responsible for ARA production. Leukocytes from fish fed the VO diets showed lower concentrations of EPA when compared with those from fish fed the FO diet. This

resulted in fish fed the VO diets, having a slightly higher EPA/ARA ratio compared to fish fed the FO diet. Thus, feeding the VO diets decreased ARA production and in turn altered the range of eicosanoids produced by the fish, in particular, reduction of $PGE_2$ was observed in fish fed the VO diets. Suppression of ARA-derived eicosanoids by dietary ALA has been observed in mammals (Marshall & Johnston, 1982). ARA is the principal precursor of eicosanoids in mammals (Horrobin, 1983), however, EPA and 20:3(n-6) are also eicosanoid precursors producing 1- and 3-series prostaglandins, although both are poorer substrates than ARA for prostaglandin synthetase (Crawford, 1983). Competition exists between EPA, 20:3(n-6) and ARA for prostaglandin production and the substrates can act as competitive inhibitors of ARA for the enzyme binding sites (Willis, 1981). Therefore dietary supplementation with 20:3(n-6), EPA or their C18 precursors can reduce the production and efficacy of ARA-derived eicosanoids (Willis, 1981; Weber, 1990).

The overall histological appearance of sea bass sampled from all of the dietary treatments was normal with very few differences observed between the groups. The only major difference was in the levels of absorptive vacuoles present in the proximal intestine. Sea bass fed the VO diets showed elevated numbers of absorptive vacuoles compared to fish fed the FO diet. The presence of increased absorptive vacuoles tends to suggest an "active" mucosa, however with increased mucosal vacuolation this could, in turn, leave the intestinal mucosal membrane more vulnerable to sloughing and breakdown.

In summary, FO may be partially replaced by diets containing blends of VO in diets for sea bass without compromising growth, non-specific immune function and overall histological

appearance. Further investigation needs to be carried out to assess the impact of VO on the process of macrophage respiratory burst and the production of reactive oxygen species.

# Chapter - 7 Culture of Atlantic cod (*Gadus morhua*) juveniles and Arctic char (*Salvelinus alpinus*) on diets containing echium oil: Effects on fish health and immune function.

# 7.1 General Introduction

## *7.1.1 Atlantic cod (Gadus morhua)*

Atlantic cod (*G. morhua*) is one of the most widely recognised fish consumed in the Western world. The species is distinguished by a distinctive barbel that hangs from the lower jaw and it has three dorsal fins, two anal fins and a broom shaped tail. The colourisation ranges from shades of grey to reddish brown and is speckled with spots. Cod has been identified as having considerable potential for intensive farming in northern Europe and Canada. Moreover, over-exploitation of wild cod has led to a sharp increase in its market value and has stimulated great interest in the farming of this species. Stocks have declined to unprecedented levels and are now considered to be below safe biological levels in many areas. The intensive farming of cod began in the mid-1980s although commercial production has failed to reach its potential due to technical difficulties, notably the insufficient production of juveniles. Initially the larvae are too small and undeveloped to feed on commercial pellets so rotifers and Artemia are used as a food source. Cod can spawn in tanks and so there is no need to strip them of their eggs like salmon. The market size for farmed cod is between 2-5 kg with Canada, Iceland, Norway, UK and USA being the major exporting countries. While only small amounts of farmed cod have been produced in the UK, production in the next ten years is forecast to increase dramatically.

Fish farmers throughout the world are familiar with using high-energy feeds for producing salmon, but there are a number of key differences in the nutritional requirements of cod, some of which present production challenges for feed producers. For example, cod require

very low levels of oil in feed. Cod is a lean fish, with less than 2 % fat in the muscle and the main storage facility for surplus fat is therefore the liver, which can comprise up to 23 % of the total body weight. Cod liver oil is of course a potentially valuable resource, rich in vitamins A and D and high in n-3 PUFA. However, in order to optimise fillet yield, cod need low oil diets to avoid excessive processing loss. Cod have similar muscle protein levels to salmon and therefore similar dietary protein levels can be used.

Several studies have emphasised the importance of investigating the immune system of any "new" species before it is used in commercial aquaculture (Press & Jϕrgensen, 1998). In this context studies on the immune system of cod are highly relevant. Atlantic cod is an economically important fish species, however, its immune system has not been extensively studied. The literature has indicated that cod serum contains a relatively high level of natural antibodies (Magnadottir *et al.*, 1999; Pilstrom & Petersson, 1991) and a high concentration of IgM (Israelsson *et al.*, 1991; Magnadottir *et al.*, 1999). The role of these natural antibodies in fish is not fully understood however it has been suggested that natural antibodies may recognise cellular self structures and hence be involved in natural viral defence (Gonzalez *et al.*, 1988). Some studies of the immune system of cod have indicated some unusual features compared to other teleosts species, in the fact that they are apparently unable to mount a specific antibody response to immunisation (Magnadottir *et al.*, 2001). This may perhaps be due to suppressed or poorly developed immune regulatory mechanisms or the lack of important cytokines or cell surface receptors.

### *7.1.2 Arctic char* (*Salvelinus alpinus*)

Arctic char (*Salvelinus alpinus*) is a member of the Salmonidae family and is closely related to both salmon and trout and has many characteristics common to both. They are variable in colour depending on the environmental conditions and time of year. Arctic char are an anadromous species, that in the wild can grow to salmon size but can live up to twenty five years as they do not die after spawning like most salmon. This species is captured from both the wild and as farmed fish, and cultured char can reach between 1.8 and 2.25 kg in less than thirty months. The typical market size of the fish is between 1 and 3.5 kg but because it is less widely available and takes longer to grow to an edible size, farmed Arctic char is generally more expensive than salmon. Iceland produces most of the world's supply of Arctic char along with other countries such as Norway, Sweden and Canada. Due to the fact that in some geographical locations, Arctic char appears to be in decline (Maitland, 1992), interest in farming this species has arisen from the point of restocking and for the development of commercial farming for human consumption.

The basic nutritional requirements for Arctic char are similar to those of other salmonids, although char differ in a number of ways. Char require higher levels of ALA, higher levels of carotenoids and higher quality proteins than other salmonids. As in other cultured salmonids, Arctic char require dietary ALA for optimal growth and feed conversion (Yang & Dick, 1994b) and char can convert ALA or LA into long chain PUFA (Olsen *et al.*, 1991; Olsen *et al.*, 1998). In a study carried out by Yang *et al.*, (1994) Arctic char fed diets containing 1 % LA or 0.1 % ALA had much lower haematocrit and haemoglobin values

compared to those given diets containing > 0.1 % n-3 PUFA, differing from reports published on rainbow trout (*O. mykiss*) (Castell *et al.*, 1972a, b, c). From the same study Yang *et al.*, (1994) concluded that Arctic char require 1-2 % dietary ALA or 20–40 % of dietary lipids. The requirement of LA is not fully clear, but some evidence suggests that levels <0.7 % of the total diet are beneficial to the growth of Arctic char (Yang *et al.*, 1994). Higher levels of LA inhibit growth once the requirements of ALA are met (Yang & Dick, 1994b).

In common with other salmonids, the Arctic char deposits large quantities of storage lipid in the muscle, and the fatty acid composition of these neutral lipid reserves is largely determined by the fatty acid composition of the diet (Olsen *et al.*, 1991). Among salmonid fishes the Arctic char is well adapted to cold waters (Johnson, 1980) and the optimum temperature for growth of farmed char is between 13 and 14 °C (Jobling *et al.*, 1993). Cultured Arctic char appear more tolerant to many common viruses and bacterial agents compared with other salmonids (Robbins *et al.*, 1990). They are also less susceptible to stress even when stocked at high densities, which may perhaps make them more able to fend off diseases than other salmonids. However, they are still susceptible to bacterial infections such as furunculosis, bacterial kidney disease, viral infections and sea lice infestation.

### *7.1.3 Echium oil*

Echium oil is produced from the plant seed of *Echium plantagineum*, also known as purple vipers bugloss. The plant is approximately two feet tall, with blossom varying in colour from white to pink to blue (Figure 7.1). The plant is a member of the Boraginaceae family and despite being regarded as a weed in some parts of the world for example in Australia, it has been domesticated for agricultural use in the UK and Europe. The echium oil is obtained by mechanical (cold) pressing of the seeds of the *Echium plantagineum* followed by a refining process. Echium contains two fatty acids which are not normally found in one singular natural seed oil, namely GLA (18:3(n-6)) and stearidonic acid (SDA) (18:4(n-3)), which gives echium oil the potential to be a very valuable product. Recent research has revealed multi-beneficial effects of SDA and GLA on human health (Fan & Chapkin, 1998; Kokura *et al.*, 1997; Zurier *et al.*, 1996; Horrobin *et al.*, 1989; Coupland *et al.*, 1996). SDA is the most highly unsaturated fatty acid found in terrestrial plants and is the Δ6-desaturase product of ALA and an intermediate in the biosynthesis of higher PUFA such as EPA and DHA. The SDA content of echium oil naturally varies between about 9 % and 14 %, but by conventional plant breeding techniques, cultivars with much higher SDA contents have recently been developed. SDA has been shown to possess both anti-inflammatory and anticancer properties and to be of value in reducing the harmful effects of UV radiation. As mentioned previously, SDA and GLA are post Δ6 metabolites and since Δ6 is the rate limiting step in the conversion of dietary EFA to higher PUFA, oils containing these fatty acids provide an excellent alternative to both FO and evening

# Chapter 7 - Culture of Atlantic cod (*Gadus morhua*) juveniles and Arctic char (*Salvelinus alpinus*) on diets containing Echium oil: Effects on fish health and immune function.

Figure 7.1 The Echium plant (*Echium plantagineum*).

primrose oil. The recent availability of echium oil in commercial quantities provides a viable land-based food source of the n-3 PUFA SDA that may be more readily converted to EPA and DHA than ALA. GLA is a commonly known fatty acid found in oils such as echium, evening primrose and starflower (borage). The body, however, has difficulty in converting LA into GLA, which would normally be obtained from a balanced diet of vegetables, fish and meat products. The normal production of EFA, via the metabolic pathway shown in Figure 7.2, can be influenced by many factors, some of which are outlined in Table 7.1 However, by introduction of GLA containing oils such as echium it can bypass these blockages. GLA can be further converted to DHGLA and then to $PGE_1$ which also has a number of beneficial effects in the body such as inhibition of cholesterol synthesis and inflammation, lowering of arterial pressure and it regulates production of saliva and tears to mention a few (Fan & Chapkin, 1998; Kokura *et al.*, 1997; Zurier *et al.*, 1996).

Two separate trials investigating the immune status of Atlantic cod and Arctic char fed diets containing either FO or echium oil are presented in this chapter. A number of non-specific immunological parameters including macrophage function and lysozyme activity were determined. In addition, samples of liver, heart and intestine were collected for assessment of histopathological lesions.

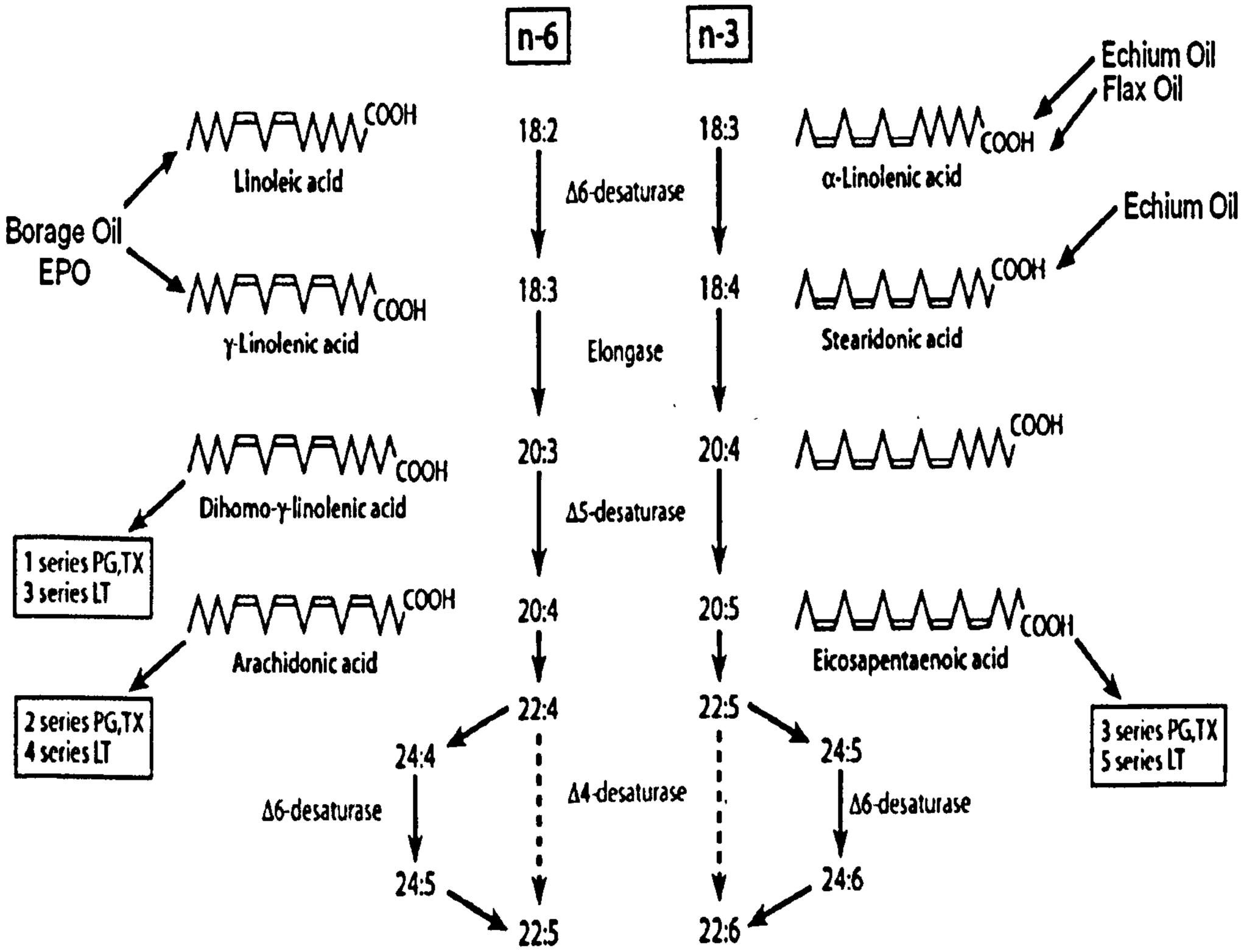



Figure 7.2 Desaturation & elongation pathways of n-3 and n-6 PUFA.

Table 7.1 Ways in which the normal production of GLA can be blocked

| |
|---|
| Too much dietary saturated fat |
| Too much dietary cholesterol |
| Trans fatty acids |
| Too much sugar consumption |
| Stress hormones |
| Diabetes |
| Too much alcohol consumption |
| Viral infections |
| Atopic conditions |
| Radiation |
| Cancer |
| Zinc deficiency |
| Aging |

## 7.2 Materials and Methods

### *7.2.1 Culture of Atlantic cod (G. morhua) on diets containing echium oil – Trial 1*

#### 7.2.1.1 Dietary groups

Duplicate groups of Atlantic cod juveniles (initial weight approximately 4 g) held at the Scottish Executive Environment and Rural Affairs Department (S.E.E.R.A.D) Fish Cultivation Unit, Aultbea, Wester Ross were fed fish meal based diets containing 55 % crude protein and 16 % crude lipid for a period of 18 weeks. The fish were fed a fixed ration of between 2.5 and 4 % of biomass per day. The added lipid component was either Northern hemisphere FO or echium oil and the dietary formulations are shown in Table 7.2. Diets were prepared by the Lipid Nutrition Group, Institute of Aquaculture, University of Stirling, using a California Pellet Mill (model number. labmill CL2). The fatty acid composition of the dietary treatments is shown in Table 7.3. The FO diet contained high levels of the long chain monoenoic fatty acids, 20:1(n-9) and 22:1(n-11), and the n-3 HUFA EPA and DHA. The echium oil diet contained reduced levels of these four fatty acids as well as reduced saturated fatty acids and ARA. However, the echium oil diet had increased levels of LA, GLA, ALA and SDA, compared to the FO diet.

Table 7.2 Diet formulation.

| Component | g $kg^{-1}$ |
|---|---|
| Fish meal [1] | 530 |
| Casein[2] | 180 |
| Starch[3] | 133 |
| Mineral mix ($M_2$)[4] | 24 |
| Vitamin mix (complete)[5] | 10 |
| Arginine[2] | 4 |
| Methionine[2] | 3 |
| Cystine[2] | 2 |
| Leucine[2] | 4 |
| Echium oil or Fish oil[6] | 110 |
| Antioxidant mix[7] | 0.4 |

[1] LT94 low temperature fish meal (Ewos Ltd., Bathgate, Scotland, U.K.)

[2] Sigma

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

[4] Supplied (kg $diet^{-1}$): $KH_2PO_4$, 22 g; $FeSO_4.7H_2O$, 1 g; $ZnSO_4.7H_2O$, 0.13 g; $MnSO_4.4H_2O$, 52.8 mg; $CuSO_4.5H_2O$, 12 mg; $CoSO_4.7H_2O$, 2 mg; KI, 2 mg.

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

[6] Echium oil – John King and Son Ltd. Fish oil – United Fish Products, Aberdeen, U.K.

[7] dissolved in propylene glycol and containing (g $L^{-1}$): butylated hydroxyanisole, 60; propyl gallate, 60; citric acid, 40.

Table 7.3 Fatty acid composition of experimental diets (g / 100 g total fatty acids).

| Fatty acid | Fish oil diet | Echium oil diet |
|---|---|---|
| 14:0 | 5.4 | 2.2 |
| 16:0 | 14.7 | 9.6 |
| 18:0 | 2.5 | 3.1 |
| Total saturates[1] | 23.1 | 15.1 |
| 16:1n-7 | 4.6 | 1.4 |
| 18:1n-9 | 13.3 | 13.4 |
| 18:1n-7 | 2.7 | 1.1 |
| 20:1n-9 | 8.6 | 4.4 |
| 22:1n-11 | 12.5 | 5.3 |
| 24:1 | 1.0 | 0.5 |
| Total monoenes[2] | 45.1 | 27.1 |
| 18:2n-6 | 1.9 | 12.8 |
| 18:3n-6 | 0.2 | 7.2 |
| 20:4n-6 | 0.7 | 0.1 |
| Total n-6[3] | 3.4 | 20.3 |
| 18:3n-3 | 1.7 | 20.4 |
| 18:4n-3 | 2.8 | 9.0 |
| 20:4n-3 | 0.8 | 0.2 |
| 20:5n-3 | 8.0 | 2.6 |
| 22:5n-3 | 1.2 | 0.3 |
| 22:6n-3 | 12.9 | 4.8 |
| Total n-3 | 27.6 | 37.4 |
| Total PUFA[4] | 31.8 | 57.8 |
| n-3/n-6 | 8.1 | 1.8 |

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

### 7.2.1.2 Collection of samples for immunological studies

### 7.2.1.3 Haematology and Macrophage function

After 18 weeks of feeding the experimental diets, blood samples were taken from 16 fish dietary treatment$^{-1}$ (8 fish per tank) as described in Section 2.3.2. From the same fish, samples of head kidney were removed as outlined in Sections 2.3.4 and 2.3.7 for macrophage activity analysis in the NBT reduction assay (Section 2.4.2) and phagocytosis assay (Section 2.4.3). The methods outlined in Sections 2.3.7 and 2.4.2 were followed, but certain changes to the protocol were implemented. Instead of culturing cod head kidney macrophages at 18 °C, the cells were cultured at 4 °C according to a study by Steiro *et al.*, (1998) who found that the cultivation of cod macrophages is optimal at low temperatures (4 – 6 °C).

### 7.2.1.4 Serum lysozyme activity

A second aliquot of blood was allowed to clot overnight and serum collected (Section 2.3.3) for analysis of lysozyme activity (Section 2.4.5).

### 7.2.1.5 Histopathology

Sections of heart, liver and intestine were sampled according to Sections 2.3.5 and 2.7.1 from 16 fish dietary treatment$^{-1}$ and processed and assessed according to the methods outlined in Section 2.7.2.

#### 7.2.1.6 Statistical analysis

All data are presented as means ± SEM (n = 16). Significant differences between dietary treatments were determined by Student's t-test using $p<0.05$ as a cut off for significance depending on the number of dietary groups. Differences between means were determined by Newman-Keuls post-test.

### *7.2.2 Culture of Arctic char (Salvelinus alpinus) on diets containing echium oil – Trial 2*

#### 7.2.2.1 Dietary Groups

This project was a joint study involving the Institute of Aquaculture, University of Stirling, Croda Universal Ltd. and John K. King and Son Ltd. Two diets were prepared using a base extrusion supplied by BioMar Ltd. containing 10 % of either Northern hemisphere FO or echium oil. The oils were sprayed onto the base extrusion feed to give a control FO and test echium oil diet comprising 47 % protein, 20 % oil and 8.7 % moisture. The fatty acid composition of the diets is shown below in Table 7.4.

These diets were fed to two different strains of Arctic char, in duplicate. The two strains of char came from a) Loch Coulin, a shallow loch in Wester Ross with a natural diet of zooplankton and insects and b) Loch Rannoch, a deep loch where char are predominately demersal and pisciverous. The reason for choosing these two strains was due their

Table 7.4 Fatty acid compositions of the fish oil and echium oil diets. Values are weight % of total fatty acids (n = 2).

| Fatty Acid | Fish Oil Diet | Echium Oil Diet |
|---|---|---|
| 14:0 | 5.7 | 3.5 |
| 16:0 | 15.5 | 12.8 |
| 18:0 | 2.7 | 3.1 |
| Total saturates[1] | 24.7 | 20.1 |
| 16:1 n-7 | 5.5 | 3.8 |
| 18:1 n-9 | 12.8 | 14.1 |
| 18:1 n-7 | 2.7 | 1.8 |
| 20:1 n-9 | 8.2 | 5.4 |
| 22:1 n-11 | 11.1 | 6.6 |
| 24:1 | 0.5 | 0.4 |
| Total monoenes[2] | 40.8 | 31.9 |
| 18:2 n-6 | 3.7 | 9.7 |
| 18:3 n-6 | 0.7 | 4.5 |
| 20:2 n-6 | 0.3 | 0.2 |
| 20:3 n-6 | 0.1 | 0.0 |
| 20:4 n-6 | 0.6 | 0.3 |
| 22:5 n-6 | 0.2 | 0.1 |
| Total n-6 | 5.5 | 14.7 |
| 18:3 n-3 | 2.7 | 12.6 |
| 18:4 n-3 | 3.4 | 6.8 |
| 20:4 n-3 | 0.7 | 0.4 |
| 20:5 n-3 | 8.2 | 5.1 |
| 22:5 n-3 | 0.9 | 0.6 |
| 22:6 n-3 | 11.7 | 7.4 |
| Total n-3 | 27.6 | 32.6 |
| n-3/n-6 | 5.0 | 2.2 |

[1] Includes 15;0, 17:0 and 20:0.

[2] Includes 16:1 n-9, 20:1 n-11, 20:1 n-7 and 22:1 n-9.

differing ability to convert $C_{18}$ PUFA to their long chain products, which was identified, in a previous study at the Institute of Aquaculture (Tocher *et al.*, 2001).

The trial started in October 2000 and ended in February 2001. Thirty fish were placed into eight 1m diameter tanks supplied with freshwater at a rate of 5 L $min^{-1}$. Fish were fed the diets by automatic feeders at a rate of 3 % biomass $day^{-1}$ and the ration adjusted every 28 days according to increase in biomass. The water temperature was maintained at 10 °C by heating the water. The immune status of the two different strains of Arctic char was observed after feeding the dietary treatments for 16 weeks.

### 7.2.2.2 Collection of samples for immunological studies

### 7.2.2.3 Haematology and Macrophage function

After 16 weeks of feeding the experimental diets, 8 fish dietary $treatment^{-1}$ from each strain of Arctic char were sampled. The methods outlined in Sections 7.2.1.3 and 7.2.1.5 were followed to assess immune function and assess pathological changes in tissues. Due to the small size of the fish sampled, and limited volumes of blood collected, serum lysozyme activity was not measured in this trial.

#### 7.2.2.4 Statistical analysis

All data are presented as means ± SEM (n = 8). Significant differences between dietary treatments were determined by ANOVA using $p<0.05$ as a cut off for significance depending on the number of dietary groups. Differences between means were determined by Newman-Keuls post-test.

## 7.3 Results

### *7.3.1 Trial 1 – Replacement of dietary FO with echium oil in Atlantic cod*

#### 7.3.1.1 Growth and flesh fatty acid composition

Growth and the flesh fatty acid composition of the fish were determined by Bell *et al.*, (personal communication) and the results are summarised below. No differences in growth were observed in fish after feeding them for 18 weeks with the experimental diets. Cod maintained on the FO diet had an average weight of 54.15 g and the fish fed the echium oil diet had an average weight of 55.15 g (n = 90-95). There were very few differences in lipid class composition in the flesh of cod fed the different diets. Feeding the echium oil diet, significantly reduced the percentages of 14:0, 16:0, total saturates, 16:1(n-7), 18:1(n-7), 20:1(n-9), 22:1(n-11), 24:1, total monoenes, 20:2(n-6), ARA, 20:4(n-3), EPA, 22:5(n-3), DHA, total n-3 PUFA and the n-3/n-6 PUFA ratio, compared to cod fed FO. By contrast, feeding echium oil significantly increased the percentages of LA, GLA, 20:3(n-6), ALA, SDA and total n-6 PUFA, compared to fish fed FO.

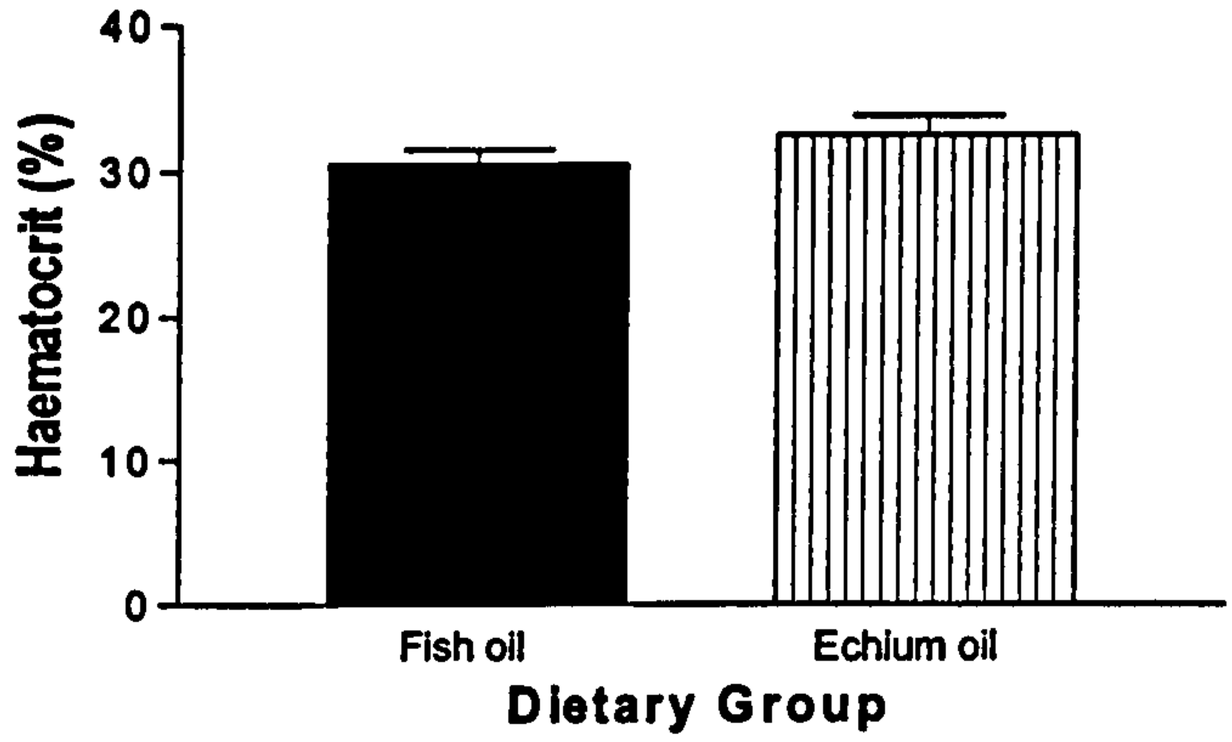
### 7.3.1.2 Haematology and Macrophage function

No differences were found in the haematocrit value between fish fed the experimental diets as shown in Figure 7.3a. Fish fed the FO diet had haematocrit values averaging 30.6 %, whereas fish maintained on the echium oil diet had an average value of 32.6 %. Fish fed the echium oil diet showed a marginally higher leukocyte count but this again was not found to be significant (Figure 7.3b). No differences were found between fish fed the different dietary treatments when measuring the total number of circulating erythrocytes as shown in Figure 7.3c. When measuring macrophage function by the production of superoxide anion by the NBT method, feeding echium oil to Atlantic cod resulted in a significant reduction in macrophage activity compared to fish maintained on a FO diet as shown in Figure 7.4 ($p=0.003$). No significant differences were observed in the phagocytic capacity of head kidney macrophages fed the experimental diets (Table 7.5).

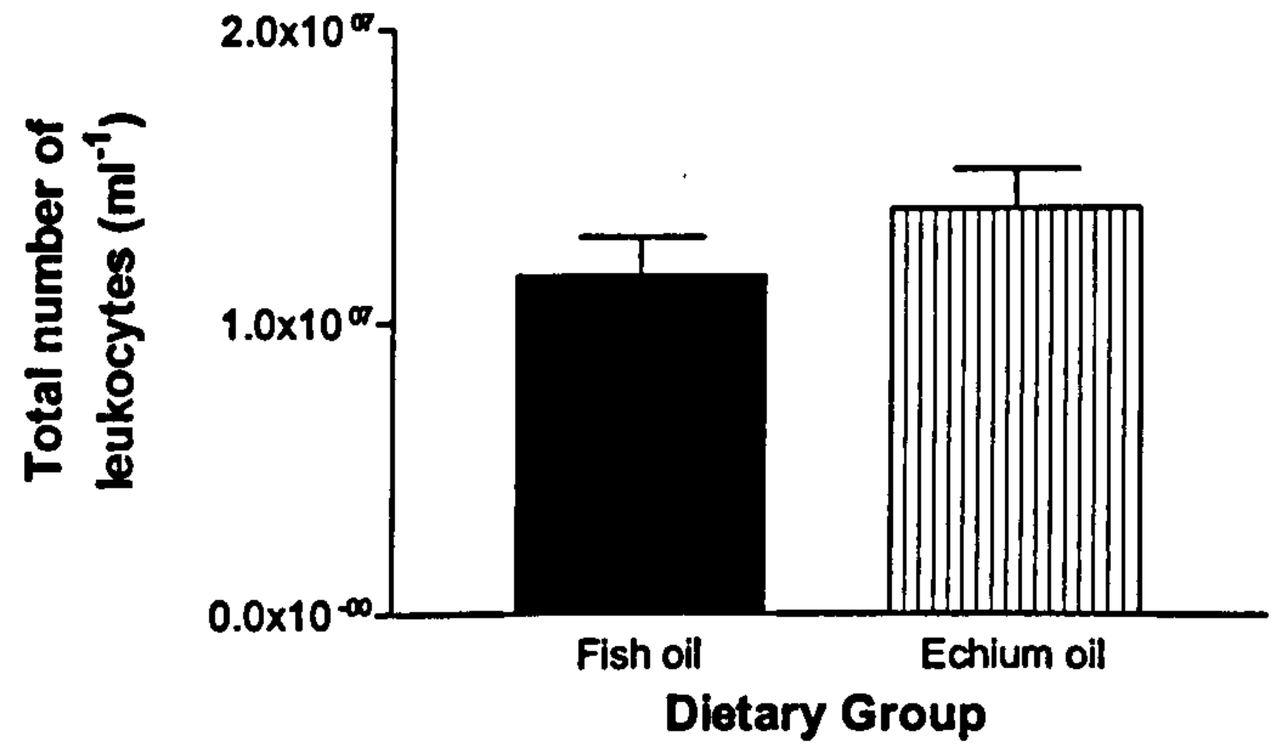
### 7.3.1.3 Serum lysozyme activity

Serum lysozyme activity was not influenced by feeding echium oil when compared to fish fed the FO diet (Figure 7.5). Atlantic cod fed the FO diet had a lysozyme concentration of 285.3 U $ml^{-1}$ $min^{-1}$ compared to 294.2 U $ml^{-1}$ $min^{-1}$ for echium oil fed fish.

(a)




(b)




(c)

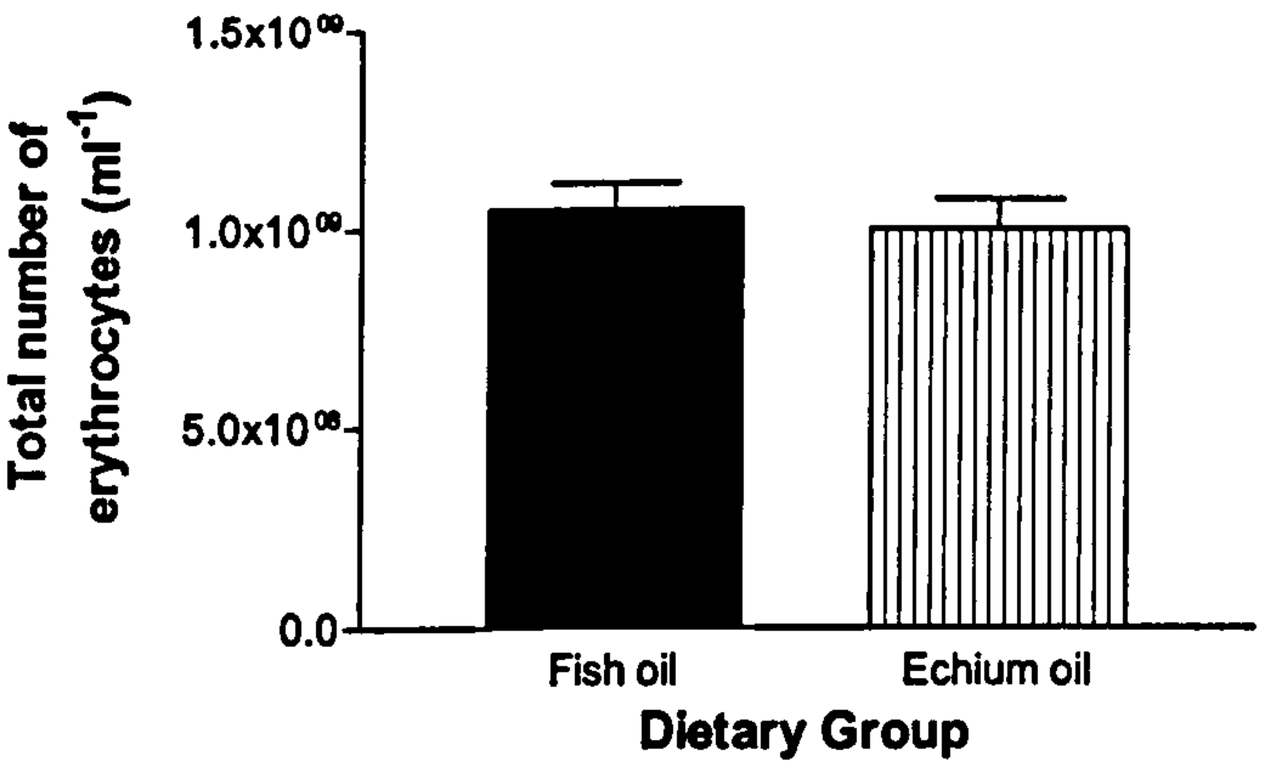



Figure 7.3 Effect of feeding the experimental diets for 18 weeks on Atlantic cod (a) % haematocrit (b) total number of leukocytes (c) total number of erythrocytes. Values are means ± SEM, n = 16.

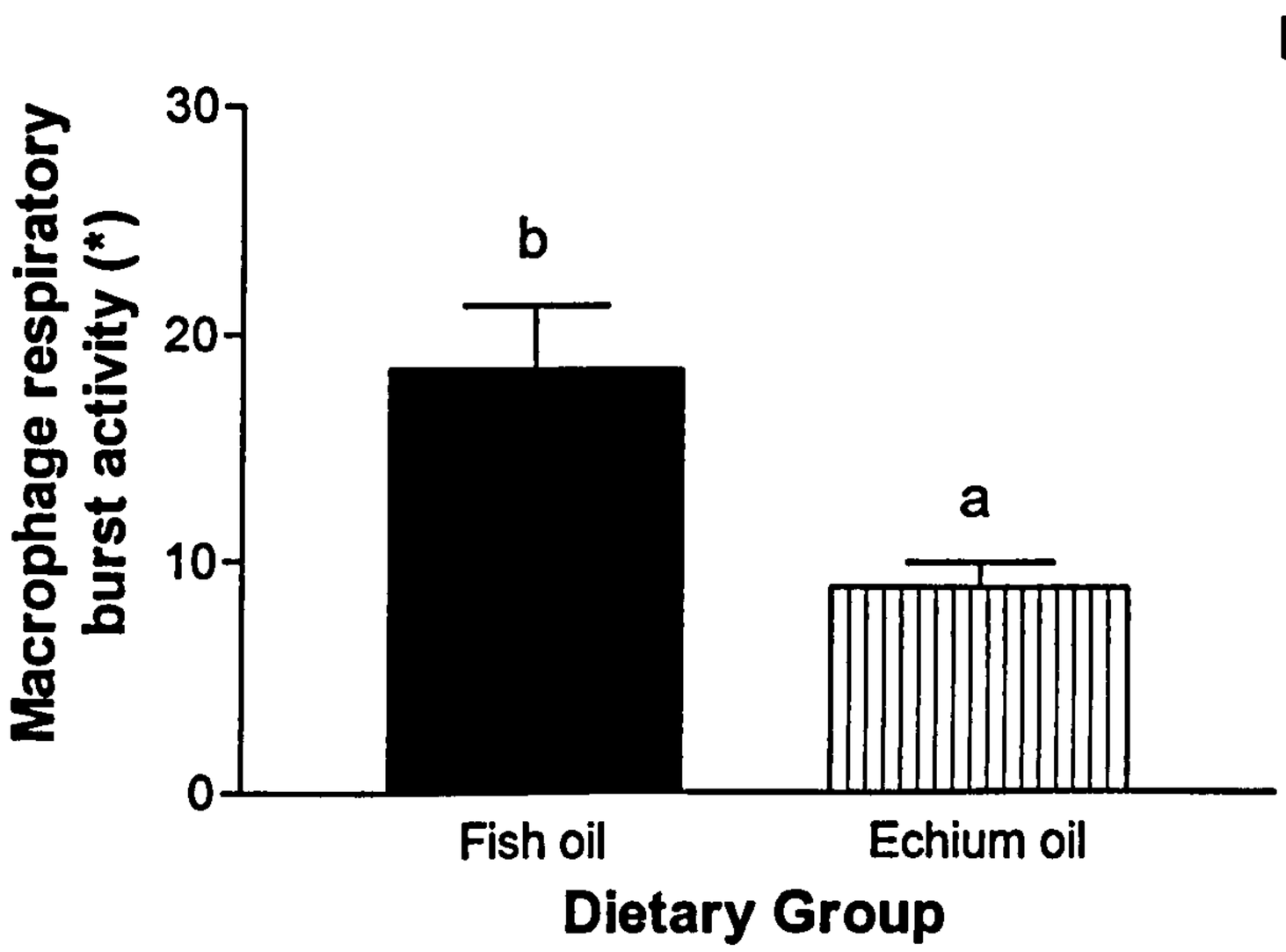



Figure 7.4 Effect of feeding the experimental diets for 18 weeks on head kidney macrophage respiratory burst activity (NBT reduction). Values are means ± SEM, n =16. Values having different column letters are significantly different ($p<0.05$). * = (Absorbance per $10^5$ cells x 100).

Table 7.5 Phagocytic capacity (% of macrophages which have phagocytosed yeast particles) of Atlantic cod fed either a FO diet or echium oil diet for 18 weeks.

| Diets | Phagocytic capacity (%) |
|---|---|
| Fish oil | 47.6 ± 21.3 |
| Echium oil | 43.8 ± 11.6 |

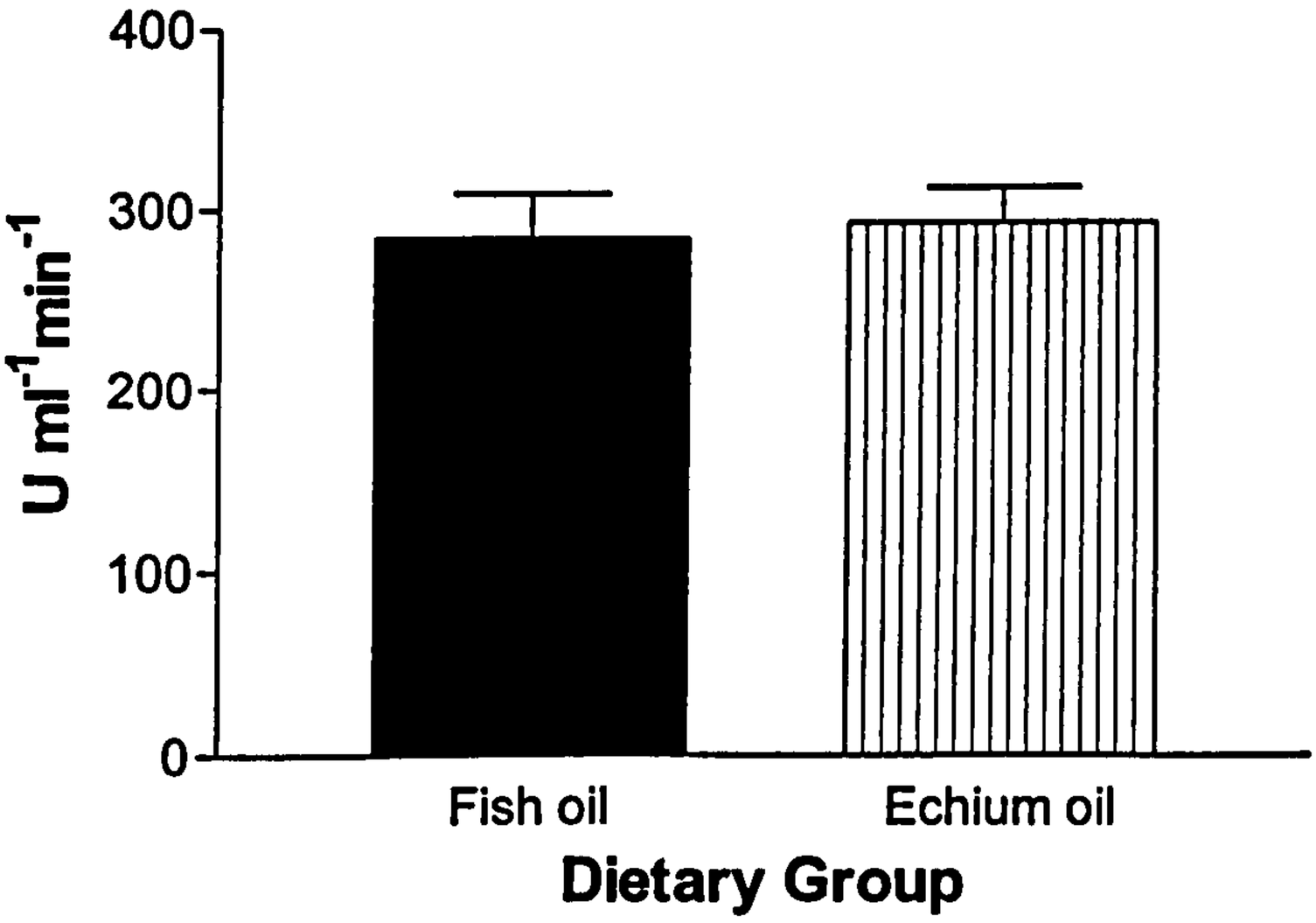
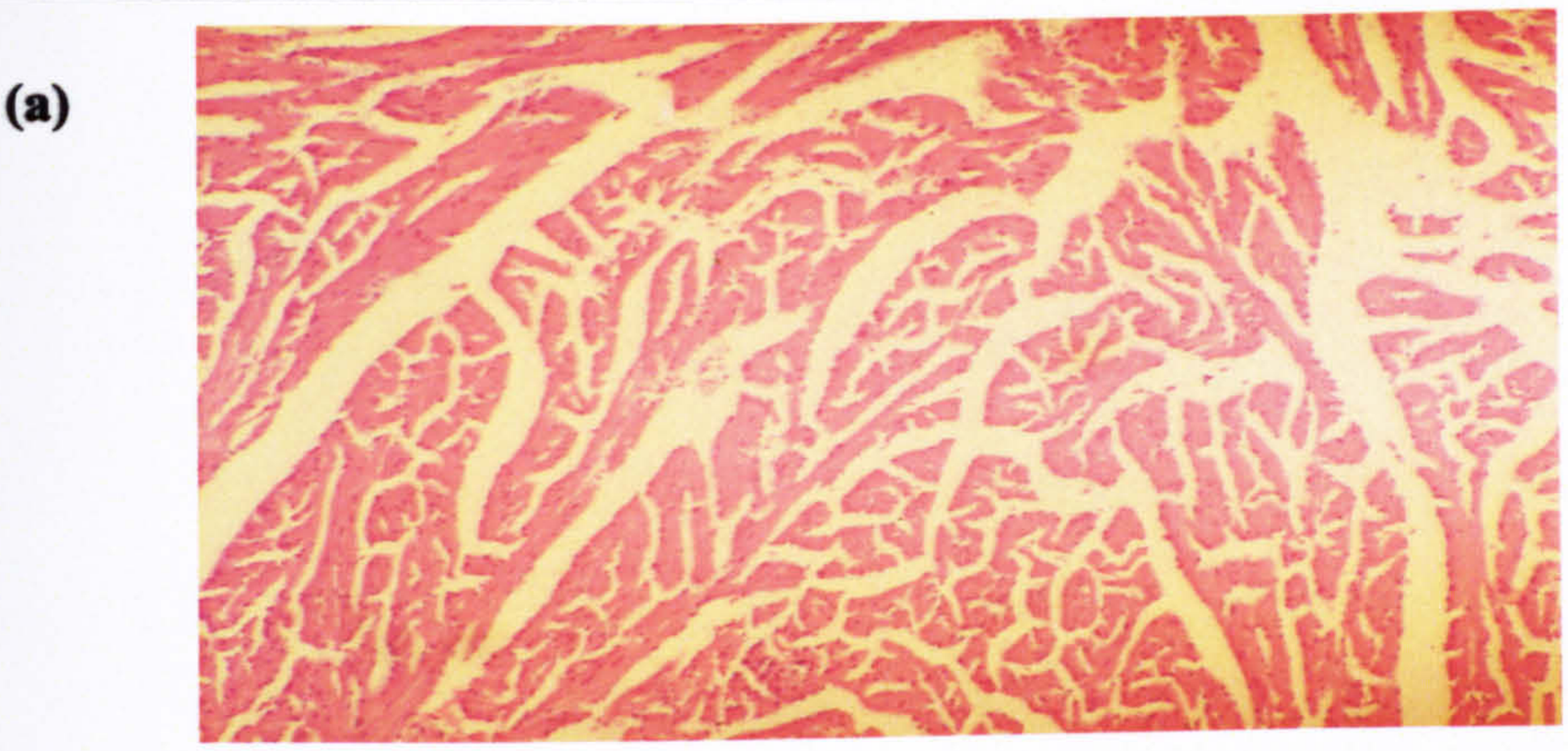
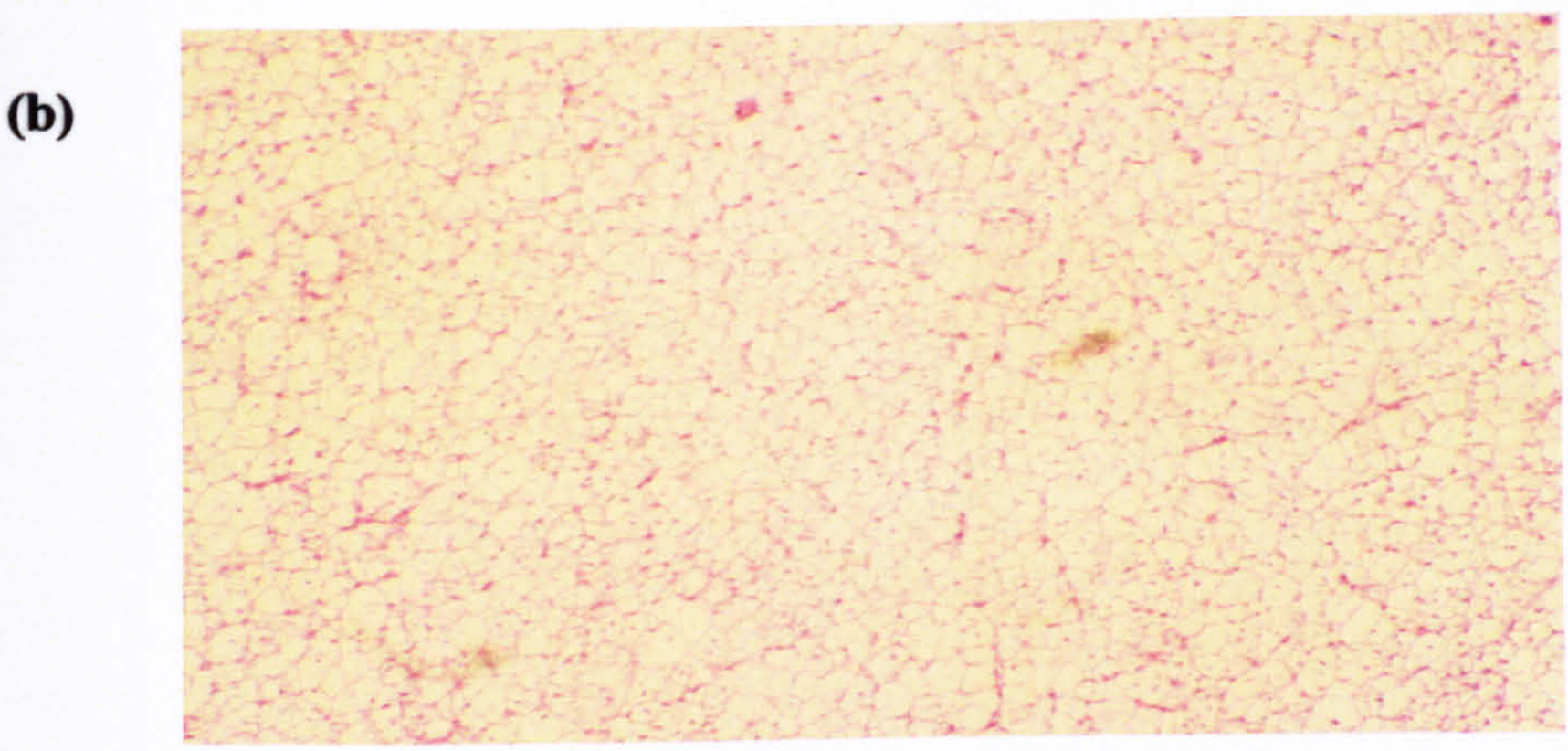



Figure 7.5 Effect of feeding the experimental diets for 18 weeks on cod serum lysozyme activity. Values are means ± SEM, n =16.

### 7.3.1.4 Histopathology

It was noted that the histological appearance of the Atlantic cod intestinal tissue tended to differ from Atlantic salmon in that they seem to have a more refined mucosal structure than the large absorptive vacuoles seen in the mucosa of Atlantic salmon. When comparing the different dietary groups very few pathological changes or differences between the groups were reported. Fish fed the FO diet showed normal histological appearances of the heart and liver sections examined as illustrated in Figure 7.6a and 7.6b. All dietary groups had similar liver fat levels and some of the livers from both the FO diet and echium oil diet had small patchy inflammatory lesions, but no necrosis or peri vascular cuffing (Figures 7.6c and 7.7a). Heart sections taken from fish fed the echium oil diet appeared normal, showing no pericarditis and only a few sections had small patches of minor endocarditis but no necrosis or other muscle pathology was found (Figure 7.7b). No intestinal pathology was reported, just slight mucosal sloughing in the proximal and mid sections of fish fed the echium oil diet as shown in Figures 7.7c and 7.7d. It was thought that these were most likely processing artefacts rather than true pathology. Mucus levels and vacuolation appeared normal and similar for both dietary groups.



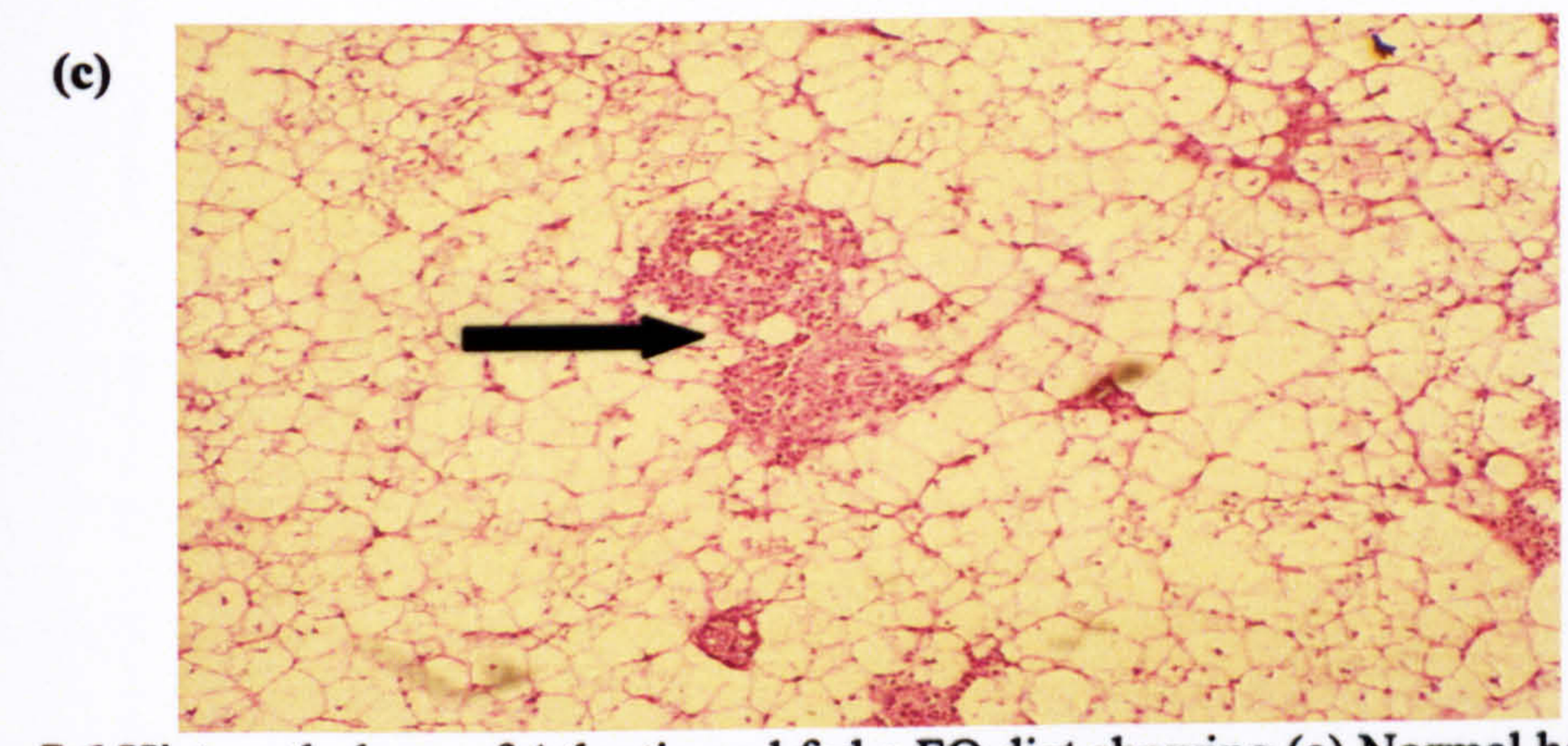




Figure 7.6 Histopathology of Atlantic cod fed a FO diet showing (a) Normal heart (mag. x 175) (b) Liver showing a regular structural appearance (mag. x 175) (c) Liver showing minor patches of inflammation (mag. x 175).

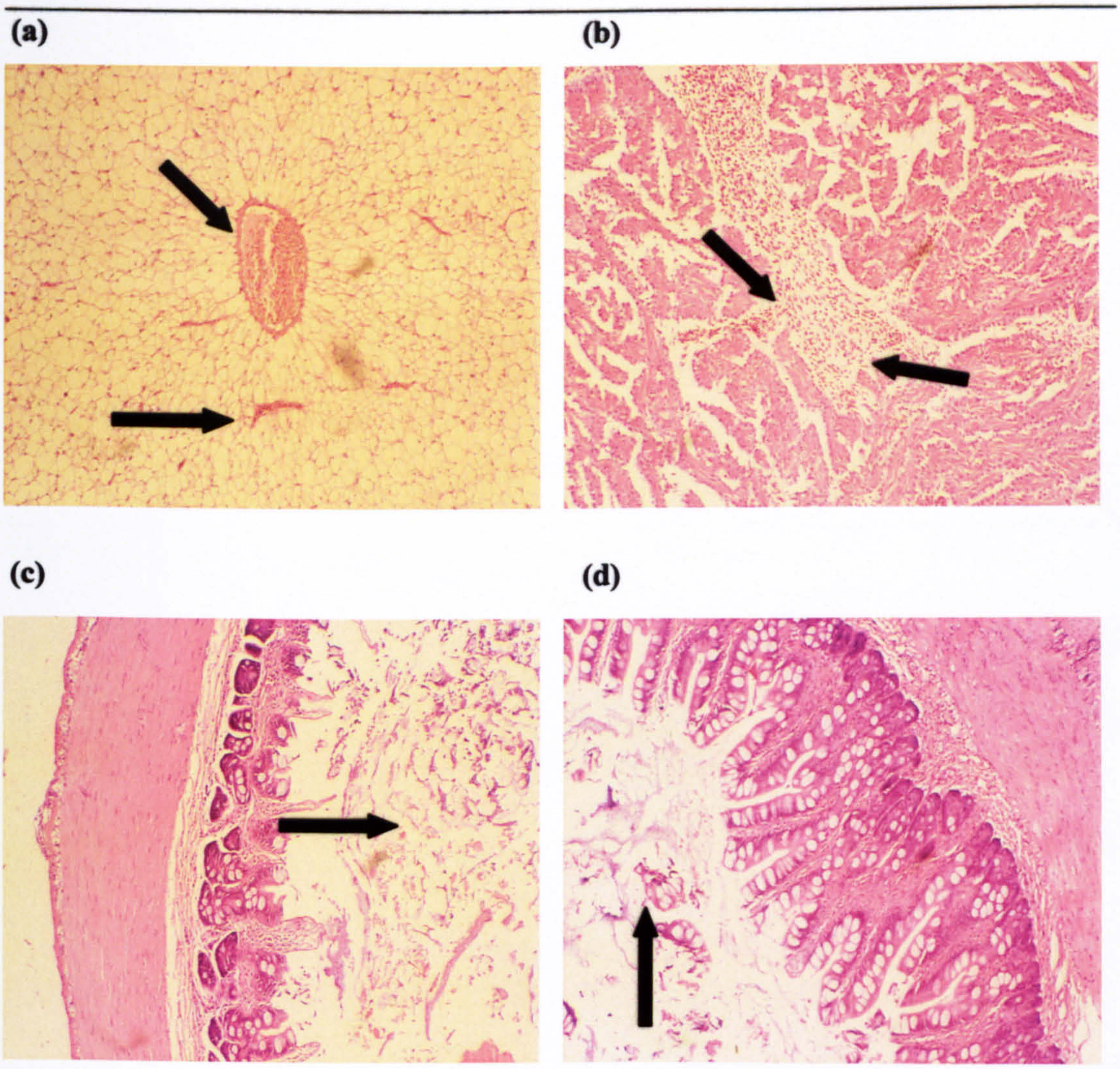



Figure 7.7 Histopathology of Atlantic cod fed an echium oil diet showing (a) Liver with a slight inflammatory response (mag. x 175) (b) Heart showing patches of minor endocarditis but no necrosis or other muscle pathology (mag. x 175) (c) Proximal intestine showing mucosal sloughing which may be a processing artefact rather than true pathology (mag. x 175) (d) Mid intestine showing slight mucosal sloughing which may be a processing artefact rather than a dietary effect (mag. x 175).

### *7.3.2 Trial 2 – Replacement of dietary FO with echium oil in Arctic char*

#### 7.3.2.1 Growth, tissue lipid content and flesh fatty acid composition

Growth and the flesh fatty acid composition of the fish were again determined by Bell *et al.*, (personal communication) and the results are summarised below. No significant differences were observed in growth between the different strains of Arctic char fed the two experimental diets. The Coulin strain fed either the FO diet or echium oil diet showed slightly higher weights compared to the Rannoch strain, but no significant differences were found. The flesh fatty acid composition of the Coulin strain of char fed the echium oil diet had significantly increased levels of 18:0, LA, GLA, ALA, SDA, 20:3 n-6, total n-6 and total PUFA compared to fish fed the FO diet. In addition, the Coulin strain fed the echium oil diet showed significantly reduced levels of 14:0, 15:0, 16:0, 16:1 n-7, 18:1 n-7, 20:1 n-9, 20:1 n-7, ARA, EPA, 22:1 n-11, 22:1 n-9, 22:5 n-3, total saturates and total monoenes when compared to fish fed the FO diet. The flesh fatty acid composition found significantly increased levels of 18:0, oleic acid, LA, GLA, ALA, SDA, 20:3 n-6, total n-6 PUFA and total PUFA and decreased levels of 16:0, 16:1 n-7, 18:1 n-7, 20:1 n-9, EPA, 22:1 n-11, 22:5 n-3, total saturates and total monoenes in the Rannoch strain of char fed the echium oil diet when compared with those fed the FO diet.

#### 7.3.2.2 Haematology and Macrophage function

With regard to immune function studies, feeding the echium oil diet had no effect on the non-specific immune system of Arctic char as shown in Figures 7.8 and 7.9 and Table 7.6.

The only significant difference that was found was in the Coulin strain of Arctic char fed the FO diet, which had reduced numbers of circulating leukocytes, compared to all other dietary groups (Figure 7.8b).

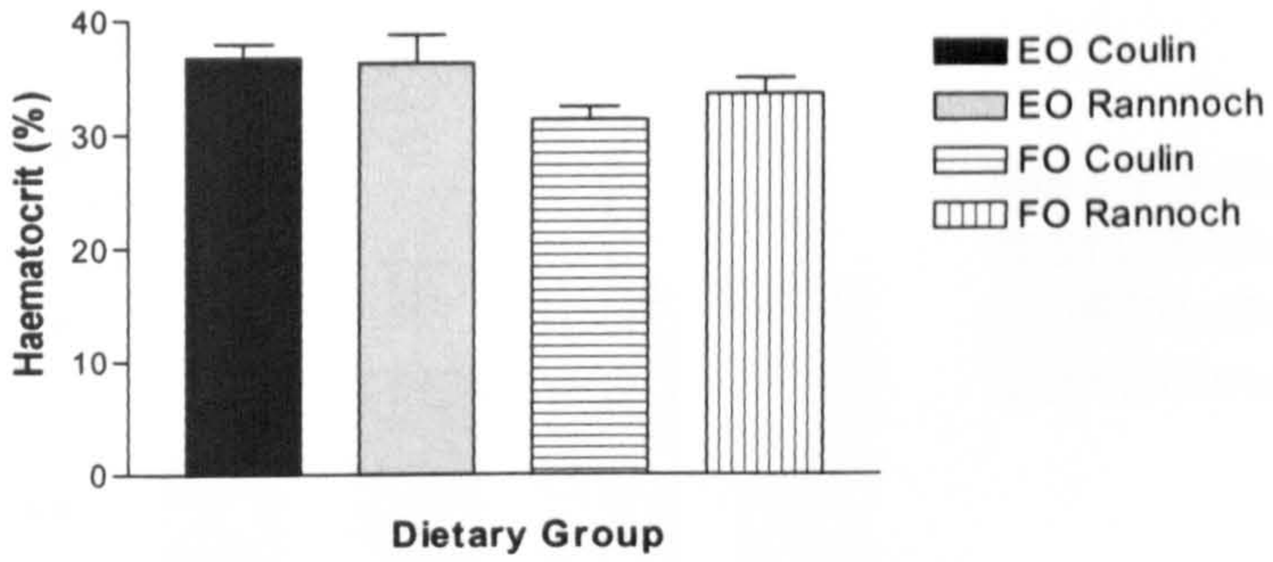
### 7.3.2.3 Histopathology

The Coulin Arctic char strain fed the FO diet showed no abnormalities in the heart sections examined. The livers had an overall moderate fat content with single, regular vacuoles, which were evenly distributed throughout the tissue as illustrated in Figure 7.10a. The intestine appeared, in general, normal with no pathology detected and only some sloughing of the mucosa in the mid sections (Figure 7.10b).

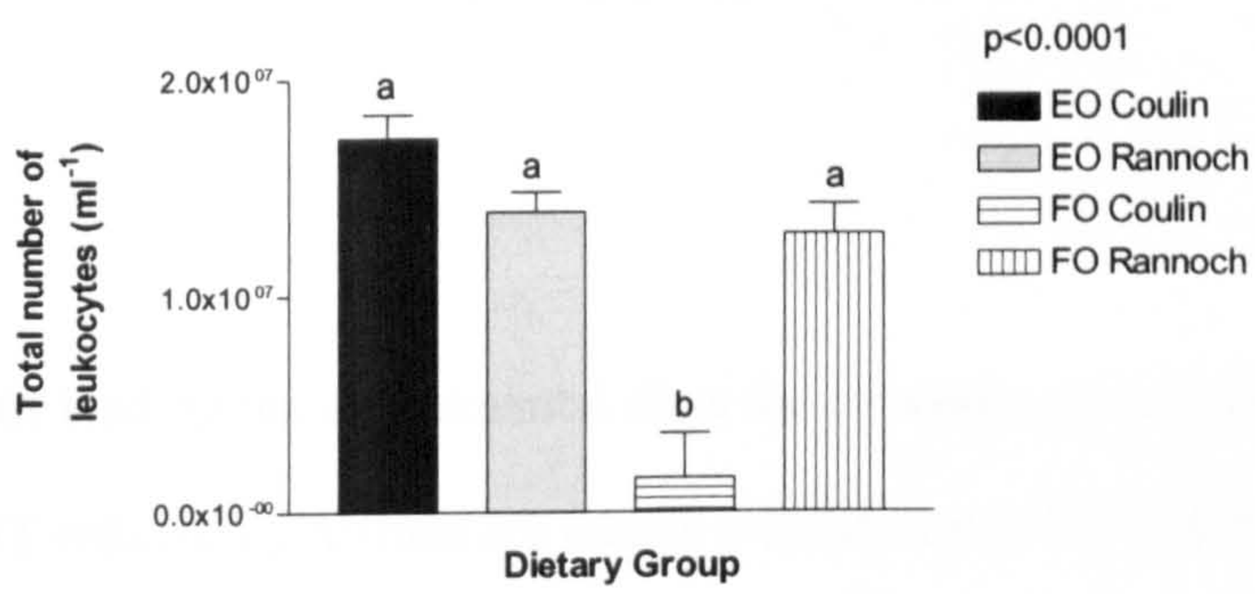
Hearts from the Rannoch Arctic char strain fed the FO diet appeared normal. There was a low to moderate fat content within the livers with single, regular hepatocytes (Figure 7.11a). No pathology was evident in the intestines examined although a few had signs of enterocyte breakdown in the distal section as shown in Figure 7.11b.

After feeding the echium oil diet, the heart and intestinal sections sampled from the Coulin strain of char were normal. The distal segments of the intestines were highly vacuolated and the livers had a moderate fat content with mostly single vacuoles that were variable in size (Figure 7.12). In addition, there were some minor patches of single cell necrosis and some loss of the general architecture of the liver but overall changes were minimal.

(a)




(b)




(c)

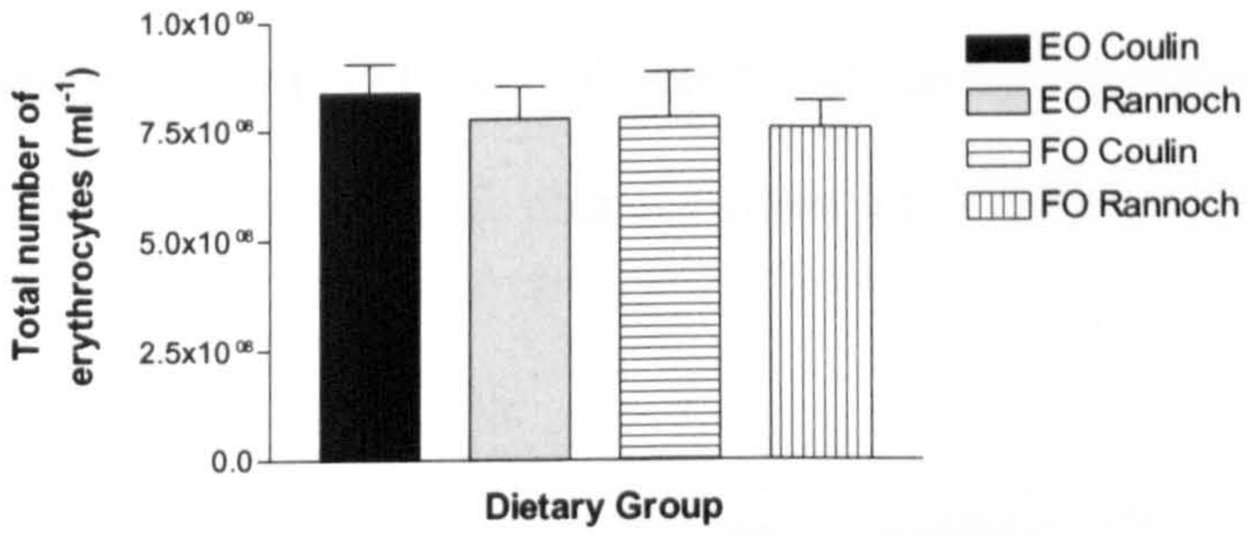



Figure 7.8 Effect of feeding the experimental diets for 16 weeks to Coulin and Rannoch strains of Arctic char on (a) % haematocrit (b) total number of leukocytes (c) total number of erythrocytes. Values are means ± SEM, n = 8. Values having different column letters are significantly different ($p<0.05$). FO = fish oil diet, EO = echium oil diet.

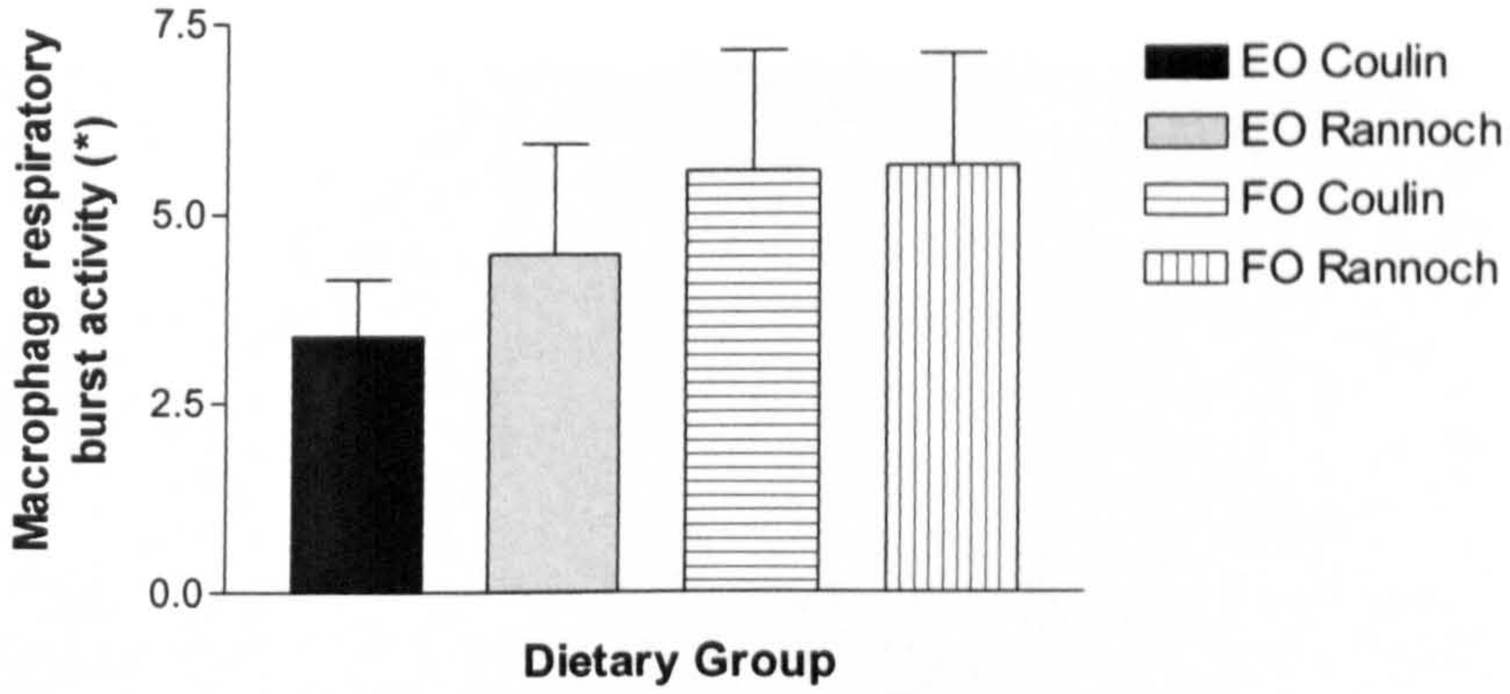



Figure 7.9 Effect of feeding the experimental diets for 16 weeks on macrophage respiratory burst activity (NBT reduction). Values are means ± SEM, n = 8. FO = fish oil diet, EO = echium oil diet. * = Absorbance per $10^5$ cells x 100.

Table 7.6 Phagocytic capacity (% of macrophages which have phagocytosed yeast particles) of the different strain of Arctic char fed either a FO diet or echium oil diet for 16 weeks.

| **Dietary Group** | **Phagocytic capacity (%)** |
|---|---|
| Echium oil Coulin strain | 55.8 ± 10.2 |
| Echium oil Rannoch strain | 65.0 ± 14.8 |
| Fish oil Coulin strain | 63.2 ± 8.8 |
| Fish oil Rannoch strain | 69.4 ± 17.6 |

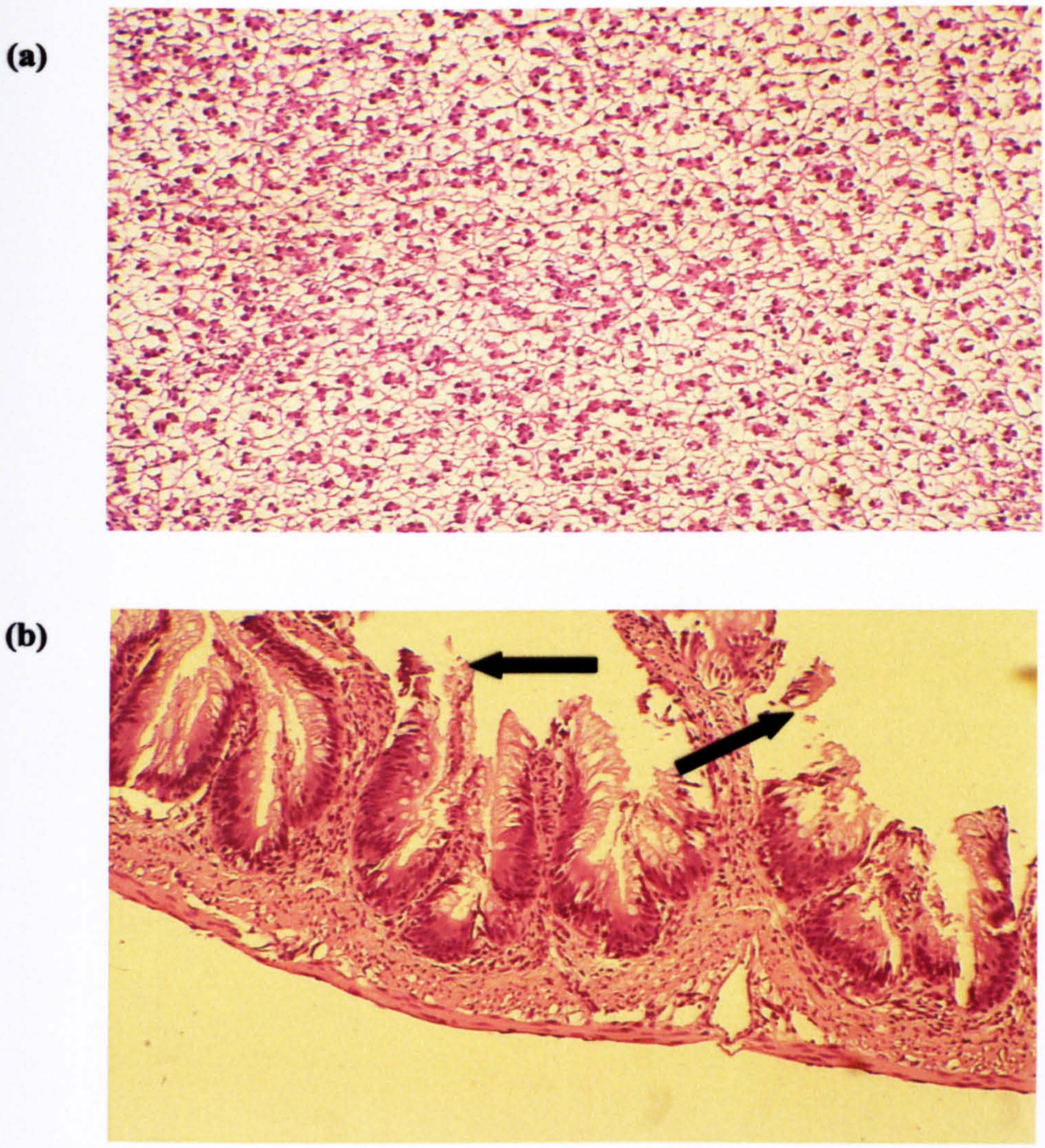
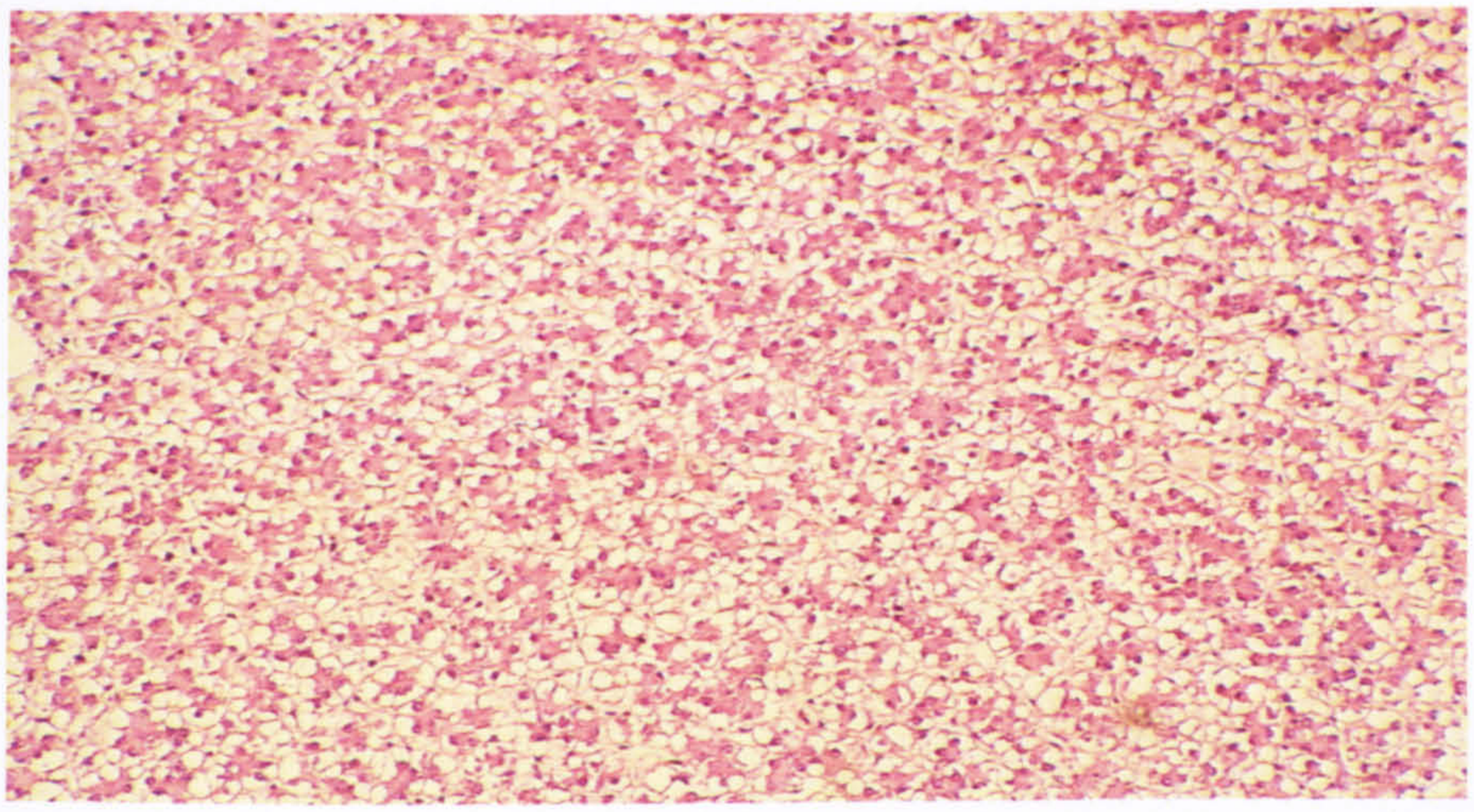



**Figure 7.10 Histopathology of the Coulin strain of Arctic char fed a FO diet showing (a) normal liver appearance with regular vacuoles and normal fat content within the hepatocytes (mag. x 175) (b) mid intestine showing some degree sloughing of the mucosal folds (mag. x 175).**

(a)



(b)

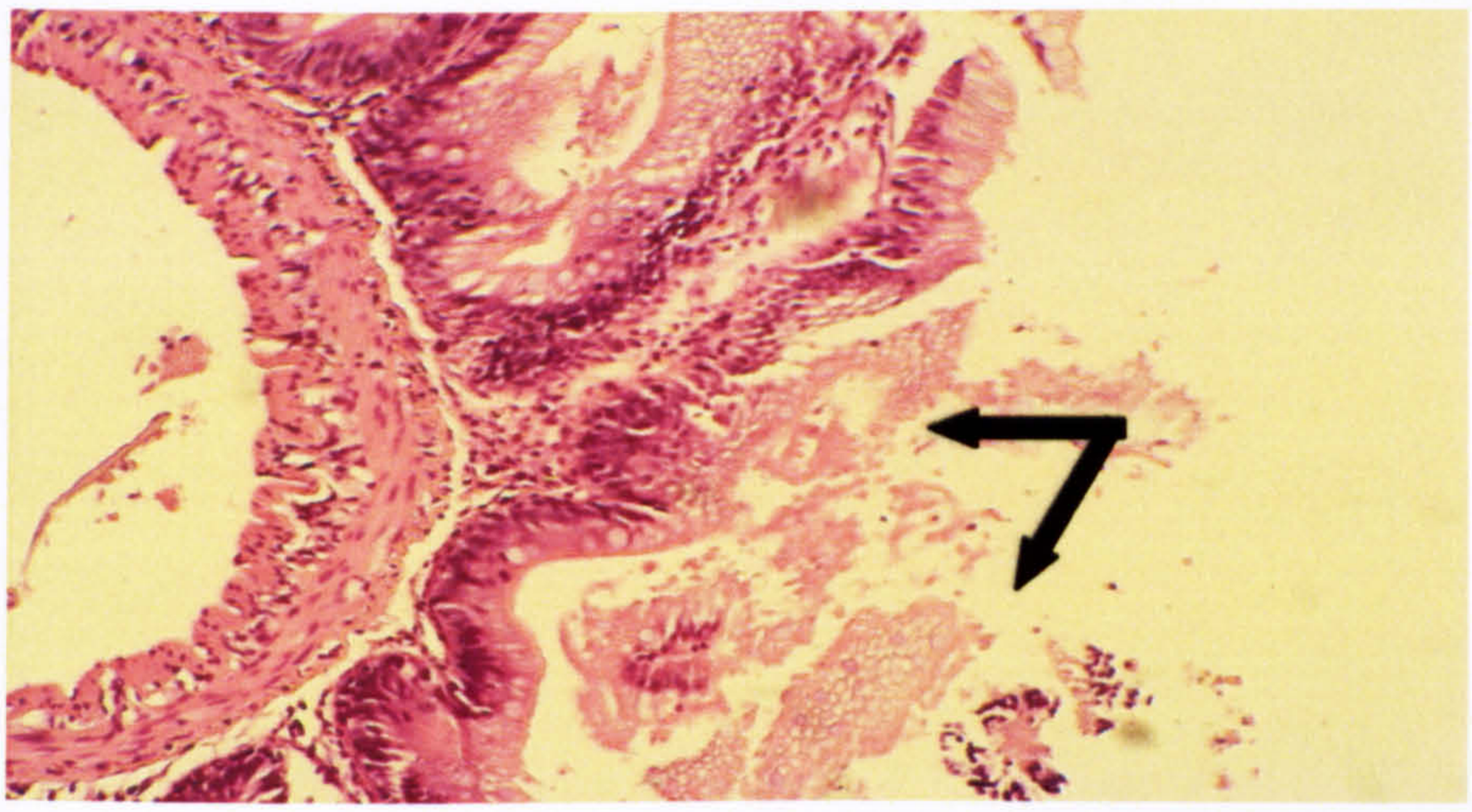


Figure 7.11 Histopathology of the Rannoch strain of Arctic char fed a FO diet showing (a) normal liver appearance with regular vacuoles and normal fat content within the hepatocytes (mag. x 175) (b) distal intestine showing some degree of sloughing of the mucosa and enterocytes breakdown (mag. x175).

Heart sections from the Rannoch char strain showed no signs of pathological change. The livers resembled those of the Coulin strain with some granular cytoplasm and irregular shaped vacuoles (Figure 7.13a) and a minor scattering of single necrotic cells as shown in Figure 7.13b. Overall the intestine appeared normal with only the distal segment showing some necrotic mucosa (Figure 7.13c).

## 7.4 Discussion

Numerous studies have shown that salmonids can effectively utilise VO, with no apparent adverse effects on growth and overall performance, provided that the oils contain sufficient ALA to satisfy the fishes EFA requirements (Polvi & Ackman, 1992; Bell *et al.*, 1993a, b; Guillou *et al.* 1995; Dosanjh *et al.*, 1998). From a review of the literature available, no other study has investigated the potential use of echium oil in diets for fish. The main reasons for investigating the effect of echium oil inclusion in aqua-feeds were firstly, because it contains GLA and ALA which can by-pass Δ6-desaturase, this may lead to increased conversion to EPA and DHA in Arctic char and secondly, elongation to 20:3(n-6) and 20:4(n-3) in Atlantic cod. Both these changes would have implications for eicosanoid production and hence immune response.

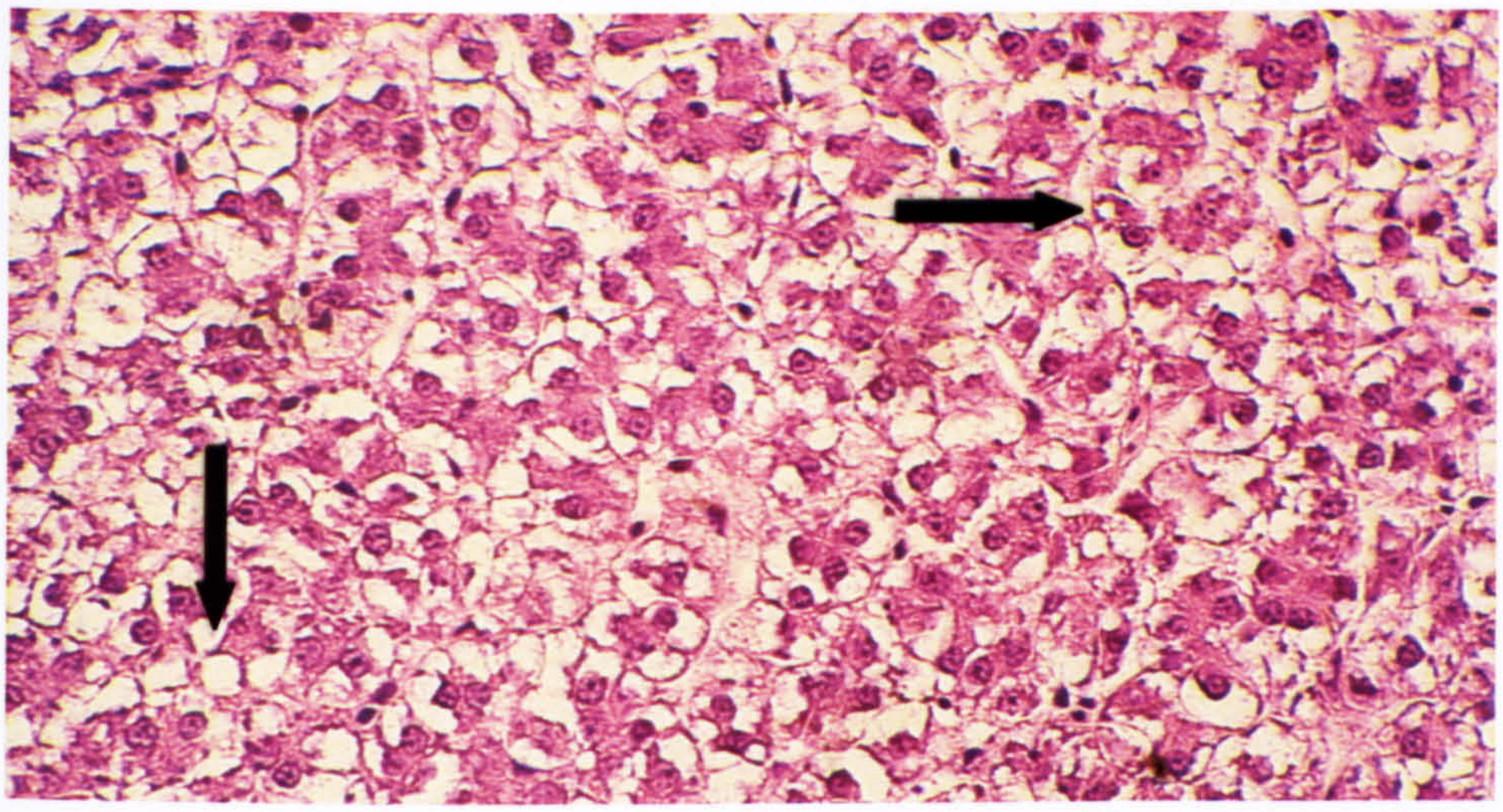
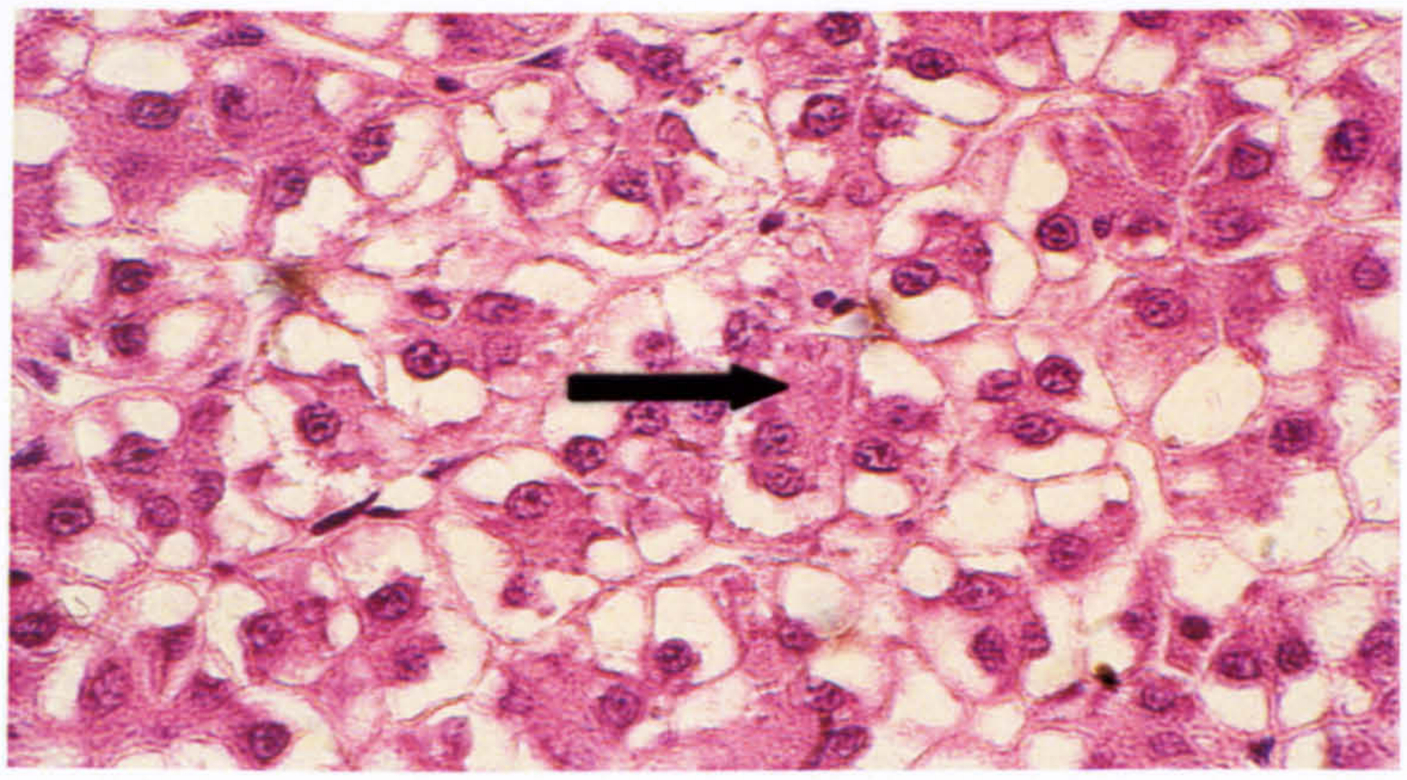
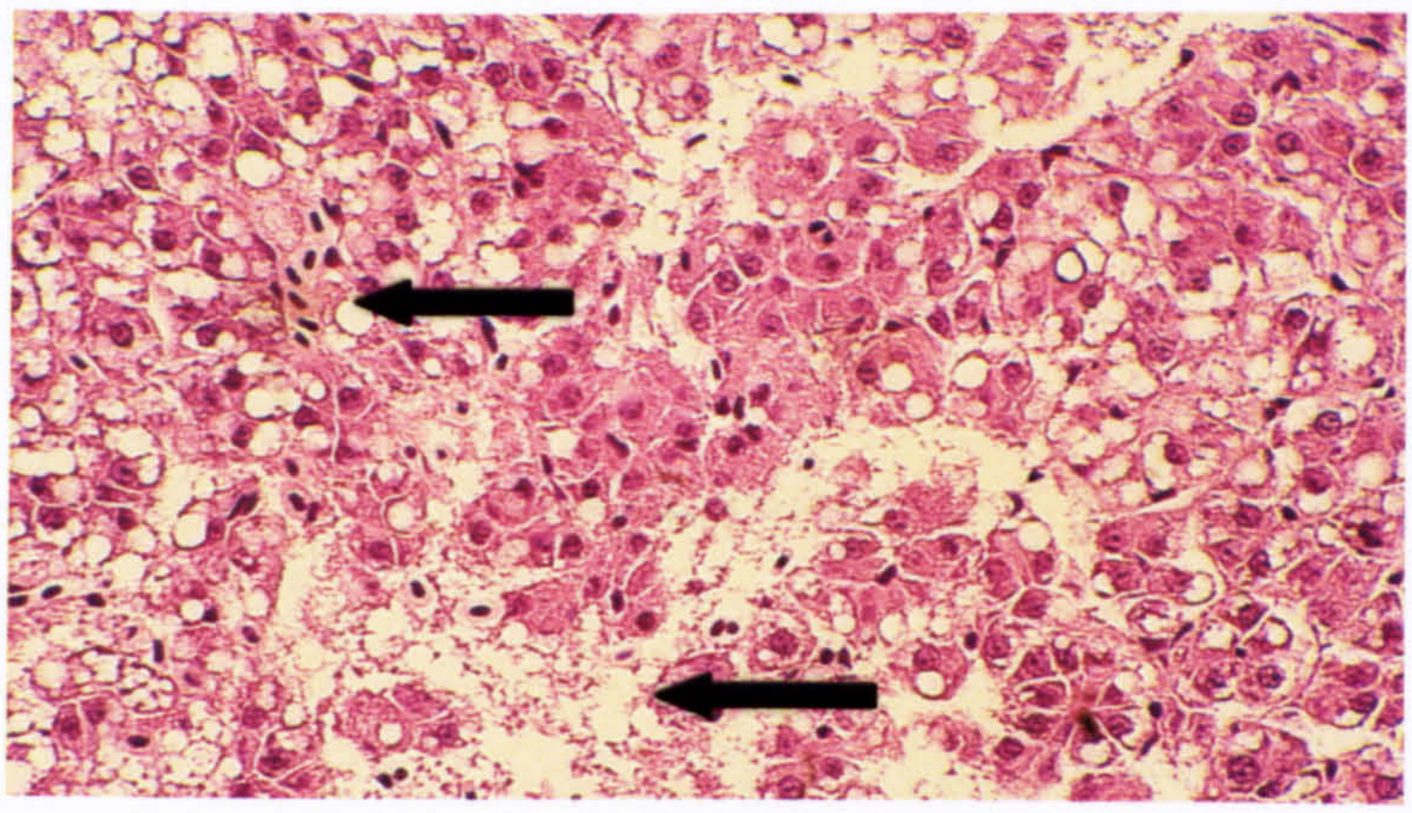


Figure 7.12 Histopathology of the Coulin strain of Arctic char fed an echium oil diet showing a liver with a moderate fat content with mostly single vacuoles, variable in size (mag. x 175).

(a)



(b)



(c)

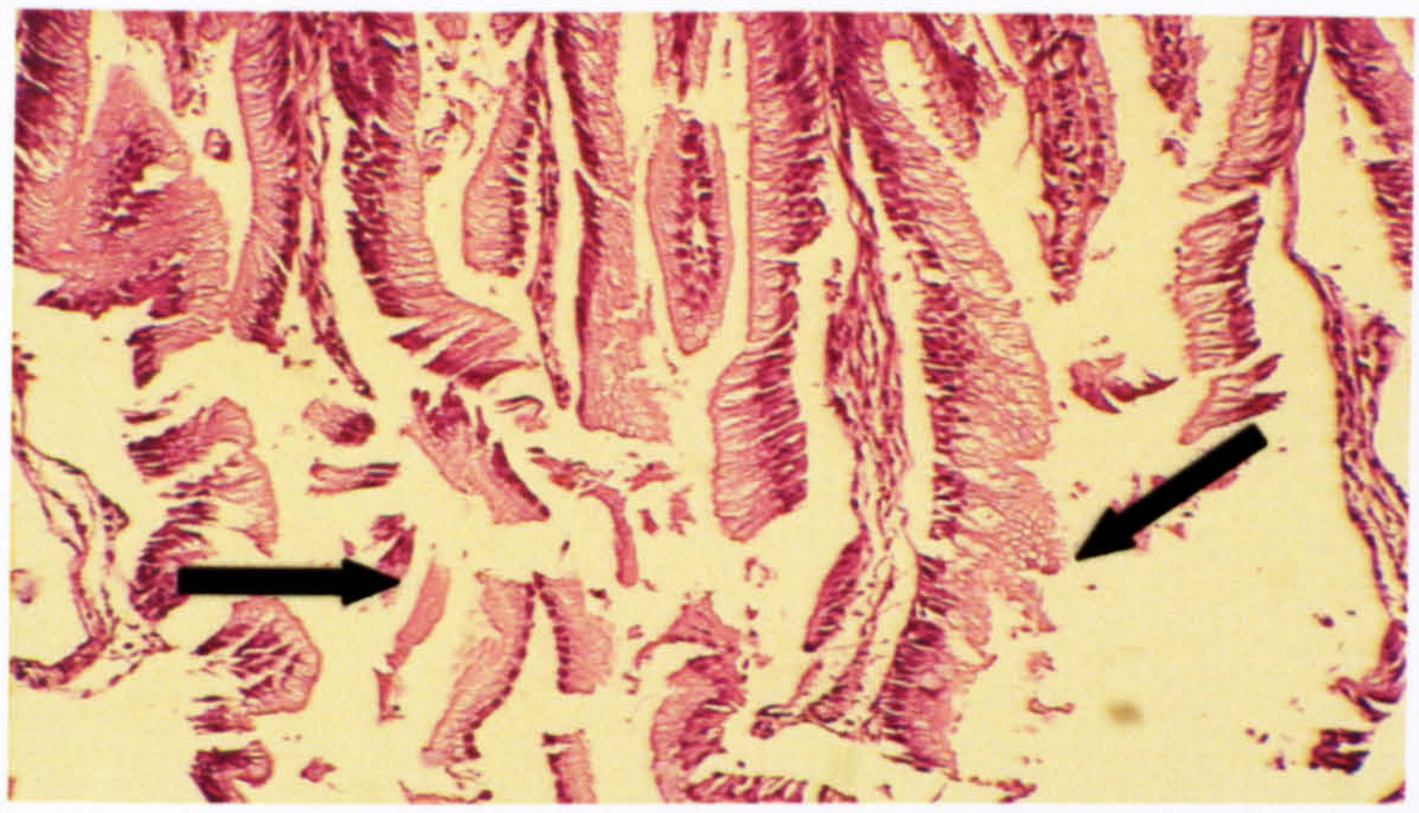


Figure 7.13 Histopathology of the Rannoch strain of Arctic char fed an echium oil diet showing (a) Liver similar in appearance to the Coulin strain with some granular cytoplasm and irregular shaped vacuoles (mag. x 430) (b) liver showing some single cell necrosis and general loss of structure (mag. x 175) (c) distal intestine with some necrotic mucosa (mag. x 175).

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The principal aim of Trial 1 was to investigate how echium oil would impact on selected aspects of the innate immune response in Atlantic cod. From the data presented in this chapter it would seem that feeding echium oil diets to cod had no significant effect on the immune parameters measured when compared to cod fed a FO diet.

As evident from previous trials (Chapters 3-5) the only immunological parameter affected by dietary manipulation with VO appears to be macrophage respiratory burst activity. In Trial 1, Atlantic cod fed with the echium oil diets had significantly reduced macrophage respiratory burst activity compared to fish fed the FO diet. Interestingly, no significant differences were found when measuring the phagocytic capacity of head kidney macrophages to engulf yeast particles. In a study by Waagbϕ *et al.*, (1995) the authors found that adult cod fed with a soybean oil-containing diet, had lower haematocrit and haemoglobin levels compared to cod fed with a capelin oil-containing diet.

Lysozyme activity has been detected in fish with levels varying between species (Mock & Peters, 1990). It has been previously shown that its concentration in fish depends on several environmental factors, including the presence of bacteria and water temperature (Fletcher & White, 1976). It is also known that even minute variations in the fish's environment may influence the level of lysozyme (Grinde *et al.*, 1988). Rainbow trout has a twenty times greater activity of the enzyme than Atlantic salmon. Grinde *et al.*, (1988) tested lysozyme activity from the head kidney of twelve different species of fish. The authors found that within a species, there was typically a 5 to 10 fold variation in activity (rainbow trout 5877 U $ml^{-1}$, Atlantic salmon 413 U $ml^{-1}$, cod 33 U $ml^{-1}$ and sea char 2094 U

ml$^{-1}$). Activities varied considerably both between and within species. Rainbow trout showed the highest activity of kidney lysozyme, approximately fourteen times higher than Atlantic salmon. Lie *et al.*, (1989) found that the main lysozyme activity in Atlantic salmon was associated with a molecular weight of ca 12000 to 15000 Daltons, which corresponds to the classical type lysozyme found in birds and mammals. The differences in lysozyme activity between species may be due either to isozymes with different specific activities or to the concentration of lysozyme, or to a combination of the two. Within a species variation is probably due to different concentrations of lysozyme molecules. Lysozyme distribution is associated with leucocytes-rich tissues, with kidney having the highest levels of activity in Atlantic salmon then the alimentary canal, spleen, skin, mucus, serum, gills, liver and muscle (Lie *et al.*, 1989). Some authors have reported that lysozyme concentration increases during infection and injection of foreign matter (Fletcher & White, 1973; Siwicki & Studnicka, 1987) and the close association with cells of the immune system indicates that the enzyme contributes to defence against infectious diseases.

Previous studies have failed to demonstrate lysozyme activity in cod (Fletcher & White, 1973; Fange *et al.*, 1976; Magnadottir, *et al.*, 1999). In the present study however, low levels were detected which is in agreement with Lie *et al.*, (1989) who reported concentrations of kidney lysozyme at 150 lysozyme units g $^{-1}$ and Grinde *et al.*, (1988) who reported a concentration of 33 U ml $^{-1}$. The concentration of lysozyme seems to be much higher in salmonids than in members of the Gadoid family where it is relatively low (Grinde *et al.*, 1988; Lie *et al.*, 1989). This is not due to an environmental effect because high concentrations have been recorded for other marine fish in similar habitats. In the

present study, the same pH of sodium phosphate buffer was used (pH 5.8) for salmon as for cod, however it has been shown that there are possibly differences in the optimal pH between fresh water and marine fish.

A study carried out by Rϕed *et al.*, (1993) revealed that there was significant individual and family variation in Atlantic salmon with regard to lysozyme activity. Subsequently, studies nowadays are trying to improve lysozyme concentration in certain species by employing genetic improvements through selective breeding. Species with low levels of lysozyme, like cod, are ideal candidates for the transfer of lysozyme genes from within a species or between other species potentially improving their bacterial defence system. Employment of new techniques such as recombinant DNA in transgenic experiments aimed at creating enhanced or optimised lysozyme activity in fish, would ultimately help them to withstand stressful culture conditions and increase their bacterial defence system.

It was thought that the elongation activity, present in cod tissue, might lead to increased deposition of the elongation products of GLA and SDA, namely 20:3(n-6) and 20:4(n-3). According to Bell *et al.*, (personal communication) this did not occur to a significant extent in flesh, liver or gill tissue. Feeding cod the echium oil diets resulted in cod liver with a unique fatty acid profile enriched with n-3 HUFA and GLA, ALA and SDA fatty acids. Bell *et al.*, (personal communication) also examined the phospholipid composition of cod flesh in fish fed echium oil and found high levels of DHA and EPA and increased levels of GLA, ALA and SDA. It is believed that phospholipids are more effectively absorbed and

incorporated compared to the same PUFA supplied as TAG, therefore the flesh fatty acid profiles of echium oil fed cod could be important in delivering essential PUFA to human consumers.

Very few histopathological differences were observed after feeding either echium oil or FO diets to Atlantic cod in Trial 1. In a study by Morais *et al.*, (2001) the authors confirmed previous observations in cod and other species (Lie *et al.*, 1988; Jobling *et al.*, 1991; Lanari *et al.*, 1999; Peres & Oliva-Teles, 1999; Santinha *et al.*, 1999; Nanton *et al.*, 2000), in which increases in the hepatosomatic index and liver lipid content were observed with increasing levels of dietary fat. Morais *et al.*, (2001) also studied the effect of diets containing two protein (48 % and 58 %) and two lipid levels (12 % and 16 %) on cultured cod liver histology. In many cases they found, the presence of numerous lipid droplets in hepatocytes, which are a physiological response to excess lipid. In addition, there were signs of increased vacuolation within the hepatocytes but no evidence of impaired liver function or pathology, as described by Mosconi-Bac (1987) in sea bass. A study by Caballero *et al.*, (1999) reported that seabream fed a low lipid level (15 %) showed numerous small lipid droplets within liver hepatocytes compared to large sized lipid droplets within the hepatocytes of fish fed a lipid level of 22 %. When fish were fed a diet containing 27 % lipid, hepatocytes appeared swollen with displaced nuclei and cellular organelles. Mosconi-Bac (1987) and Caballero *et al.*, (1999) suggested that this fat accumulation may only be a mechanical consequence of increasing dietary lipid content and is not related to a nutritional pathological disorder.

In conclusion, with respect to immune function and histological appearance, replacement of FO with echium oil in diets for Atlantic cod is acceptable, however further work is needed to investigate the effects on macrophage respiratory burst activity and overall disease resistance.

In Trial 2 the only significant difference found when assessing the immune status of Arctic char fed the experimental diets was a reduction in leukocyte numbers in the Coulin strain fed the FO diet. There is no conclusive explanation as to why this observation was made, perhaps genetic variation within the species contributed to the differences observed. Perhaps these tanks of fish were previously stressed prior to, or during the sampling regime. One of the main mechanisms involved in stress induced reduction in disease resistance is a suppressive effect on numbers and function of circulating lymphocytes (Ellsaesser & Clem, 1986). It is doubtful that the reduction in leukocyte numbers was caused by a dietary effect. Although both strains of char fed the echium oil diet exhibited lower respiratory burst activity than fish fed the FO diet, no significant differences were seen.

Similar to the results found with Atlantic cod, very few pathological changes were found after feeding the echium oil diet to Arctic char. Both strains of char fed the echium oil showed granular cytoplasm and some irregular shaped vacuoles within the hepatocytes, however it is doubtful if these changes would affect the overall performance of the fish and its health status. In contrast to this, Olsen *et al.*, (1999, 2000) found a significant impact on

the gastrointestinal tract function and integrity when feeding large amounts of LO to Arctic char. Feeding with LO promoted lipid droplet accumulation in the enterocytes, which resulted in epithelial damage and lipid droplet and cell debris in the intestinal lumen. Olsen *et al.*, (1999, 2000) concluded that feeding experimental diets containing high levels of PUFA and low amounts of saturated fatty acids promotes the accumulation of lipid droplets that induce a pathogenic state. It is worthwhile noting, however, that the diets fed in the Olsen studies were purified diets that contained no phospholipids and Liu *et al.*, (2002) found that dietary phospholipids greatly affect lipid digestion, absorption and transport in cultured fish, and its absence in the diet may lead to morphological changes in gut epithelia. No intestinal damage was observed in seabream fed LO diets which had similar levels of phospholipids (Caballero *et al.*, 2003).

Nutritional status is one of the most important factors that determines the ability of fish to resist disease, and some information is available on the relationship between dietary lipid and disease resistance (Erdal *et al.*, 1991; Li *et al.*, 1994; Thompson *et al.*, 1996; Hardy 1997). Lϕdemel *et al.*, (2001) fed diets containing LO, soybean oil and FO to groups of Arctic char and found enhanced survival of char fed the soybean oil and LO diets compared to those fed the marine FO diet. These results are in accordance with results cited by Hardy (1997) in which channel catfish disease resistance was enhanced by feeding VO diets, but contradicts the findings by Li *et al.*, (1994) (outlined in Chapter 5).

From the results presented in Trial 2, it is concluded that under these experimental conditions, Arctic char can be fed diets containing echium oil without adverse effects on

immune function and histological appearance. Future investigation into the relative survival of Arctic char fed echium oil diets after bacterial challenge would be beneficial to measure overall disease resistance.

# Chapter - 8 General Discussion and Future Prospects

A large proportion of the world's production of FO is used in the production of fish feed for the aquaculture industry. The demand for FO by this industry will probably exceed available resources in the coming years (Tacon, 1998). It is therefore essential to search for alternative lipid sources, to guarantee the future growth of the industry. Vegetable oils are the obvious candidates for FO replacement and global production of these has increased in recent years, therefore the price of VO has been relatively constant. Successful replacement of FO by VO would reduce both the absolute dependence on FO as a raw material and its related cost. Finding alternatives to FO has become the focus of much research with the aim of producing sustainable supplies of commercial fish diets at reduced costs. However, it is important that the commercial producers realise the influence that dietary fatty acids have on the composition of storage lipids, especially because of the current attention focusing on the value of fish and fish products as a source of bioactive PUFA in human nutrition. This focus is no longer just on promoting good growth in fish but also on the production of fish with good nutritional properties. The awareness of the relationship between fish consumption and human health issues, for example in the prevention of heart disease and reduction of inflammatory disorders, has instigated public interest in the nutritional value of n-3 PUFA, especially EPA and DHA (Sargent, 1999).

The need for optimised diets which improve health and prevent diseases of farmed fish is widely recognised (Kiron *et al.*, 1995; Waagbϕ *et al.*, 1993a, b; Li *et al.*, 1999; Sealey & Gatlin, 1999; Sitja-Bobadilla & Perez-Sanchez, 1999; Kanazawa, 2001; Gannam & Schrock, 2001, Irianto & Austin, 2002). Numerous studies have reported various effects of dietary lipids on immune functions in both mammals (Calder, 1999; Hummell, 1993; Thies *et al.*, 2001) and fish (Bell *et al.*, 1996a; Blazer *et al.*, 1989; Wise *et al.*, 1993). Variations

in the dietary fatty acid profiles caused by the inclusion of dietary VO may alter fish metabolism, which might in turn affect fish health and stress resistance. The inclusion of VO can produce sub-optimal ratios of n-3 and n-6 fatty acids, which could affect fish health by altering the synthesis of eicosanoids (Fracalossi *et al.*, 1994). Changes in the fatty acid composition of the dietary lipids have been shown to affect both innate defence mechanisms (Sheldon & Blazer, 1991; Obach *et al.*, 1993, Waagbϕ *et al.*, 1993a, 1995), the adaptive immunity (Erdal *et al.*, 1991; Waagbϕ *et al.*, 1993b, 1995; Fracalossi & Lovell, 1994; Thompson *et al.*, 1996) and resistance to infectious diseases (Salte *et al.*, 1988; Erdal *et al.*, 1991; Waagbϕ *et al.*, 1993a; Fracalossi & Lovell, 1994; Thompson *et al.*, 1996). However, the role of n-3 and n-6 fatty acids in the fishes immune response is unclear, and reports are not conclusive and are very often contradictory.

The purpose of the trials carried out in this study was to assess the effect of partial replacement of FO, by several VO sources, in feeds for farmed fish on immune function, histopathological changes and disease resistance. The effects of dietary VO on innate immune responses such as, serum lysozyme activity, phagocytosis, respiratory burst response of head kidney macrophages, white and red blood cell numbers and haematocrit were examined. The specific antibody response to vaccination and protection elicited by the vaccine when fish were fed the experimental diets was also studied. While considerable data have been accumulated on the effects of different dietary lipids on tissue fatty acid compositions, for both mammals and fish, the effects of different dietary lipids on fish health and immune function are less well documented. Fatty acids have diverse roles in all cells. They are important as a source of energy, as structural components of cell membranes and as signalling molecules. In mammalian studies, dietary fatty acids may be

able to modulate the immune system through several mechanisms that include reduction of lymphocyte proliferation, reduction of cytokine synthesis, phagocytic activity and modification of natural killer cell activity (de Pablo & Cienfuegos, 2000). The main event involved in the modulation of immune functions may be associated with changes in the cell membrane due to dietary fatty acid manipulation. Fatty acids are incorporated into the plasma membrane after dietary lipid is given, so that the composition of lipids in the cellular membrane reflects the composition of dietary lipids (Clamp *et al.*, 1997). It is probable that modulation of the overall immune system occurs as a result of alterations in membrane fluidity, lipid peroxidation, eicosanoid production or regulation of gene expression.

Phagocytosis is the process whereby cells internalize, kill and digest invading microorganisms. *In vivo* and *in vitro* studies have shown that monocytes/macrophages and granulocytes are phagocytic and will ingest a wide range of inert and antigenic particles. Phagocytes are able to kill pathogens using a variety of killing mechanisms that can broadly be categorized as oxygen dependent or oxygen independent. Fish tissues and cell membranes including phagocytic cells (macrophages, neutrophils) contain relatively high concentrations of n-3 PUFA and their composition can be altered by changes in dietary lipid composition (Bell *et al.*, 1996a). Briefly, the respiratory burst is a complex metabolic pathway that produces highly reactive antimicrobial oxidants such as the superoxide anion, hydrogen peroxide, and hydroxyl radicals by the partial reduction of oxygen. The detection of the superoxide anion is dependant upon its reduction of nitroblue tetrazolium to an insoluble formazan. The amount of formazan produced (as measured by the intensity of colour of the solubilised product) is directly proportional to the "state of readiness" or

potential capacity to deal with infections. Membrane-mediated processes are so central to normal cell functions that membrane modification is an essential feature of adaptation to an altered cellular environment. Phagocytosis and the production of oxygen free radicals via respiratory burst (Sharp & Secombes, 1993) are important events in bactericidal pathways. Specific macrophage functions may also be altered by lipids, mainly due to changes in membrane fluidity. Calder *et al.*, (1990) reported that unsaturated fatty acid incorporation is associated with an increase in the phagocytosis of zymosan particles. They also noted that the correlation between phagocytosis and membrane phospholipid was very high. Perhaps this increase may be due to an increase in the production of hydrogen peroxide, which enhances the release of superoxide anion. Membrane fluidity plays an important role in phagocytosis. If fluidity is altered by the fatty acid composition, then potentially several aspects of phagocyte function may be affected including phagocytosis and eicosanoid production. Fatty acids may influence the process of attachment or the ingestion of the pathogen by altering the formation of a phagosome or by affecting the actual killing of the pathogen involving both oxygen-independent (i.e. lysozyme secretion) and dependent (respiratory burst) processes. As a result of changes in the fluid state of the membrane, changes in membrane-associated proteins such as receptors and ion channels and those involved in enzymatic functions may also be altered. Thus binding of cytokines and other such like molecules to their respective cell surface receptors can also be altered (Stubbs & Smith 1984). 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 and is in turn affected by dietary lipids.

As a consequence of changes in the phospholipid fatty acid composition, due to dietary lipid manipulation by VO, the fluidity of the cell membrane may change. Fatty acids from dietary lipid may be incorporated into phospholipids within the plasma membrane and can therefore be altered by the availability of dietary lipids. Changes in the fatty acid composition of the cell membrane may in turn affect the activity of proteins associated with the membrane, which act as receptors, form ion channels or be related to functions such as phagocytosis and respiratory burst. Membrane PUFA composition is known to affect the activity of a number of ion pumps and membrane-bound enzymes including Na+, K+-ATPase (Spector & Yorek, 1985; Gerbi *et al.*, 1994). Binding of cytokines to their respective receptors on the cell membrane surface may also be altered (Stubbs and Smith, 1984). Processes such as intercellular communication, nutrient transport across membranes, signal transduction and expression of membrane receptors have been shown to be altered due to changes in the fatty acids within the cell membrane (Kelley, 1996; Miles & Calder, 1998; Calder, 1998a, b).

The release of ARA from the membrane phospholipids by the action of phospholipase $A_2$ ($PLA_2$) has been demonstrated to be essential in Fc-receptor mediated phagocytosis (Lennartz & Brown, 1991). ARA seems to be necessary for fusion of electron lucent vesicles with the plasma membrane under laying the particle to be phagocytosed (Karimi & Lennartz, 1995). The phagocytic process has been extensively studied in mammals, and found to involve both opsonin-dependent and independent receptors on the cells. In teleosts, neither opsonin-dependent nor independent phagocytic receptors have been thoroughly characterised (Frϕystad *et al.*, 1998). In higher vertebrates, macrophages are known to have Fc receptors and receptors for complement and other factors which are

involved in the phagocytic process. In a study carried out by Frϕystad *et al.*, (1998) who investigated the involvement of these receptors in rainbow trout head kidney macrophages, they reported that neither the Fc nor the complement-receptor were important for phagocytosis but that scavenger receptors are involved in the phagocytic process. Scavenger receptors are cell surface proteins expressed by monocytes and macrophages, among other cell types, and exhibit distinctive ligand-binding properties, recognising a wide range of ligands that include microbial surface constituents and microbes (Pearson, 1996).

A major finding of the studies presented here was the reduction in phagocytic capacity and head kidney macrophage respiratory burst activity of some fish species fed VO diets (Chapters 3-7). A significant reduction in respiratory burst activity was most pronounced in Atlantic salmon and sea bass fed high levels of RO or RO-containing diets (Chapters 3, 4, 5, 6 - Trial 3). In addition, RO and OO inclusion in the diets of salmon and sea bass significantly reduced the head kidney macrophage phagocytic capacity to engulf yeast particles as found in Chapters 4 (post-seawater transfer) and 5. These differences in macrophage function are probably due to the altered membrane fatty acid composition, physical properties and the activity of membrane-associated receptors.

Since macrophage function was affected in the majority of trials conducted here, more detailed immunological studies are necessary to determine the mechanisms responsible for these findings. Similar to the findings reported in previous chapters, Montero *et al.*, (2003) found reduced macrophage activity in sea bream fed diets with high levels of oleic acid. In addition, Sheldon and Blazer (1991) found that channel catfish macrophage killing activity

was positively correlated to the dietary content of n-3 PUFA at two temperatures. The authors found that phagocytosis of live *E. ictaluri* by catfish head kidney macrophages was not significantly affected by feeding soybean oil compared to fish fed menhaden oil or beef tallow. However, feeding fish soybean oil did significantly reduce the ability of macrophages to kill engulfed bacteria compared to macrophages from the group fed menhaden oil. Macrophages from the latter group had a significantly higher killing index than macrophages from fish fed soybean oil or beef tallow diets. A study by Waagbϕ *et al.*, (1993a) showed that Atlantic salmon fed diets rich in n-3 PUFA significantly reduced the bacterial killing ability of their macrophages at 12°C but not at 18°C indicating that temperature also influences the activity of macrophages. In contrast to the findings presented in this study, Thompson *et al.*, (1996) found no differences in phagocytosis and bactericidal activities of head kidney macrophages from Atlantic salmon fed diets enriched with either n-3 or n-6 fatty acids.

Bly *et al.*, (1986a, b, c, 1987a, b, 1988, 1990) and Bly and Clem (1988) have carried out numerous studies on the influence of temperature on processes in teleost immunity. It is well accepted that low environmental temperatures are immunosuppressive for fish (Avtalion, 1981). Analyses of T and B cell plasma membrane fluidities from catfish acclimated to various temperatures, for various periods of time, indicate that low temperature acclimation is associated with increased membrane fluidity (Bly *et al.*, 1988). It is also well established that fish can, in response to different environmental temperatures, change the fatty acid compositions of certain plasma membranes and consequently maintain an optimum fluidity for such membranes at low temperatures e.g. homeoviscous adaption (Schunke & Wodtke, 1983).

When fatty acids are incorporated or released from the plasma membrane they may undergo metabolic conversion through the eicosanoid pathways. Fatty acids can influence the activity of the enzymes involved in the synthesis of eicosanoids, and in turn alter immune function. The main eicosanoid measured throughout these studies was prostaglandin $E_2$ ($PGE_2$). It is a primary product of ARA metabolism in many cells. $PGE_2$ has been shown to be a key molecule in immune regulation in mammals and has been extensively reviewed by Goodwin & Ceuppens, (1983). Prostaglandins are produced by numerous cell types and not only affect immune functions but also exert many non-immunological functions including control of water balance, homeostasis, gut protection and reproduction (Goodwin & Ceuppens, 1983, Calder, 1998a,b; 1999, 2001a,b,c). Many beneficial effects of FO supplements in attenuating a number of atherothrombotic, autoimmune and inflammatory conditions are, in part, due to the modulation of ARA-derived eicosanoids by EPA (Weber, 1990). Suppression of ARA-derived eicosanoids by dietary ALA has been observed in mammals (Marshall & Johnston, 1982). ARA is the principal precursor of eicosanoids in mammals (Horrobin, 1983), however, 20:3(n-6) and EPA are also eicosanoid precursors producing 1- and 3-series prostaglandins respectively, although both are poorer substrates than ARA for prostaglandin synthetase (Crawford, 1983). Competition exists between EPA, 20:3(n-6) and ARA for prostaglandin production and the substrates can act as competitive inhibitors of ARA for the enzyme binding sites (Willis, 1981). Therefore, dietary supplementation with 20:3(n-6), EPA or their $C_{18}$ precursors can reduce the production and efficacy of ARA-derived eicosanoids (Willis, 1981; Weber, 1990).

In the present study, plasma $PGE_2$ levels were significantly reduced after feeding salmon with LO-containing diets (Chapter 5) or sea bass fed a blended VO diet (VO1 in Chapter 6). A reduction in prostaglandin levels was related to a reduction in macrophage respiratory burst activity in salmon fed LO diets in Chapter 5. Sea bass fed a blended VO diet of anchovy oil, LO, PO and RO (VO1) also showed significantly reduced respiratory burst activity which coincided with a reduction in plasma $PGE_2$ levels. It has also been suggested that there are species differences in eicosanoid production (Rowley *et al.*, 1995). Since the production of $PGE_2$ was found to be reduced in fish fed VO diets we can assume that the activity or expression of the cyclooxygenase enzymes are inhibited by the dietary treatment. It is possible that feeding VO for a long period of time may reduce the levels of ARA in plasma membranes and compromise immune function. Fish fed VO diets with high levels of 20:3(n-6) in their membranes increases the competition between ARA and 20:3(n-6) for prostaglandin production which could in turn affect immune cell function. Future investigations into VO substitution in aquafeeds should try to tailor the fatty acid composition so that the levels and ratios of ARA and EPA in fish macrophages are optimised for the most favourable production of eicosanoid compounds and highest immune function.

In support of the findings presented in this study, Bell *et al.*, (1993a, b; 1996a, b) also showed a reduction in the production of $LTB_4$ and $PGE_2$ by stimulated head kidney macrophages from salmon fed a diet containing LO compared to those fed with a sunflower oil or FO diet. An inhibition of eicosanoid production at high levels of LA is also in agreement with a previous study by Galli *et al.*, (1981). These authors showed that excess LA suppresses eicosanoid production by inhibiting the cyclooxygenase reaction. The

desaturation and elongation product from LA, DHGLA (20:3(n-6)) competes with ARA for cyclooxygenase, resulting in a suppression of prostaglandin formation from ARA. Eicosanoids can also affect the production of cytokines. Interleukin-1 (IL-1), IL-2, IL-6, TNF-α and IFN-γ have been described to be reduced particularly by n-3 HUFA (from FO) after incorporating the lipids into the diets (Endres *et al.*, 1989; Yaqoob & Calder, 1995). Cytokines such as IL-1 and TNF-α are important mediators of inflammation and fatty acids have been found to be able to reduce the pro-inflammatory response induced by IL-1 and TNF-α (Endres, 1996). However, many studies have reported contradictory effects of dietary fatty acids on pro-inflammatory cytokine production (Endres, 1996, Blok *et al.*, 1996). FO and OO are capable of suppressing IL-1 production after 4 weeks of dietary supplementation in rats, whereas OO enhances IL-1 production after 8 weeks (Tappia & Grimble, 1994). Cytokines were not measured in the present study due to a lack of probes necessary for their measurement, but with the advances in fish immunology progressing rapidly this may be a possible area of future work when investigating the effect of dietary VO on the fish immune system.

Reduced lysozyme activity was found in Chapters 4 (post-seawater transfer) and 5 – Trial 1 in salmon fed diets containing a blend of LO/RO (1:1) or high levels of LO. Changes in macrophage function in these trials may in turn be a factor which causes a reduction in lysozyme activity observed. Although lysozyme differences were found in some of the dietary trials presented here, many other reports from the available literature found no significant differences of lysozyme activity in fish fed VO diets (Bell *et al.*, 1996a; Montero *et al.*, 2003).

Blaxhall and Daisley (1973) found the normal physiological ranges, in terms of haematological values of fish, were wider than those for humans. It is important to note that haematological values for fish are influenced by temperature (Houston, 1980; Dunn *et al.*, 1989; Lie *et al.*, 1989). Leukocyte numbers can also be reduced by cortisol secretion. Leucocytes are much less numerous than erythrocytes in blood and respond to specific stimuli and signals. As previously mentioned, leucocytes are composed of functionally and morphologically distinct populations of cells. In mammals, macrophages spend around thirty hours as monocytes in the blood before entering into the tissues and differentiating into macrophages. Neutrophils have a life span of two days (Wintrobe, 1974) whereas T lymphocytes by comparison have a life span of several years. Differences in fatty acid metabolism by these various populations may reflect differences in their life spans.

Erythrocyte numbers were reduced in Chapters 4 (pre-vaccination) and 5 - Trial 1 and 2 in fish fed with either LO or RO or an equal blend of both oils. Montero *et al.*, (2003) found that seabream fed a FO diet had higher numbers of erythrocytes compared to fish fed a LO or soybean oil diet which may be related to a higher oxygen requirement due to higher peroxisomal β-oxidation (Waagbϕ *et al.*, 1995). Leray *et al.*, (1986) found that the fatty acid composition of erythrocyte membrane phospholipids isolated from trout can be profoundly altered by feeding various oils. This is in contrast with results from mammalian erythrocytes where only minor changes were detected in the membrane fatty acid pattern (Vajreswari *et al.*, 1983; Benga *et al.*, 1984). Trout fed with a coconut oil diet showed increased levels of n-9 fatty acids in the phospholipids and erythrocytes showed shape changes and a 30 % more shrunken appearance than fish fed with a normal commercial diet. Perhaps by feeding high levels of VO in the diets of farmed fish the reduced

haematocrit levels seen in some trials (Chapter 4 (pre-vaccination and post seawater transfer)) Chapter 5 (Trial 1 and 2) may be linked with a shrunken erythrocyte shape causing a lower volume of packed red blood cells.

With regard to total leukocyte counts, some species fed a VO diet showed reduced numbers compared to those fed a FO diet, however not all differences were found to be significant. (Chapter 3 (pre-vaccination), Chapter 5 Trial 2 and 3, Chapter 6 Trial 3). Perhaps signals such as growth factors or cytokines for cell differentiation are different or suppressed or not recognised to the same extent in VO fed fish compared to FO fed fish and therefore do not produce leukocytes or erythrocytes to the same extent. In addition, cell surface receptors on stem cells may be different or not expressed to the same extent due to the differences in cell membrane lipid composition, caused by VO inclusion, or perhaps even protein synthesis necessary for cell production is affected in some way as a result of feeding different fatty acids. Further investigation into the differences found in haematology parameters is therefore required.

The lipid composition of monocytes, macrophages, lymphocytes and polymorphonuclear cells (PMNs) reflect the fatty acid composition of dietary lipids (Meade & Mertin, 1978; Johnston & Marshall, 1984). Manipulation of the fatty acid composition of lymphocytes *in vitro* can alter immune functions (Traill & Wick, 1984) and enhancement and suppression of T- and B-cell responses may occur depending upon the concentrations and type of fat presented to these cells in culture.

An alteration in the fatty acid composition of immunoactive cells could perhaps change the activity of membrane associated receptors and thereby change cellular activity. Johnston and Marshall (1984) showed that the fatty acid composition of lymphocytes, thymocytes, splenocytes and mast cells are altered by dietary fatty acids. In several studies carried out by Johnston and Marshall (1984), rats were fed various levels of LA and ALA in the form of either corn oil, soybean oil, soybean/LO mix or LO containing diets. All the isolated cell populations responded to the diets in a similar way, namely, as the ratio of ALA to LA ratio increased the levels of ARA decreased while EPA increased. However, it has been noted that different cell populations respond to different degrees (Marshall *et al.*, 1983).

Studies by Waagbϕ *et al.*, (1995), Farndale *et al.*, (1999) and Montero *et al.*, (2003) reported that the dietary oils administered determines the fatty acid profile of macrophages and immune cells in fish species. Montero *et al.*, (2003) reported a selective incorporation of certain fatty acids in head kidney macrophages of seabream. DHA was found to be preferentially incorporated and retained in this cell type. Similarly Meade & Mertin (1978) and Johnston (1992) found that the lipid composition of leukocytes was determined by the fatty acid composition of the dietary oil. Bell & Raynard (1990) fed diets containing FO or sunflower oil and found that leukocytes from fish given the sunflower oil diet showed increased amounts of LA, GLA, 20:2(n-6), ARA compared with those given the FO diet. It was also noted that the sunflower oil fed fish had decreased levels of n-3 HUFA as well as decreased monoenes. Bell & Raynard (1990) reported that the occurrence of increased levels of $C_{20}$ (n-6) fatty acids indicates considerable Δ6 and Δ5 desaturase activity in Atlantic salmon.

In all trials where the fatty acid composition of fish PBLs were investigated the resulting leukocyte fatty acid profile resembled that of the dietary treatment administered. This has also been observed in other species (Bell & Raynard, 1990; Bell *et al.*, 1992; Waagbϕ *et al.*, 1995; Farndale *et al.*, 1999; Montero *et al.*, 2003). Generally overall, fish fed with a VO-containing diet had increased levels of oleic acid, LA, ALA and total n-6 PUFA and decreased levels of EPA, DHA, total n-3 PUFA and a lower n-3/n-6 ratio than fish fed with a FO diet. Fish fed a FO diet showed the highest n-3 HUFA proportion in all trials performed. Interestingly in Chapter 6 Trial 1, salmon fed with a carefully blended VO diet showed a leukocyte fatty acid composition similar to salmon fed a FO diet. The only significant difference in fatty acid content was in the levels of saturated fatty acids found.

With regard to disease resistance after bacterial challenge, conflicting results were found. In Chapter 4 salmon were challenged with *A. salmonicida* and no significant differences in cumulative mortalities were found between fish fed a FO diet (low and high lipid levels) and fish fed a VO diet (LO/RO (1:1) low and high lipid levels). However, in Chapter 6, salmon fed either a FO diet or one containing 75 % blended VO (RO/LO/PO) did show significant differences in mortalities. Increased numbers of mortalities were found in VO fed fish. Examination of the literature shows that changing the concentration of dietary n-3 PUFA in fish feeds can have both detrimental and beneficial effects on disease resistance (Li *et al.*, 1994; Erdal *et al.*, 1991; Fracalossi & Lovell, 1994, Thompson *et al.*, 1996; Bransden *et al.*, 2003; Lϕdemel *et al.*, 2001). In the present study, resistance to *V. anguillarum* was significantly impaired in salmon fed a blended VO diet (FO/LO/PO/RO), although it remains unclear as to why no differences in mortalities were found after salmon fed a mix of LO/RO were challenged with *A. salmonicida*. Thoughts on the reasons why

reduced resistance in fish fed VO diets challenged with *V. anguillarum* were observed may be a result of the role of the microbiota in the gut. Perhaps the growth of *V. anguillarum* is inhibited to a greater extent by antimicrobial compounds in FO fed fish compared with VO fed fish.

Diet composition may modify the microorganisms in the gastrointestinal tract and the integrity of the intestinal epithelium. Oxidised lipids and anti-nutritional factors in plant products can affect the gut physiology and microfloral population. Only one study to date (Ringϕ *et al.*, 2002) has focused on the effect of VO on the gut microbiota before and after challenge with *A. salmonicida*. The authors reported that the microbiota of hindgut (distal) gut of Arctic char fed FO was completely different from that of char fed either LO or soybean oil diets prior to bacterial challenge with *A. salmonicida*. After challenge, the bacterial population levels were reduced and differences in the predominant species of bacteria were identified between fish fed the different dietary oils. The reduction in bacterial levels after bacterial challenge may be explained by the infection with *A. salmonicida* leading to increased mucus production. Carnobacteria was recovered only from fish fed the VO diet (LO and soybean oil) and not from fish fed the FO diet. Clear differences were also observed in the position of bacterial colonisation. After feeding the soybean oil diet, some enterocytes were heavily colonised by bacteria, whereas in fish fed the LO diet, most bacteria were associated with the apical brush border of the cell. Apart from the study by Ringϕ *et al.*, (2002) there is no other available information about the effect of dietary lipid on intestinal microbiota and their influence on fish disease susceptibility. Even so, the study by Ringϕ *et al.*, (2002) clearly shows differences in the gut microbiota of fish fed different oils before and after challenge. In future dietary trials

involving VO diets and disease resistance this may be a possible area for further investigations.

Histologically the major differences between groups of fish fed a FO diet and those fed with a VO-containing diet was in the appearance of the liver. With increasing replacement levels there was a tendency for increased lipid deposition leading to "foamy hepatocytes", variability of vacuole size and in some cases general loss of the regular architecture and liver structure. From all the trials conducted few histological changes were observed in heart sections sampled from fish fed a VO diet. This is in contrast to a number of earlier studies where cardiomyopathy in relation to nutrition has been reported (Fjϕlstad & Heyeraas, 1985; Ferguson *et al.*, 1986). A number of mammalian studies have also demonstrated that the PUFA composition of heart membrane lipids can be influenced by modifying the dietary lipid intake (Swanson & Kinsella, 1986; Leonardi *et al.*, 1987). Bell *et al.*, (1991a, 1993a) reported that Atlantic salmon fed a sunflower oil diet developed cardiac lesions ranging from mild to severe. However, a recent study by Grisdale-Helland *et al.*, (2002) reported no heart histopathologies in salmon fed a 100 % soybean oil diet.

In Chapters 3 – 5 the trials involved feeding different combinations of FO, LO and RO to either salmon or sea bass which resulted in some distinct histopathological changes in the liver and intestinal sections examined. A major finding in the liver was the hugely variable lipid deposition within the hepatocytes. As the levels of VO inclusion in the diet increased, the lipid deposition became more pronounced with the presence of "foamy" hepatocytes and signs of "un-metabolised" fat. Most fish fed a VO diet showed a high degree of variability, with vacuoles ranging from small, single ones, to multiple, large vacuoles

containing varying amounts of lipid. From the results found in Chapter 4, hepatocytes of salmon fed the lower dietary lipid levels showed a greater number of small lipid droplets in comparison to fish fed the higher lipid levels showing a higher lipid droplet diameter. These findings are supported by the results of Caballero *et al.*, (1999, 2002) who found similar results when feeding seabream and trout VO containing diets. However, Mosconi-Bac (1987) suggested that the presence of numerous and voluminous lipid droplets in hepatocytes may be a response to excess lipid and represent an energy storage rather than a pathological condition. It is also likely that these effects observed are due to impairment in lipoprotein synthesis and the transport of lipids.

In Chapter 4, fish fed a LO/RO (1:1) diet at a high dietary lipid level (25 %) showed low mucus activity and low numbers of vacuoles in the proximal intestine which may be a consequence of the diet with the gut becoming less responsive and having a low absorption capacity. The proximal intestine including the pyloric caecae is the major site for lipid absorption in teleost fish (Ostos Garrido *et al.*, 1993; Olsen *et al.*, 1999). The enterocytes of the intestine absorb the mono and diglycerides, which result from the hydrolysis of TAG and phospholipids. Following absorption, the end products are resynthesized into lipoprotein particles which are transported in the bloodstream. Therefore, the structure of the intestinal cells rapidly and reversibly respond to dietary changes. Another noticeable change was in the distal intestine of VO fed fish where in some trials increased cellular infiltration, sloughing of the mucosal folds and general loss of intestinal mucosal integrity was evident. The distal intestine of VO fed fish appeared highly vacuolated which will make the mucosal membrane more prone to degradation. In Chapters 3, 4 and 5, fish fed a RO containing diet showed noticeable histological changes in the distal section of the

intestine. There appeared to be more breakdown of the mucosal folds in these fish compared to those fed a FO diet. In some cases (Chapter 4 post vaccination) this was thought to be a response to the vaccination rather than a true pathological finding. An interesting observation was found in Chapter 6. When the dietary fatty acid composition of the blended VO was changed to reflect the fatty acid composition of a FO diet, very few histological changes were found between the two treatments. The physiological significance of the histological changes found in the present study are difficult to interpret and would require further investigation. For future studies involving FO replacement it may even be beneficial to attempt to identify the component/s in the oils responsible for the histological observations seen in the liver and to a lesser extent in the intestine.

Caballero *et al.*, (2003) found large accumulations of lipids in the basal zone of enterocytes from fish fed a 60 % and 80 % soybean oil diet. The authors believed that this was perhaps due to impairment in the transit capacity through the lamina propria, due to the high synthesis of VLDL particles. The authors reported that the presence of VLDL in fish fed the soybean oil diets may be related to a higher reacylation of LA compared to other $C_{18}$ fatty acids. They therefore concluded, that LA seems to promote the synthesis of surface materials for lipoprotein formation, which is known to result in smaller lipoprotein-like VLDL (Sire *et al.*, 1981). In another study by the same author, Caballero *et al.*, (2002) found similar lipid vacuoles in rainbow trout fed soybean oil, RO and PO diets compared to those fed a FO diet. In mammals, dietary fatty acid type has also been found to interfere with intestinal lipoprotein assembly, affecting lipid transport and esterification into TAG and phospholipids (Van Greevenbroek *et al.*, 1995). There is limited information on the importance of the intestine and how their physiological functions can be affected by

different sources of lipids. Olsen *et al.*, (2000) suggested that certain fatty acids, such as 16:0, are required to maintain cellular intestinal PC synthesis and thereby, a high lipoprotein synthesis, avoiding the accumulation of absorbed lipids in enterocytes. Some lipids may be stored in the epithelial cells (Bergot, 1981), but most of them are initially transported to the liver (Sargent *et al.*, 1989). Most VO contain lower levels of 16:0 than FO. However, in Chapter 6 the diets containing blends of VO including PO had 16:0 values similar to the FO diet. Few pathological changes in the intestines of fish fed the diets in Chapter 6 were observed suggesting that the intestinal PC and lipoprotein synthesis was unaffected by this dietary treatment.

There is also a food safety argument for using alternative oil sources because FO and fish meals have relatively high levels of dioxins and PCB contaminants, compared to terrestrial raw materials, and the EU has limited the maximum dioxin concentration in fish meal and FO as well as in farmed fish and fish feed. Highly chlorinated compounds are lipophilic and accumulate in the food-chain by depositing in the lipids of fish. When fish for feed production are caught, the FO extracted may be contaminated with chlorinated hydrocarbons (Jacobs *et al.*, 1997, 1998, Ministry of Agriculture Fisheries and Food, 1997). Although the industry still favours the use of FO in aquaculture feeds it is likely that these oils are contributing significantly to the contamination of farmed fish by PCDDs PCDFs and PCBs. Therefore the use of alternative oils will reduce the levels of these organochlorine toxins in fish benefiting the producer, consumer and the environment. Studies by Bell *et al.*, (Aquaculture, submitted manuscript) have shown the benefits of reducing FO intake and subsequent reductions in dioxins/PCBs. Salmon fed either a low or high FO diet or VO diet (LO/RO, 1:1) contained dioxin levels below the 2.25 ng $kg^{-1}$ Toxic

Equivalents (TEQ) limit in fish feed and 4.0 ng $kg^{-1}$ TEQ in fish for human consumption set by EU legislation. Fish fed the high FO diet had higher concentrations of dioxins compared to the other diets, with the high VO diet giving the lowest dioxin values. This research also showed that the benefit of using VO diets for most of the growth cycle, followed by a period of wash out with a high FO diet, to restore fatty acid compositions, is reflected in significantly lower flesh dioxin and dioxin-like PCB levels in fish previously fed VO.

The health effects of VO substitution in aquafeeds is an area which requires more research to determine the immunosuppressive effects seen in the chapters presented here. It would be interesting to investigate whether dietary supplementation of EPA, DHA or ARA would prevent a reduction in some immune parameters found. In addition, there are new oils available, both from genetically modified (GM) and non-GM sources that, while not produced in large quantities at the present time, may be better suited to fish nutrition than those currently available on the market, at a suitable price. In the past decade, genetic engineering and biotechnology have been used to enhance both crop yields and product quality in order to offer nutritional benefits to a growing population. In the case of fats and oils, genetic researchers have studied the genes responsible for the synthesis of omega-3 fatty acids in plants, and through genetic modification; have been able to alter the fatty acid composition of the world's major oil crops. Genetic researchers have examined different ways of adjusting the plant's lipid biosynthetic pathway to create plants that produce healthier fatty acid compositions. Genetic engineering has also been used to modify the plant lipid biosynthetic pathway in other ways, tailoring the fatty acid profile produced by oilseed crops, making plant oils as healthy as possible. Plants that are modified to produce

high levels of EPA and DHA would benefit the aquaculture industry greatly. However, even although GM oils may offer many potential benefits, as with every good thing, there are potential problems with them as well. In addition to the concerns of all GM crops, for example, gene flow and adverse environmental effects, when the natural biochemical pathways of a plant are modified, as they are in creating these GM oils, there is the danger of unintentionally introducing allergens and other antinutritional factors into the food chain (Monsanto, 2002).

As this PhD was sponsored by EWOS it may be interesting in the future to investigate the use of alternative oil diets in conjunction with EWOS "Boosterfeed" diet which is a dietary supplement that provides fish with additional nucleotides and nucleotide precursors. The diet has been proven to enhance various immune responses, disease resistance, and improve several other cell dependent biological processes such as increasing the intestinal villus height which in turn increases nutrient absorption and can increase growth. It is possible that feeding Boosterfeed to fish maintained on VO diets may help to alleviate some of the pathological conditions found in the intestine and improve overall immune function.

From a human health perspective, replacing FO with VO may have profound consequences. The high concentrations of DHA and EPA in FO have proven to be very effective in patients with coronary heart disease, rheumatoid arthritis and psoriasis (Sardesai, 1992; De Deckere *et al.*, 1998; Horrocks & Yeo, 1999; Simopoulos, 1999; Connor, 2000). The levels of EPA and DHA within the muscle of fish are also changed in direct relation to the dietary levels. Since these fatty acids are considered important for

human nutrition, their concentrations within the final aquaculture product should also be maintained at satisfactory levels through dietary manipulation. In the final stages of production it is therefore recommended that fish previously fed with a VO diet be switched to a FO-only diet for an appropriate period of wash-out or dilution to restore the flesh fatty acid composition and, thereby, increase the levels of n-3 PUFA and decrease those of the n-6 series. In this way consumers will still be provided with a healthy product with all the added beneficial properties attributed to high n-3 PUFA. Results from Bell *et al.*, (2003a, b) have shown that after a 20- week wash-out phase with FO, the DHA and EPA levels are restored to values similar to those in fish fed FO throughout the production cycle. However, LA values did remain elevated, supporting the fact that VO used for FO replacement should ideally contain minimal levels of LA.

The reported effects of vegetable lipids on fish immune functions provides a new insight into how dietary fatty acids might play a critical role in fish health and disease. Accordingly, the study of dietary lipids and their influence on the immune response will allow a better understanding of the possible effects of these substances on the host natural resistance to pathogenic agents. Potential does exist to replace FO with VO in feeds for farmed fish however, it is important to establish that the dietary lipid is not only supplied in the correct quantities and balance for optimal growth and feed conversion but can maintain proper immune function and prevent infection from pathogens. Results from Chapter 6 suggest that a possible way to maintain proper immune function while replacing dietary FO is by using an appropriately blended source of various VO rather than just one individual oil. The first commercial production of VO-containing salmon is already a reality in Norway and will soon be produced in Scotland. Consequently, there will undoubtedly be

further questions to answer if these new products are to be fully accepted by retailers and consumers. Most of the major aquafeed companies now have a commercial feed available which contains vegetable oil products for example EWOS "OilMIX" and Skretting "Lite" which contains a combination of selected marine and alternative oils.

In conclusion, feeding VO in replacement of FO does appear to affect some immune parameters, histological appearance and in some cases, disease resistance to bacterial challenge in the fish species studied. Additional studies are clearly required to establish the role of VO dietary lipids on immune response and disease resistance in fish and their possible immunosuppressive effects.

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## Appendix 1- Buffers and Reagent Formulations

Alcian blue

Dissolve 1 % (w/v) alcian blue dye in sterile distilled water. Filter prior to use.

Anti-Atlantic salmon IgM monoclonal antibody

Each vial contains 200 µg of lyophilised protein prepared from bovine free culture medium which is sufficient for three 96-well ELISA plates. The powder was reconstituted by adding 1 ml of PBS to the vial then transferring the contents of the vial into 32 ml of antibody buffer so that the total volume equals 33 ml. For prolonged storage the monoclonal antibody should be stored at –20 °C.

Antibody buffer

Add 1 g of BSA to 100 ml of PBS (i.e. 1 % BSA solution)

Benzocaine/ Ethyl -3-aminobenzoate methanesulfonate salt (MS222)

| | | |
|---|---|---|
| Benzocaine/MS222 | 20 g | Dissolved in 500 ml of methanol |
| | | To make a 40 mg $ml^{-1}$ solution |

Chloroform:methanol (2:1) + Butylated hydroxytoluene (BHT)

| | |
|---|---|
| Butylated hydroxytoluene | 30 mg |
| Methanol | 100 ml |
| Chloroform | 200 ml |

Chromogen (ELISA Substrate)

3'3'5'5'-Tetramethylbenidine dihyrdochloride (TMB -0.0394 g = 42 mM) was added to 1:2 acetic acid: distilled water. Usually 3 ml is prepared but more can be made if stored in a foil wrapped universal in the fridge. 150 µl of this solution was added to 15 ml of substrate buffer (therefore alter amounts according to how much substrate is needed.

Chromatography Running Solution

| | |
|---|---|
| Isohexane | 90 ml |
| Diethyl ether | 10 ml |
| Acetic acid | 1 ml |

Coating buffer

Carbonate-bicarbonate solution (pH 9.6)

1 carbonate bicarbonate buffer tablet in 100 ml distilled water and 1ml poly L lysine

Complement inactivation of foetal calf serum (FCS) and foetal bovine serum (FBS)

FCS completely thawed, was placed in a water bath at 56 °C for 30 min, mixing periodically. It was aliquoted into 10 ml and 20 ml volumes and stored at –20 °C.

Concanavalin A (ConA)

Sterile stock solution 1 mg $ml^{-1}$

Con A was dissolved in an appropriate volume of Leibowitz L-15 medium containing 10 % FBS/FCS (10 % L-15) (e.g. 5 ml medium containing 5 mg Con A). The solution was filter

sterilised through a syringe tip filter. Working solution previously determined to be optimal (25 μg $ml^{-1}$) was prepared by diluting the stock in PBS

Conjugate buffer

Add 1 g of bovine serum alubumin (BSA) to 100 ml of low salt wash buffer (LSWB)

10% Neutral Buffered Formalin (500 ml)

| | |
|---|---|
| Formalin | 50 ml |
| 1 X PBS | 450 ml |

Heparin

10 ml of PBS to one vial of heparin containing 25,000 U then sterilised through a membrane filter and stored at 4 °C in 5 ml aliquots.

1 % Iodine

| | |
|---|---|
| Iodine | 1 g |
| Chloroform | 99 ml |

Leibowitz L-15 medium containing 5 % FCS

| | For 500 ml |
|---|---|
| Leibowitz L-15 base medium | 456.5 ml |
| Heat-inactivated FBS/FCS | 25.0 ml |
| Penicillin/Streptomycin solution | 5.0 ml |
| Gentamycin | 5.0 ml |

| | |
|---|---|
| 0.01M 2-ME | 2.5 ml |
| Hepes buffer | 6.0 ml |

Leibowitz L-15 medium containing 0.1 % FCS

| | For 500 ml |
|---|---|
| Leibowitz L-15 base medium | 481.0 ml |
| Heat-inactivated FBS/FCS | 0.5 ml |
| Penicillin/Streptomycin solution | 5.0 ml |
| Gentamycin | 5.0 ml |
| 0.01M 2-ME | 2.5 ml |
| Hepes buffer | 6.0 ml |

Lipopolysaccharide of E. coli 0127:B8 (LPS)

Sterile stock solution 1 mg $ml^{-1}$

LPS was dissolved in an appropriate volume of Leibowitz L-15 medium containing 10 % FBS/FCS (10 % L-15) (e.g. 5 ml medium containing 5 mg Con A). The solution was filter sterilised through a syringe tip filter. Working solution previously determined to be optimal (100 $\mu g\ ml^{-1}$) was prepared by diluting the stock in PBS.

Macrophage Lysis Buffer

| | | |
|---|---|---|
| Citric acid powder | 0.0021 g | |
| Tween 20 | 1 ml | Dissolved in 100 ml of distilled water |
| Crystal Violet | 0.05 g | Store at 4 °C |

0.01Molar 2-Mercaptoethanol (ME)

| | |
|---|---|
| 2-ME | 78 µl |
| Distilled water | 100 ml |

Filter sterilise using a membrane filter and aliquot into 5ml amounts.

34 % Percoll

| | |
|---|---|
| Neat Percoll | |
| (100 % non-isotonic stock) | 34 ml |
| 10 X PBS | 10 ml |
| Deionised water | 56 ml |

51 % Percoll

| | |
|---|---|
| Neat Percoll | |
| (100 % non-isotonic stock) | 51 ml |
| 10 X PBS | 10 ml |
| Deionised water | 39 ml |

Phorbol Myristate Acetate (PMA)

PMA ampoule was solubilised with DMSO so that the final concentration of the stock was 1 mg $ml^{-1}$.

Phosphate Buffered Saline (PBS)

| | | |
|---|---|---|
| $NaH_2PO_4.2H_2O$ | 0.876 g | Dissolved in 1L distilled water |

| | | |
|---|---|---|
| $Na_2HPO_4.2H_2O$ | 2.56 g | adjust to pH 7.2 |
| NaCl | 8.77 g | |

Alternatively, dissolved 5 PBS buffer tablets in 1 L distilled water

<u>10 x Phosphate-buffered saline (10 x PBS)</u>

| | |
|---|---|
| NaCl | 80.0 g |
| KCl | 2.0 g |
| $Na_2HPO_4$ | 11.5 g |
| $KH_2PO_4$ | 2.0g |

Dissolved in 1L of distilled water

<u>2 % Potassium hydrogen carbonate</u>

| | |
|---|---|
| Potassium hydrogen carbonate | 2 g |
| Distilled water | 100 ml |

<u>0.88 % Potassium Chloride</u>

| | |
|---|---|
| Potassium chloride | 0.88 g |
| Distilled water | 100 ml |

<u>Sodium Phosphate Buffers</u>

| | | |
|---|---|---|
| $NaH_2PO_4.2H_2O$ | 31.20 g (0.2 M) | Dissolved in 1 L distilled water (Stock A) |
| $Na_2HPO_4.2H_2O$ | 35.59g (0.2 M) | Dissolved in 1 L distilled water (Stock B) |

92 ml of Stock A was added to 8 ml of Stock B to achieve a solution of 0.2 M, pH 5.8. This solution was diluted to give a 0.1 M and further diluted to make a 0.04 M sodium phosphate buffer

Substrate Buffer (Sodium acetate/citrate acid buffer)

| | |
|---|---|
| Citric acid | 21.0 g |
| (pH adjusted to 5.4 with 1 M NaOH) | |
| Sodium acetate | 82.2 g |

Add 5 µl of $H_2O_2$ for every 15 ml of substrate buffer used

Stop reagent

2 M $H_2SO_4$ in distilled water

1% concentrated sulphuric acid in methanol

| | |
|---|---|
| Concentrated sulphuric acid | 1 ml |
| Methanol | 99 ml |

Trypan Blue

| | | |
|---|---|---|
| Trypan blue | 0.4 g | Dissolved in 100 ml of PBS |

Allow solution to dissolve overnight and filter through filter paper to remove deposits

Tryptone soya agar (TSA)

| | | |
|---|---|---|
| TSA | 40 g | Dissolved in 1 L distilled water |

Boil to dissolve and sterilise by autoclaving at 121 °C for 15 min

Tryptone soya broth (TSB)

| | | |
|---|---|---|
| TSB | 30 g | Dissolved in 1 L distilled water |

Sterilise by autoclaving at 121 °C for 15 min

Wash Buffer (x1 0) (low salt) (LSWB)

| | | |
|---|---|---|
| Trisma base | 24.2 g | Dissolved in 1 L distilled water |
| NaCl | 222.2 g | pH adjusted to 7.2 with conc. HCl |
| Tween 20 | 5 ml | Diluted 1/10 prior to use |

Wash Buffer (x 10) (high salt) (HSWB)

| | | |
|---|---|---|
| Trisma base | 24.2 g | Dissolved in 1 L distilled water |
| NaCl | 292.2 g | pH adjusted to 7.2 with conc. HCl |
| Tween 20 | 10 ml | Diluted 1/10 prior to use |

## Appendix 2: List of Chemical and Equipment Suppliers

| Equipment | Supplier |
|---|---|
| Autoclave | Astell Swiftlock 6000 autoclave, Kent, UK |
| Automatic tissue processor | Shandon, Citadel, CHADIE-VMG, Germany |
| Balances | Ohaus GA200D Balance, Oxford, UK; Tecastor 61110 balance |
| Capillary column (30 m x 0.32 id silica wall) CP Wax 52CB | Chrompak, London, UK |
| Centrifuges | Joucan benchtop centrifuge C412, Virginia USA; Heraeus 1.0R refridgerated laboratory bench centrifuge, Germany |
| Counting chamber | Improved Neubauer counting chamber, Hawksley, UK |
| Cristaseal | Hawksley & Son Ltd, West Sussex, UK |
| Chromcard for Windows, version 1.19 PC package | Themoquest Italia, S.oA, Itay |
| ELISA plate reader | Dynatech MRX 1.2 ELISA reader, UK |
| Gas Chromatography (GC) | Carlo Erba GLC8000 Vega series 2, Itlay |
| GC column CP Wax 52CB | Chrompak, London, UK |
| Haematocrit centrifuge | Hawksley and Son Ltd., Lancing, UK |
| Histoembedder | Leica, UK |
| Hot Block | Stuart Scientific, Redhill, UK |
| Incubator | RS Biotech Galaxy CO2 Cooled incubator, Wellingborough, UK |
| Laminar flow cabinet | Gelaire BSB Class II Laminar Air-Flow Cabinet, Kirkham, UK |
| Leica 2035 BIOCUT microtome | Leica, UK |
| Microscopes | Leitz Wetzlar Ortholux, Merck, Germany; |

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| | Olympus CH2, Merck, Germany. |
| Nitrogen evaporator (N-Evap 112) | Organomation Associates Inc., Berlin, MA, USA |
| Pan jet inoculator | Wright Health Group Ltd., Dundee, UK |
| PAP pen | AGAR Scientific, Essex UK |
| Sep-Pak mini column | Waters Corporation, Milford, Massachusetts, USA |
| Spectrophotometer | Cecil CE 2041 2000 Series spectrophotometer |
| Sterile sieves | Homemade – 100 μm 'Spectramesh' nylon mesh |
| TLC plates (20 x 20cm Silica gel 60) | Merck, Darmstadt, Germany |
| 96-well ELISA plates | NUNC, Merck, UK |
| 96-well tissue culture plates (lysozyme assay) | Greiner Bio-One, Cellstar®, Austria |
| Vacutainers, needles and connectors | Becton Dickinson, Oxford, UK |
| Vortex mixer | Yellow-Line KKO-WORKS, Wilmington USA |
| EIA $PGE_2$ Kit | Cayman Chemicals, Ann Arbor MI |

| Chemical | Supplier |
|---|---|
| Acetic acid | Fisher Scientific, Loughborough, UK |
| Alician blue | SIGMA, Poole, UK |
| Alphaject 1200 furunculosis vaccine | Alpharma, Hampshire, UK |
| Anti-Atlantic salmon IgM monoclonal antibody | Aquatic Diagnostics Ltd., Institute of Aquaculture, Stirling, UK |
| Benzocaine | SIGMA, Poole, UK |
| BHT | Fisher Scientific, Loughborough, UK |
| Bovine serum albumin | SIGMA, Poole, UK |
| Carbonate-bicarbonate tablets | SIGMA, Poole, UK |
| Chloroform | Fisher Scientific, Loughborough, UK |
| Chromogen/substrate | SIGMA, Poole, UK |
| Citric acid | SIGMA, Poole, UK |
| Con A | SIGMA, Poole, UK |
| Crystal violet | SIGMA, Poole, UK |
| Diethyl ether | Fisher Scientific, Loughborough, UK |
| DMSO | SIGMA, Poole, UK |
| Ethanol | Fisher Scientific, Loughborough, UK |
| Ethyl-p-aminobenzoate | SIGMA, Poole, UK |
| Foetal bovine/calf serum | SIGMA, Poole, UK |
| Formalin | SIGMA, Poole, UK |
| Gluteraldehyde | SIGMA, Poole, UK |
| $H_2SO_4$ | SIGMA, Poole, UK |
| Heparin | SIGMA, Poole, UK |
| Hepes | SIGMA, Poole, UK |
| Histopaque® | SIGMA, Poole, UK |
| Horse radish peroxidase | SIGMA, Poole, UK |
| Hydrogen peroxide | SIGMA, Poole, UK |
| Iodine | SIGMA, Poole, UK |

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| Isohexane | Fisher Scientific, Loughborough, UK |
| $KH_2PO_4$ | SIGMA, Poole, UK |
| L-15 Leibowitz medium | SIGMA, Poole, UK |
| LPS | SIGMA, Poole, UK |
| 2-ME | SIGMA, Poole, UK |
| Merthiolate | SIGMA, Poole, UK |
| Methanol | SIGMA, Poole, UK |
| Micrococcus lysodeiticus | SIGMA, Poole, UK |
| MS-222, tricaine methanesulphonate | SIGMA, Poole, UK |
| $Na_2CO_3$ | SIGMA, Poole, UK |
| $Na_2HPO_4.2H_2O$ | SIGMA, Poole, UK |
| $NaH_2PO_4.2H_2O$ | SIGMA, Poole, UK |
| $NaHCO_3$ | SIGMA, Poole, UK |
| Nitroblue tetrazolium salt tablets | SIGMA, Poole, UK |
| Percoll | Amersham Pharmacia Biotech AB, Uppsala, Sweden |
| PHA | SIGMA, Poole, UK |
| Phosphate buffered saline | SIGMA, Poole, UK |
| PMA | SIGMA, Poole, UK |
| Poly-L-lysine | SIGMA, Poole, UK |
| Potassium chloride | SIGMA, Poole, UK |
| Potassium hydrogen carbonate | SIGMA, Poole, UK |
| Potassium hydroxide | SIGMA, Poole, UK |
| Prostaglandin $E_2$ EIA Kit | Cayman Chemicals, Ann Arbor, MI |
| Pyceze® | Norvartis Animal Health UK Ltd. |
| RapiDiff Staining system | R A Lamb, SIGMA Poole, UK |
| SDS | SIGMA, Poole, UK |
| Sheep Anti-mouse IgG HRP conjugate | Diagnostics Scotland, Edinburgh, UK |
| Sodium acetate | SIGMA, Poole, UK |
| Sodium bicarbonate | SIGMA, Poole, UK |
| Sodium chloride | SIGMA, Poole, UK |

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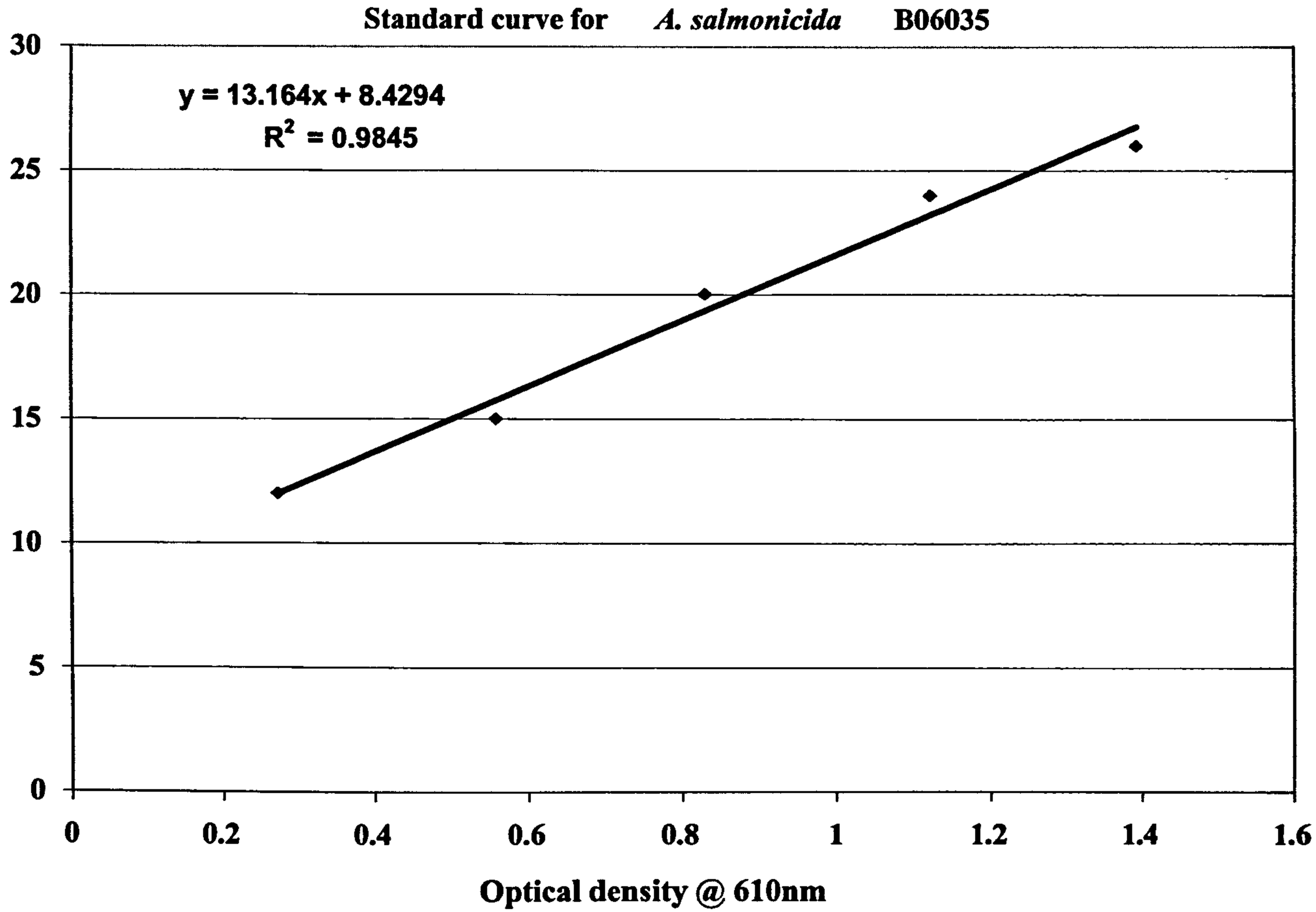
| Sodium hydroxide | SIGMA, Poole, UK |
| Sulphuric acid | SIGMA, Poole, UK |
| Superoxide dismutase | SIGMA, Poole, UK |
| TMB | SIGMA, Poole, UK |
| Trisma base | SIGMA, Poole, UK |
| Trypan blue | SIGMA, Poole, UK |
| Tween 20 | SIGMA, Poole, UK |
| Tryptone soya agar | Oxoid, Hampshire, UK |
| Tryptone soya broth | Oxoid, Hampshire, UK |
| Yeast | SIGMA, Poole, UK |

## Appendix 3: Preparation of bacterial standard curve

The OD of the bacterial suspension was plotted against the number of colony forming units resulting in a standard curve subsequently used for all estimations of bacterial concentration of *Aeromonas salmonicida*

| Concentration of *Aeromonas salmonicida* (%) | OD at 610nm | Colony forming units ($1 \times 10^8$ $ml^{-1}$) |
|---|---|---|
| 100 | 1.392 | 26 |
| 80 | 1.122 | 24 |
| 60 | 0.829 | 20 |
| 40 | 0.554 | 15 |
| 20 | 0.270 | 12 |

TEXT BOUND INTO THE SPINE




Standard curve of the number of colony forming units against the OD at 610nm of *Aeromonas salmonicida*

Calculation of total magnification using a Leitz Orthloux microscope and camera

x Mag. = Objective mag. X Tube mag. X Eyepiece mag. X Camera factor x Print mag

Where:

Objective mag. = x 4; x 10; x 25; x 40 etc.

Tube mag. = 1.0

Eyepiece mag. = x 12.5

Camera factor = x 0.32

Print mag. = x 4.3 (35mm to 6" x 4")

} 17.2

With x 4 Objective, total magnification = x 68.8 (**x70**).

With x 10 Objective, total magnification = x 172 (**x175**).

With x 25 Objective, total magnification = x 430 (**x430**).

With x 40 Objective, total magnification = x 688 (**x700**).