Effects of substitution of dietary fish oil with a blend of vegetable oils on liver and peripheral blood leukocyte fatty acid composition, plasma prostaglandin E_2 and immune parameters in three strains of Atlantic salmon (*Salmo salar*)

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Abstract

Duplicate groups of three genetic strains of Atlantic salmon smolts were cultured on diets containing either fish oil (FO) or a blend of vegetable oils (VO). Fatty acid compositions of liver and peripheral blood leukocytes were compared. The effect of different strains and diets on innate immune parameters and plasma prostaglandin E_2 were also measured. Two strains were selected as being either "fat" or "lean" in terms of muscle adiposity. The third strain was a commercial stock (MH). Total replacement of dietary FO with VO resulted in reduced docosahexaenoic (DHA; 22:6*n*-3) and eicosapentanoic acids (EPA; 20:5*n*-3) in liver, while oleic (18:1*n*-9), linoleic (18:2*n*-6) and α-linolenic (18:3*n*-3) acids were all increased in VO-fed fish. Fatty acid compositions of blood leucocytes showed similar changes. Evaluation of innate immune function showed that in the fat strain circulating leucocytes were significantly lower in VO fish. The lean strain also had significantly higher serum lysozyme activity than MH fish. Reduced haematocrit was seen in VO lean fish compared to FO lean fish. This study provides evidence of strain-induced differences in liver and leukocyte fatty acid compositions and innate immunity in Atlantic salmon fed either FO or VO-based diets.

Introduction

The production of aquafeeds, especially for carnivorous fish such as Atlantic salmon (*Salmo salar*), is currently dependent on the use of marine fish oils (FO). In recent years FO production from feed-grade fisheries has declined as a result of over fishing and natural phenomena such as El Nino, and this, coupled with increased demand, has resulted in higher market prices. In addition, there is increasing global awareness about ethical issues, including sustainability of our oceans and the need to secure natural resources for future generations (FAO, 2006). As the supply of FO becomes a limiting factor in finfish aquaculture development, there is an urgent need for diversification of the raw materials used in aquafeed production. Global production of the major seed oils has increased over recent years and the price of vegetable oils (VO) has been relatively constant as a result (Mourente & Bell 2006). A number of VO including soybean, linseed, rapeseed, palm and olive oil have been investigated as alternative lipid sources for salmonids (Rosenlund *et al.* 2001; Ng *et al.* 2004; Bell *et al*. 2004; Torstensen *et al.* 2005).

Lipids provide essential fatty acids (EFA) and energy in fish diets. Fish require three highly unsaturated fatty acids (HUFA) namely, eicosapentaenoic (20:5n-3; EPA) and docosahexaenoic acids (22:6n-3; DHA), of the n-3 series, and arachidonic acid (20:4n-6; ARA) of the n-6 series (Sargent *et al.* 2002) for normal growth, development and reproduction. The physiological functions of these EFA in all vertebrates, including fish are: (a) maintaining the structural and functional integrity of cell membranes and (b) precursors of eicosanoids (prostaglandins and leukotrienes), which are the key cellular messengers in biological processes, including inflammation (Sargent *et al*., 1999).

Vertebrates, including fish, cannot synthesise long chain (C_{20} and C_{22}) polyunsaturated fatty acids (PUFA) *de novo*, and they or their C_{18} precursors must be provided in the diet (Lall *et al*., 2002). Generally, dietary EFA requirements of freshwater

64 fish can be met by supplying linoleic acid (LA; 18:2n-6) and □-linolenic acid (ALA;
65 18:3n-3) (Lall *et al*., 2002). Freshwater fish, including anadromous salmonids, can
66 elongate and desaturate 18:3n-3 to 22:6n-3, but the process is too slow to meet HUFA
67 requirements (Sargent *et al*. 2002) and thus, these species show higher growth performance
68 when fed diets rich in the n-3 HUFA, EPA and DHA (Sargent & Tacon, 1999).
69 Previous studies have shown that up to 100% of FO in aquafeeds can be replaced
70 with VO in salmonids, provided EFA requirements are met by inclusion of dietary
71 fishmeal (Bell *et al.* 2004; Torstensen *et al.* 2005; Richard *et al.* 2006). However,
72 replacement of FO with VO results in lower levels of n-3 HUFA in tissues of salmonid and
73 marine species (Torstensen *et al*., 2005; Mourente *et al*., 2005).
74 The nutritional status of an organism, including fish, is well-known to influence the
75 immune system and resistance to disease (Blazer 1992; Calder 2001). Therefore,
76 replacement of FO by VO could impact on the immune system of the fish as well as their
77 resistance to infectious diseases, especially if the VOs are rich in n-6 fatty acids. In fish,
78 introduction of n-6 fatty acid enriched diets alters the dietary n-3/n-6 ratio and can
79 influence the composition of fish immune cells, including blood leukocytes (Thompson *et*
80 *al.* 1996; Mourente *et al*., 2005).
81 The aim of this study was to determine possible immunological effects caused by
82 substitution of FO with a blend of VO in three strains of Atlantic salmon. As the inclusion
83 of VOs might alter the tissue n-3/n-6 fatty acid ratio, eicosanoid production and health
84 status of the fish, we assessed their immune response by measuring plasma lysozyme
85 activity, macrophage function (respiratory burst) and red and white blood cell counts.
86 Samples of liver and blood leucocytes were also analysed for fatty acid composition and
87 prostaglandin E_2 (PGE_2) determined in plasma.
88

Materials and methods

Fish and diets

In the present study three strains of Atlantic salmon were fed two experimental diets, where the added oil was either FO or a blend of VOs. Two of the chosen strains were genetically-defined families supplied by Landcatch Natural Selection (LNS, Ormsary, Scotland, UK), which had been selected as being "fat" or "lean" in terms of their muscle fat storage. The third strain was a commercial production stock supplied by Marine Harvest (MH; Fort William, Scotland, UK). The three strains of Atlantic salmon smolts were transferred to the Marine Harvest Fish Trials Unit (Ardnish, Lochailort, by Fort William, Scotland, UK) in May 2006. Each strain was fed one of two experimental diets produced by Skretting ARC, Lerang, Norway, and had reduced levels of fish meal compared to current commercial formulations, [~25% of dry weight (dw)] with additional protein being supplied by a blend of plant meals (~40% dw). They contained either FO or a blend of VOs supplied as 100% of added lipid. The blend of VOs comprised of palm oil, rapeseed oil and Camelina (*Camelina sativa sp*.) oil. Diet formulation, proximate composition and total lipid fatty acid composition are shown in Tables 1 and 2, respectively. Fish were initially fed 3 mm extruded pellets, then 6 mm and finally 9 mm pellets over the course of the trial. To summarise, six duplicate treatments (3 strains x 2 diets), placed in twelve cages and designated as MH FO, MH VO, LNS lean FO, LNS lean VO, LNS fat FO and LNS fat VO were used in the study.

Sample collection

Fish were sampled in February 2007, after 42 weeks from the onset of feeding the diets, when they had reached an average weight of ~2 kg. Fish were killed by a sharp blow to the head following anaesthesia using MS222 (Sigma Aldrich). Blood samples were taken to

assess various haematological parameters and for peripheral blood leukocyte lipid analysis. Five ml of blood was collected from the caudal vein in heparinised syringes from six fish per treatment and transferred into plastic labelled tubes, and kept on ice. Immediately after bleeding, the ventral side of the animal was opened with a sterile scalpel, taking care not to puncture the intestine. A small sample of the liver was removed into a sterile bijou tube and this was placed on dry ice. Samples were transferred to the Institute of Aquaculture, University of Stirling for further analysis. The head kidney was then dissected out and processed as described below for macrophage isolation.

Separation of peripheral blood leucocytes (PBL) for lipid analysis

PBL were isolated from whole blood from three fish per treatment. 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 band of leukocytes was collected using a Pasteur pipette. If erythrocyte contamination of PBL was considered to be excessive (>2%) then the PBL fraction was centrifuged again on 4 ml of fresh Histopaque®. The cells were washed twice in 0.1% L-15, at 600 x g for 10 min and stored at -20^{o}C for lipid extraction.

Lipid analysis of PBL and liver

Total lipid was extracted from fish PBL by a modification of the method of Folch *et al*., (1957). During the procedure the tubes were held on ice. KCl (1 ml, 0.88%) was added to each leukocyte suspension and the solutions were vortexed. Each leukocyte suspension was washed again with KCl (1 ml, 0.88%), mixed and transferred to a labelled glass tube. Chloroform:methanol (8 ml, 2:1 v/v) was added with a glass Pasteur pipette to each tube, vortexed and kept on ice for 30 min. Liver samples (approximately 1g of each) were

extracted using a similar procedure according to Folch *et al.* (1957) and Christie (1982). The tubes or vials were finally reweighed and the lipids were redissolved in chloroform:methanol (2:1, □/□) + 0.01% (w/□) BHT at a concentration of 10 mg ml^{-1}.

The entire lipid extract of PBL or 1 mg of liver lipid was transferred to 15 ml stoppered glass tubes. For liver samples only, heptadecanoic acid (17:0) was added as internal standard, at 10% of the total lipid mass (0.1 mg). For both PBL and liver the organic solvent was evaporated under nitrogen and 1 ml of toluene and 2 ml of the methylating reagent (1% v/v sulphuric acid in methanol) was added prior to the samples being flushed with nitrogen and incubated for 16 h at 50°C in a hot-block. The tubes were removed from the hot-block and were left to cool, before adding 2% (w/v) $KHCO_3$ (2 ml) and 5 ml iso-hexane: diethyl ether (1:1, □/□) + 0.01% (w/□) □□□. After vigorous mixing and centrifugation at 350-400 x g for 2 min, the upper layer was transferred to a clean tube. The sample was re-extracted with a further 5 ml of iso-hexane:diethyl ether (1:1, □/□), centrifuged and the upper layer was added to the first extract.

The solvent was evaporated under nitrogen, and the FAME re-dissolved in 100 □l of iso-hexane. The methyl esters were purified by thin layer chromatography (TLC) using iso-hexane:diethyl ether:acetic acid (90:10:1, □:□:v). The FAME bands were visualized after spraying the edges of the plates with 1% (w/□) iodine in chloroform. The FAME bands were then scraped from the TLC plate into test tubes and eluted from the silica with iso- iso-hexane:diethyl ether + BHT 1:1 (□:□). After removing the silica by centrifugation, the solvent was placed in a clean test tube and evaporated under nitrogen. FAMEs were separated by gas-liquid chromatography (CARLO ERBA GC6000 VEGA Series 2, Milan, Italy) using a 30 m x 0.32 mm i.d. column (CP Wax 52CB, Chrompak, London, UK) with an on-column injection system and flame ionization detection (FID). The injector temperature was 50°C and the FID temperature was 250°C. Hydrogen was used as carrier

gas with a thermal gradient from an initial 50^{o}C to 150^{o}C at 40^{o}C min^{-1} and then to a final temperature of 225^{o}C at 2^{o}C min^{-1}. Individual FAMEs were identified by comparison with known standards and by reference to published data (Ackman 1980).

Plasma lysozyme activity

Lysozyme activity was assayed by using a modified turbidimetric microtitre plate technique according to Ellis (1990) following the method outlined by Mourente *et al*. (2007). The results are given as units (U) ml^{-1} min^{-1} (1U=the amount of sample causing a decrease in absorbance of 0.001 min^{-1}).

Haematology

Blood was used immediately for haematological studies. A heparinised capillary tube was inserted into the sampled blood 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 3 min at 8000 x g. Haematocrit levels were measured using a micro-haematocrit reader (Hawksley, UK) and results were expressed as the percentage packed red blood cell volume in relation to the whole blood volume.

Total erythrocyte and total leukocyte counts, including thrombocytes, were made from blood diluted 1/1000 and 1/100 with phosphate buffered saline (PBS) respectively using an improved Neubauer haemocytometer (Hawksley, UK). Cell numbers were expressed as the number of cells/mm^{3}.

Differential leukocyte counts were determined by staining blood smears with a commercially available Wright-Giemsa staining kit (RapiDiff) according to the manufacture's instructions. Smears were examined under oil immersion (1000 x

magnification) and the percentage of different leukocyte types (lymphocytes, neutrophils, thrombocytes and monocytes) calculated by counting 100 white blood cells on each slide.

Assessment of Respiratory Burst activity by Head Kidney Macrophages

Head kidney was transferred onto a piece of sterile 100 □m nylon mesh, placed over a Petri dish containing 5 ml of L-15 medium with 20 □l of heparin. Cell suspensions were prepared by gently rubbing the tissue through the mesh and the resulting cell suspension was transferred into sterile bijoux and kept on ice. Two hundred □l of each cell suspension was placed into 8 replicate wells of a sterile 96-well microplate. The plate was covered, sealed and placed in a cooled container to avoid temperature fluctuations during the journey back to the laboratory in Stirling.

Once in the laboratory the plates were gently washed three times with L-15 to remove non-adherent cells. One hundred □l of L-15 medium containing 1 mg ml^{-1} nitroblue tetrazolium (NBT) was added to three replicate wells. One hundred □l of L-15 containing 1 mg ml^{-1} NBT and 1 □l PMA (1□g ml^{-1}) was added to another three replicate wells. The plates were sealed and incubated for 60 min at 22^{o}C. Following incubation, the reaction was stopped by adding 100 □l of methanol to each well. A further 100 □l 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 □l of potassium hydroxide and 140 □l of dimethyl sulphoxide to each well and gently mixed using a pipette. Absorbance was determined using an ELISA plate reader at a wavelength of 620 nm. The remaining wells were used to estimate the number of macrophages attached per well for each kidney sample. The results were expressed as 'macrophage activity' by estimating the mean optical density of the triplicate cultures and dividing the mean OD by the number of cells per well to obtain the OD per 10^5 cells.

Extraction and measurement of prostaglandin E_2 (PGE_2) concentration in plasma

After collection of the blood plasma fraction by centrifugation, the plasma was acidified by adding 50 µl ml^{-1} of 2M formic acid and stored at -20°C. The extraction and measurement of the prostaglandin E_2 (PGE_2) concentration in plasma of experimental fish was determined according to Mourente *et al*. (2007) using an enzyme immunoassay (EIA) kit, (SPI-Bio, Massy, France) to make the quantification.

2.6. *Statistical analysis*

All statistical analyses were performed using the statistical package, SPSS 14.0 for Windows. The graphs were created using Prism 5 (Graphpad Software Inc., San Diego, USA). The significance of treatment effects on the different parameters measured were determined by two-way ANOVA followed by Tukey's multiple comparison test where appropriate. Data which were identified as non-homogeneous (Levene's test) were subjected to either arcsine, square root or log transformation before analysis. Prostaglandin data were log transformed, before the above analysis were carried out. Differences were reported as significant if $P<0.05$ (Zar, 1984). Lipid results are reported as means ± SD (n=2) unless otherwise stated and immune parameter results as means ± SD (n=6).

Results

The total lipid content and fatty acid compositions of liver from groups of Atlantic salmon fed the two experimental diets are presented in Table 3. Liver lipid deposition was higher in fish fed VO with the highest % in the LNS fat fish and lowest in the LNS lean fish. Significant differences were seen in the fatty acid profiles of fish from the different dietary groups for all fatty acids, except 18:0 and 20:1n-9. Total saturated fatty acids (especially

14:0, 15:0 and 16:0) were significantly higher in fish fed the FO diet. Also, the proportion of 14:0 was significantly greater in the MH strain in comparison to LNS lean strain.

Total liver monounsaturated fatty acids (MUFA) were significantly higher in fish fed VO diets, due to the higher inclusion of oleic acid (18:1n-9, OA) in the VO blend. The percentage of OA was significantly greater in MH and LNS fat strains compared to LNS lean. The livers of fish fed FO have 3-fold greater levels of 22:1n-11 compared with fish fed VO, due to the higher level of this fatty acid in the FO diet. Liver LA (18:2n-6) was 4-fold greater in fish fed the VO diet for all strains. LA in liver of fish fed with the VO blend was significantly lower in the MH strain in comparison to the two LNS strains. The same pattern was seen for total n-6 PUFA since LA was the major n-6 PUFA. The levels of n-6 PUFA in livers were significantly greater in fish fed VO for all strains. The total n-6 PUFA in VO-fed fish were significantly lower in MH and LNS fat strains in comparison to LNS lean fish. For 20:2n-6 and 20:3n-6, which are intermediates in the desaturation and elongation pathway of LA to 20:4n-6, significant differences were observed with higher percentages in fish fed VO diets compared to those fed the FO diet. In contrast, arachidonic acid (ARA; 20:4n-6) was 2-fold greater in fish fed the FO diet than fish fed the VO diet. The livers of fish fed the VO blend contained 20:2n-6 as the major C_{20} n-6 PUFA, while fish fed with FO had 20:4n-6 as the major C_{20} n-6 PUFA.

The percentage of linolenic acid (ALA; 18:3n-3) in livers was 4-fold greater in fish fed the VO diet for all strains. The level of ALA in fish fed with VO was significantly lower in the MH strain in comparison to LNS lean and fat strains. The elongation product of 18:3n-3, 20:3n-3, was also significantly higher in fish fed the VO blend than in fish fed the FO diet. The 20:4n-3 concentration in the MH strain was significantly lower in fish receiving the VO diet compared to those receiving the FO diet. Among the VO groups, the level of 20:4n-3 was significantly lower in the MH strain compared to the LNS strains.

EPA content in liver showed a significant reduction (2-fold lower) in fish fed VO, compared to fish fed the FO diet. Also, for the VO groups, a significant decline in EPA was observed in the LNS fat strain compared to the MH and LNS lean strains. Percentages of DHA (22:6n-3) and total n-3 PUFA were significantly higher in fish fed the FO diet, in line with dietary values. The values of DHA and n-3 PUFA were significantly higher in the LNS lean strain for both diets. The level of total PUFA and the ratio of n-3/n-6 were significantly higher in fish receiving the FO diet.

The lipid composition of PBL is also influenced by the fatty acid composition of dietary oils although dietary induced changes were generally less than for liver. The fatty acid composition of 18:2n-6, ARA, EPA and DHA is shown in Figure 1 (a-d). The DHA and EPA levels were significantly higher in FO-fed fish, compared to fish fed VO. The level of 18:2n-6 was significantly greater in fish fed VO while ARA was not significantly different between dietary treatments. The ratio of n-3/n-6 was significantly reduced in fish fed the VO diet.

Lysozyme activity was significantly different between the MH and LNS lean strains (Fig. 2a). Fish fed the FO diet showed the highest, but not significantly different, value of lysozyme activity. In addition, the highest level of lysozyme activity was observed in LNS lean strain for both FO and VO diets. The respiratory burst results by head kidney macrophages were inconclusive and are not shown. Significant interaction was found between dietary oil and genetic strain for haematocrit values. These were significantly higher in the MH strain fed the FO diet compared to the LNS fat strain fed the FO diet. The haematocrit values were also significantly higher in the MH and LNS fat strains compared to LNS lean strain. Significant differences were also observed between LNS lean strain and LNS fat strain fed the two different diets. In the LNS lean strain the haematocrit levels were significantly higher in fish fed the FO diet, whereas haematocrit levels in LNS fat

strain were significantly higher in fish fed the VO diet (Fig. 2b). No differences were found in the total number of erythrocytes between the different treatments. However, the number of circulating leucocytes was significantly affected in the LNS fat strain, with values in fish fed VO being significantly lower than values in fish fed the FO diet (Fig. 2c).The mean number of leucocytes was higher, but not statistically different, in the MH strain compared to LNS lean strain fed either of the diets.

Percentage values of differential blood cell counts and the concentration of plasma prostaglandin E_2 (PGE_2) for each treatment are presented in Table 4. There were no significant differences in lymphocyte, monocyte and thrombocyte levels among the different groups. The large variations between individual fish resulted in no statistical differences between different treatments on plasma concentrations of PGE_2. There was, however, a significant interaction between dietary oil and strain in the number of granulocytes counted. The granulocyte value for FO groups was significantly higher in the LNS lean strain compared to the MH strain. Also, for the VO diet, granulocyte number was significantly higher in the MH strain than the LNS fat strain. In the MH strain, however, the granulocyte value was significantly higher in fish fed the VO blend in comparison to fish fed FO (Fig. 2d).

Discussion

The aim of this study was to examine the effects of two dietary oils (either FO or a blend of VO) on total lipid fatty acid composition of liver and peripheral blood leucocytes in three strains of Atlantic salmon. In addition, it was hoped to establish if the strain of Atlantic salmon had an effect on the deposition of VO-derived fatty acids and whether there were differences in retention of EPA and DHA. Finally, the potential impact of high VO diets on the health of the different strains of salmon was investigated. It is generally

312 accepted that the fatty acid composition of fish tissues is closely related to dietary fatty
313 acid composition (Bell *et al.* 2004; Menoyo *et al.* 2005). LNS lean and LNS fat strains
314 were selected for the difference in their capacity to store muscle fat.

315 In the short to medium term, the currently available high production volume VOs
316 will probably meet most of the requirements for FO substitution. These include rapeseed
317 and palm oils and also soybean oil and linseed oil as well as blends of these selected to
318 provide a balance of saturates, MUFA and PUFA that can be tailored to the specific needs
319 of individual fish species (Torstensen *et al*. 2005; Mourente & Bell, 2006). In the longer
320 term, other VO not presently produced in large tonnages may have fatty acid compositions
321 that would make them suitable for inclusion in aquafeeds. These include *Camelina sativa,*
322 a plant that is known by many names in different geographical locations (e.g. German
323 Sesame and False Flax) due to its long history as an oilseed crop. The principal fatty acids
324 are 18:3n-3 (~38%), 18:2n-6 (~18%) and interestingly 20:1n-9 (~16%) which is more
325 commonly found in high latitude fish oils and is a rich energy source in fish feeds
326 (Marquard & Kuhlmann, 1986; Sargent *et al.* 2002). In the present study, *Camelina* oil,
327 added as 6% of the diet formulation or 20% of added oil, had no obvious detrimental
328 effects on fish health.

329 The two LNS strains used in the present study were chosen due to their differential
330 deposition of muscle lipids. However, differences were also observed in deposition of lipid
331 in the liver that was affected both by strain and lipid source. The LNS lean strain showed
332 significantly lower levels of oil deposition in the liver, with both FO and VO, while lipid
333 deposition was greater when fish were fed VO compared to FO. Oil source has been shown
334 to alter liver lipid deposition in a number of species including salmon where liver lipid
335 increased with increasing replacement of FO with rapeseed oil (Bell *et al.* 2001). A similar
336 effect was seen in gilthead sea bream (*Sparus aurata)* when increasing levels of soybean

337 oil were fed but was not observed when linseed oil replaced FO (Menoya *et al.* 2004). By
338 contrast liver lipid deposition was unaffected in sea bass (*Dicentrarchus labrax)* fed 60%
339 of VO as a blend of rapeseed, palm and linseed oils (Mourente *et al.* 2007). The tendency
340 for lipid accumulation when fed VO may be related to decreased dietary n-3 HUFA, which
341 could result in depressed lipoprotein synthesis (Mourente *et al*., 2005).

342 The results of this study indicate that total replacement of dietary FO by a blend of
343 VO resulted in significant changes in fatty acid profiles of liver and peripheral blood
344 leucocytes. Percentages of EPA, DHA and total n-3 PUFA were significantly higher in
345 livers of fish fed the FO diet compared to those fed the VO diet. No significant differences
346 were observed in liver EPA and DHA content in fish fed FO between the three strains. By
347 contrast, in the VO salmon, EPA level was significantly higher in the LNS lean strain
348 compared to the LNS fat strain. However, the value of DHA was significantly greater in
349 the LNS lean strain fed both diets. This effect is probably due to the lower level of total fat
350 deposition, and probably lower triacylglycerol (TAG) content, in the LNS lean liver. In
351 general, HUFA are preferentially deposited in phospholipids compared to TAG and would
352 account for the differences seen in n-3 HUFA between the salmon strains (Sargent *et al.*
353 2002). The percentages of LA and ALA in fish fed the VO diet were not significantly
354 different between the two LNS strains.

355 In peripheral blood leucocytes (PBL), EPA and DHA values are obviously affected
356 by diet in the MH and LNS lean strains but less so in the LNS fat fish. In general, the
357 differences seen in the concentrations of essential HUFA in PBLs from the different
358 groups were not as pronounced as seen in the liver. This probably reflects the requirement
359 to preserve fatty acid compositions in highly bioactive cell types that relate to the
360 physiological functions of these cells. Fish fed with VO have a higher level of n-6 PUFA

compared with those fed with a FO diet (Bell *et al.* 1996; Thompson *et al*. 1996; Montero *et al*., 2004; Mourente *et al.* 2005, 2007).

Different types of dietary oils can influence not only the fatty acid composition of fish liver and peripheral leucocytes, but could also have negative effects on fish health due to alterations in the types and concentrations of eicosanoid compounds produced (Balfry *et al*., 2006). Dietary lipids modulate the immune response by influencing the physical properties of immune cell membranes and membrane-associated enzymes and receptor sites (Montero *et al*., 2004). Phagocytic cells such as neutrophils and macrophages contain a relatively high concentration of n-3 PUFA, and their composition can be altered by changes in dietary lipids (Mourente *et al*., 2007). Resistance to infection in Atlantic salmon was superior in fish fed a high dietary ratio of n-3/n-6 PUFA compared to fish fed a low n-3/n-6 ratio (Thompson *et al*., 1996).

Dietary fatty acids may affect the immune system through the production of eicosanoids from ARA and EPA (Mourente *et al*., 2005, 2007). Fish fed the FO diet had significantly more ARA in liver than the fish fed the VO diet. It therefore appears that feeding VO diets had an inhibitory effect on ARA synthesis and retention. The replacement of FO with VO in fish diets can effect the production of eicosanoids by influencing the availability of precursor fatty acids and particularly the EPA/ARA ratio (Yaqoob, 2004; Mourente *et al*., 2007). Nevertheless, the EPA/ARA ratio was similar between the different treatments. Only in the LNS lean strain was the ratio of EPA/ARA found to be slightly lower for both of the two dietary treatments compared to the MH and LNS fat groups. Prostaglandins, especially the ARA-derived PGE_2 are produced by monocytes and macrophages and associated with modulation of immune cell function (Bell & Sargent, 2003; Yaqoob 2004). A diet high in n-6 PUFA, produced high levels of PGE_2, whereas diets with high levels of n-3 PUFA produced more PGE_3 (Ganga *et al*., 2005).

According to Bell *et al*., (1993) Atlantic salmon fed a diet containing sunflower oil (rich in n-6 PUFA) produced more ARA-derived eicosanoids compared to fish fed diets containing linseed or a southern hemisphere FO (higher in EPA). In the present study, ARA-derived PGE_2 production in plasma was not affected by the VO diet. However, no significant differences were observed in monocyte number between different groups of fish fed either of the two diets. The production of PGE_2 was reduced in fish fed the VO diet except in the MH strain although the differences were not significant.

The inclusion of VO in fish diets could cause significant reductions in haematological and innate immune parameters such as haematocrit, total circulating leucocytes and macrophage respiratory burst (Mourente *et al*., 2005). In the present study, the only significant difference in the total number of circulating leucocytes found was in the LNS fat strain where the number of circulating leucocytes was significantly lower in fish fed the VO diet compared to fish fed the FO diet. However, no statistical differences were found in the total number of erythrocytes among the different treatments. In contrast, 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. This suggests that the reason for this reduction in the number of leucocytes may be, at least partly, due to the genetic variation between the strains.

Lysozyme activity was significantly different between the MH and LNS lean strains. Montero *et al*., (2003) reported that sea bream fed a FO diet had higher serum lysozyme activity compared to fish fed diets containing either linseed, rapeseed or soybean oil. Bell *et al*., (1996) found no differences in lysozyme concentrations between groups of salmon fed FO, linseed oil or sunflower oil diets. The highest levels of lysozyme activity were observed in the LNS lean and fat strains and especially in the lean. It is possible the significant difference in lysozyme levels between the different strains is associated with the

411 number of granulocytes measured in these strains. Neutrophils synthesize and secrete
412 lysozyme, and increases in serum lysozyme activity have been correlated with an increase
413 in neutrophil numbers (Balfry *et al*., 1997). The significant strain differences in lysozyme
414 activity found here, suggest there may be a genetic influence on variation of lysozyme
415 activity. The most likely explanation for the observed results is differences in disease
416 resistance between the strains. Strain differences in the activity of their innate immune
417 response appear to be related to inherent disease resistance because lysozyme activity tends
418 to be higher in the more disease resistant strains (Balfry *et al*., 2001).
419 There was a significant interaction between dietary oil and strain in the number of
420 granulocytes observed. Granulocyte percentage for the FO groups was significantly higher
421 in the LNS lean strain compared to the MH strain. For the VO diet, granulocyte number
422 was significantly higher in the MH strain than in the LNS fat strain. The total number of
423 granulocytes was slightly greater in the LNS lean strain compared to the MH strain and
424 much higher compared to the LNS fat group. An increase in neutrophil percentage is
425 generally associated with increased pathologies in fish (Ranzani-Paiva *et al*., 2003).
426 Significant interaction was also observed between diet and strain for haematocrit.
427 Haematocrit levels were significantly higher in the MH strain fed the FO diet, compared to
428 the LNS fat strain fed the FO diet. The haematocrit values were also statistically higher in
429 MH and LNS fat strains compared to LNS lean in fish fed the VO diet. In the present
430 paper, it was found that in the LNS lean strain, feeding the VO diet resulted in reduced
431 haematocrit compared with fish fed the FO diet. In contrast, in the LNS fat strain the
432 haematocrit value was significantly higher in VO-fed fish. A likely explanation for the
433 observed results is that the LNS fat strain can more effectively utilize the VO diet
434 compared to the LNS lean group. According to Sandnes *et al*., (1988) the normal range of
435 haematocrit value is 44-49 %. In the present study, it was found that the LNS fat strain fed

436 a blend of dietary VO resulted in a relatively higher haematocrit value while the opposite
437 was true for the LNS lean strain. Young (1949) found that haematocrit values always
438 decreased when fish lost their appetite, or when stressed or diseased. Therefore,
439 haematological parameters can be useful indicators of health status in fish. However, it
440 must be stressed that haematology is influenced not only by nutrition and genetic strain,
441 but also by water temperature and environmental stress (e.g. crowding, water quality)
442 (Sandnes *et al*., 1988) although these factors should have been consistent across all the
443 pens in the present trial.

444 In summary, the present study suggests that total replacement of FO with a blend of
445 VO has no detrimental effect on fish growth (results not shown). Liver ARA, DHA and
446 EPA values, as well as other fatty acids, are clearly affected by diet. The n-3 and n-6
447 HUFA concentrations are significantly higher in LNS lean strain fed VO diets compared to
448 MH and LNS fat strains fed the same diet. However, in PBL, EPA and DHA values are
449 obviously affected by diet in the MH and LNS lean strains but not in LNS fat strain. From
450 the present study, there is some evidence that immune function, in terms of haematocrit,
451 total number of circulating leucocytes, granulocyte number and serum lysozyme activity
452 may be influenced by the total substitution of FO with a blend of VO, especially in the
453 LNS lean strain. However, the impact of these changes on fish health requires further
454 investigation.

455

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Figure legends

Figure 1. Effects of genetic strain and replacement of fish oil (FO) with a blend of vegetable oils (VO) on fatty acid compositions of peripheral blood leukoctyes; (a) 18:2n-6; (b) 20:4n-6; (c) 20:5n-3; (d) 22:6n-3; Values are mean ± SD, (n=2).

Figure 2. Effects of genetic strain and replacement of fish oil (FO) with a blend of vegetable oils (VO) on: (a) serum lysozyme activity; (b) % haematocrit; (c) total circulating leucocytes; (d) % granulocytes. Values are mean ± SD, (n=6). For haematocrit and granulocytes significant differences ($P<0.05$) between dietary oils belonging to different strains are denoted by columns assigned different letters (lower or higher case). Dietary oils assigned an asterisk are significantly different within the same strain. Significant differences ($P<0.05$) in lysozyme activity between the strains are denoted by columns assigned different letters. Significant differences ($P<0.05$) on total circulating leucocytes among dietary oils in LNS fat strain are denoted by columns assigned different superscript letters.

604 Table 1. Formulation and proximate composition of 6 mm experimental diets (g/Kg feed)

Components	Diets	
	Fish oil	Vegetable oil
LT-fish meal[a]	250.00	250.00
Wheat[b]	78.80	78.80
Wheat gluten[c]	90.00	90.00
Soya (extracted)[d]	110.00	110.00
Soya concentrate[e]	70.00	70.00
Corn gluten[f]	56.8	56.8
Premix[g]	50.12	50.12
Fish oil (Nordic)[h]	300.00	0.00
Camelina oil[i]	0.00	60.00
Rapeseed oil[j]	0.00	150.00
Palm oil[d]	0.00	90.00
Proximate composition (%)		
Crude protein	45.46	44.71
Crude lipid	27.99	28.31
Moisture	5.29	4.96

605 [a]Consortio, Peru. [b]Statkorn, Norway. [c]Cerestar Scandinavia AS, Denmark. [d]Denofa,
606 Norway. [e]ADM, The Netherlands. [f]Cargill, USA. [g]Skretting AS, Norway. [h]Nordsilmel,
607 Norway. [i]Technology Crops Inc., USA. [j]Emmelev AS, Denmark.

608

608 Table 2. Fatty acid composition (weight % of total fatty acids) of 6 mm experimental diets.

Fatty acid %	Diets	
	Fish oil	Vegetable oil
14:0	4.9	1.2
15:0	0.5	0.1
16:0	16.5	16.8
18:0	2.3	2.7
Total saturated	26.0	22.0
16:1n-9	0.3	0.1
16:1n-7	5.3	1.2
18:1n-9	17.8	37.2
18:1n-7	2.5	2.1
20:1n-11	0.6	0.2
20:1n-9	5.4	3.9
20:1n-7	0.3	0.2
22:1n-11	7.5	1.3
22:1n-9	0.8	0.9
24:1n-9	1.1	0.3
Total monoenes	42.2	47.5
18:2n-6	4.7	15.0
20:2n-6	0.4	0.3
Total n-6 PUFA	6.2	15.5
18:3n-3	2.2	9.3
18:4n-3	2.3	0.4
20:4n-3	0.5	0.1
20:5n-3	7.7	1.9
22:5n-3	0.8	0.2
22:6n-3	10.8	2.3
Total n-3 PUFA	24.4	14.5
Total PUFA[1]	31.8	30.5

609 [1]Includes 17:0, 20:0, 22:0, 14:1, 17:1, 18:3n-6, 20:3*n*-6, 20:4n-6, 22:5*n*-6, 20:3n-3, 16:2,

610 16:3, 16:4,