

1
2 **Effects of partial substitution of dietary fish oil with blends of vegetable oils, on**
3 **blood leukocyte fatty acid compositions, immune function and histology in European**
4 **sea bass, (*Dicentrarchus labrax* L.)**

5
6 Gabriel Mourente^a, Joanne. E. Good^{b1}, Kim D. Thompson^b and J. Gordon Bell^{b*}

7
8 ^a*Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de*
9 *Cádiz, E-11510 Puerto Real (Cádiz), Spain.*

10 ^b*Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK.*

11 ¹*present address Bio-Stat Ltd., Bio-Stat House, Pepper Road, Hazel Grove, Stockport,*
12 *SK7 5BW, UK.*

13

14

15

16

17

18

19 * Corresponding author. Tel.: +44 1786 467997; fax: +44 1786 472133

20 *E-mail address:* g.j.bell@stir.ac.uk

21 Running title: Dietary oils and immune function in sea bass

22 **Keywords: European sea bass: Vegetable oils: Fatty acid compositions, Immune**
23 **function: Histology**

24

25

1 Within a decade or so insufficient fish oil (FO) will be available to meet the requirements for
2 aquaculture growth. Consequently, alternative sources are being investigated to reduce reliance
3 on wild fish as a source of FO. Vegetable oils (VO) are a feasible alternative to FO. However,
4 it is important to establish that alternative dietary lipids are not only supplied in the correct
5 quantities and balance for optimal growth, but can maintain immune function and prevent
6 infection, since it is known that the nutritional state of the fish can influence their immune
7 function and disease resistance. A way of maintaining immune function, while replacing
8 dietary FO, is by using a blend of VOs rather than a single oil. In this study, juvenile European
9 sea bass, *Dicentrarchus labrax*, were fed diets with a 60 % substitution of FO with a blend of
10 rapeseed (RO), linseed (LO) and palm oils (PO). Two oil blends were used to achieve a fatty
11 acid composition similar to FO, in terms of energy content and provide a similar balance of
12 saturates, monounsaturates and polyunsaturated fatty acids. Fish were fed the diets for 64
13 weeks, after which time growth and fatty acid compositions of liver and blood leukocytes were
14 monitored. The impact of the dietary blends on selected innate immune responses and
15 histopathology were also assessed, together with levels of plasma prostaglandin E₂. The results
16 suggest that potential exists for replacing FO with a VO blend farmed sea bass feeds without
17 compromising growth, non-specific immune function or histology.

18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34

1 Global catches from the feed grade fisheries that provide fish oil (FO) and fish meal for
2 aquafeed formulations have reached their sustainable limits¹ and it is likely that within a
3 decade or so there may be insufficient FO to meet the quantities required for current
4 aquaculture growth². Consequently, there has been considerable interest in introducing
5 sustainable alternatives to fish meal and FO that reduce reliance on marine raw materials^{3,4}. A
6 number of recent studies suggest that dietary vegetable oil (VO) inclusion does not result in
7 reduced growth performance or feed conversion in Atlantic salmon, *Salmo salar*^{5,6}, rainbow
8 trout, *Oncorhynchus mykiss*⁷ gilthead sea bream, *Sparus aurata*⁸ or European sea bass,
9 *Dicentrarchus labrax*⁹. However, at levels above 50% VO inclusion, significant accumulation
10 of fatty acids derived from VO, especially 18:2*n*-6, and reduction of eicosapentaenoic (20:5*n*-
11 3; EPA) and docosahexaenoic acids (22:6*n*-3; DHA) occurs in fish tissues^{5,6,8,9}.

12 The nutritional status of an organism, including fish, is known to influence immune
13 functions¹⁰ and the overall resistance of an organism to disease is therefore dependent on their
14 nutritional status. The first review suggesting that fatty acids might be important in immune
15 function was by Meade & Mertin¹¹ and more recent reviews have confirmed the importance of
16 the polyunsaturated fatty acids (PUFA), of both the *n*-6 and *n*-3 series, as modulators of
17 immune function^{12,13}. Fatty acids are incorporated into the plasma membrane from dietary
18 lipids, so that the fatty acid composition of cellular membranes reflects the composition of
19 dietary lipids¹⁴. In fish, dietary fatty acids and tissue fatty acid compositions are closely
20 correlated¹⁵ and changes in the dietary *n*-3/*n*-6 ratio can influence the compositions of fish
21 immune cells, including blood leukocytes.^{16,17,18}

22 Fatty acids have diverse roles in all cells. They are important as a source of energy, as
23 structural components of cell membranes and as signalling molecules. In mammalian studies,
24 dietary fatty acids may be able to modulate the immune system through several mechanisms
25 including reduction of lymphocyte proliferation, cytokine synthesis and phagocytic activity
26 and also by modification of natural killer cell activity¹⁹. The main event in the modulation of
27 immune function may be associated with changes in the cell membrane due to dietary fatty
28 acid manipulation. It is likely that modulation of the overall immune system occurs as a result
29 of alterations in membrane fluidity, lipid peroxidation, eicosanoid production or regulation of
30 gene expression²⁰.

31 In the present study, triplicate groups of juvenile European sea bass were fed diets that
32 were based on 60% substitution of FO with a blend of rapeseed (RO), linseed (LO) and palm
33 oils (PO). The level of 60% substitution was chosen as this was the maximum level of VO
34 inclusion that could be tolerated in marine fish without loss of growth performance.^{8,9} The oils

1 were blended in two different formulations to achieve a fatty acid composition as similar to
2 anchovy oil as possible, in terms of energy content and provide a similar balance of saturates,
3 monounsaturates and PUFA to that found in FO, but without highly unsaturated fatty acids
4 (HUFA). The fish were fed the diets for 64 weeks starting at an initial weight of
5 approximately 5 g. Growth parameters and the fatty acid compositions of liver and peripheral
6 blood leukocytes were monitored after 64 weeks. The impact of the dietary blends on selected
7 aspects of the innate immune response (haematological parameters, serum lysozyme activity
8 and macrophage respiratory burst activity) and histopathology were also assessed in the
9 experimental fish at this time, together with levels of plasma prostaglandin E₂.

10

11 **Materials and methods**

12 *Experimental fish and diets*

13 European sea bass (*Dicentrarchus labrax* L.), 7.9 ±0.5 cm in length and 5.2 ±1.0 g in weight
14 were purchased from MARESA, Huelva, Spain and transported to the marine aquarium facility
15 at the University of Cádiz, Faculty of Marine and Environmental Sciences, Puerto Real
16 (Cádiz). On arrival the fish were placed in nine 5000 l rectangular tanks at 600 fish per tank
17 (approx. 0.6 kg/m³), with salinity of 39‰, temperature of 20°C and saturated with oxygen.
18 Following 2 weeks acclimation (July 2002), triplicate groups of fish were fed to satiation,
19 using mechanical belt automatic feeders with three iso-energetic and iso-nitrogenous
20 experimental diets formulated to provide a constant lipid content of ~22 % (Nutreco ARC
21 Stavanger, Norway). The diets contained ~47 % protein, primarily provided by fish meal, and
22 21.4 %, 24.1 % and 21.5 % lipid for diets of pellet size, 2, 3 and 5 mm, respectively. Two
23 experimental diets contained 60 % of three VOs, LO, PO and RO, blended to provide a
24 balance of saturates, monoenes and PUFA similar to that found in FO, but without HUFA. The
25 control diet contained anchovy oil and the added oil combinations for the three experimental
26 diets were: Diet A: 100 % anchovy oil (control); Diet B: 40 % anchovy oil, 35 % linseed oil,
27 15 % palm oil and 10 % rapeseed oil; Diet C: 40 % anchovy oil, 24 % linseed oil, 12 % palm
28 oil and 24 % rapeseed oil. The formulation and proximate compositions of the experimental
29 diets are shown in Table 1, while diet total lipid content and fatty acid compositions are shown
30 in Table 2.

31

32 *Sample collection and biometric measurements*

33 After feeding the experimental diets for 64 weeks, **thirty fish per tank (i.e. 90 fish per replicate)**
34 **were sampled for length and live mass and** condition factor K and specific growth rates

1 recorded. Ten fish per replicate (i.e. 30 fish per dietary treatment) were sampled and liver (live
2 and dry mass), hepatosomatic index and flesh (live and dry mass) were recorded. Liver
3 samples for fatty acid analyses were dissected from 4 fish per replicate (i.e. 12 fish per dietary
4 treatment) and immediately frozen in liquid nitrogen and stored at -80°C until analysed. Blood
5 for eicosanoid analysis (2 ml) was collected in heparinised syringes from 6 fish per dietary
6 treatment, and centrifuged at 12 000 x g for 2 min. The plasma was collected and acidified by
7 the addition of 50 µl/ml 2 M formic acid and immediately frozen in liquid nitrogen for
8 eicosanoid analysis. Heparinised blood samples were also used for haematological analyses.
9 Live mass of the liver was determined by blotting the tissue on filter paper before weighing,
10 and dry mass determined after heating to 60°C for 24 h and cooling under vacuum before
11 weighing. Hepatosomatic index (HSI) was calculated as liver live mass * 100/fish live mass.
12 Fulton's condition factor (K) = (W/L³)*100, where W is the fish weight (g) and L the total
13 length (cm). Specific growth rate (SGR) was calculated as % weight gain/day²¹. Non-specific
14 mortality was measured at the end of the experiment and expressed as a percentage of
15 surviving fish.

16

17 *Proximate analysis of diets*

18 Moisture content was determined by thermal drying to constant weight in an oven at 110°C for
19 24 h. For the total protein content, the micro-Kjeldahl analysis method was followed, using a
20 Digestion system 40–1006 Heating Unit and a KJELTEC AUTO 1030 Analyzer. To convert
21 total nitrogen to total protein content, as a percentage of dry weight, the factor 6.25 (100/16)
22 was used. Crude fat was determined by acid hydrolysis with a Soxtec System 1047
23 Hydrolyzing Unit, followed by Soxhlet extraction using a Soxtec System HT6. Ash content as
24 % of dry weight was determined by dry ashing in porcelain crucibles in a muffle furnace, at
25 600°C overnight²².

26

27 *Lipid analysis*

28 Total lipid in samples was extracted after homogenisation, using an Ultraturrax tissue
29 disrupter, in 10 volumes of chloroform/methanol (2:1, v/v) containing 0.01 % butylated
30 hydroxytoluene (BHT) as antioxidant, basically according to Folch *et al.*²³ and essentially as
31 described by Christie²⁴.

32 Fatty acid methyl esters (FAME) were prepared from aliquots of total lipids by acid-
33 catalysed transmethylation for 16 h at 50°C, using tricosanoic acid (23:0) as internal
34 standard²⁴. FAME were extracted and purified as described previously²⁵ and were separated

1 using a Hewlett-Packard 5890A Series II gas chromatograph equipped with a chemically
2 bonded (PEG) Supelcowax-10 fused silica wall coated capillary column (30 m x 0.32 mm i.d.,
3 Supelco Inc., Bellefonte, USA), using an "on column" injection system and flame ionisation
4 detection. Hydrogen was used as the carrier gas with an oven thermal gradient from an initial
5 50°C to 180°C at 25°C/min and then to a final temperature of 235°C at 3°C/min, with the final
6 temperature maintained for 10 min. Individual FAME were identified by comparison with
7 known standards and quantified by means of a direct-linked PC and Hewlett-Packard
8 ChemStation software.

9

10 *Extraction and measurement of prostaglandins E₂ concentrations in plasma*

11 The frozen acidified plasma samples were thawed and centrifuged at 12000 x g for 2 min to
12 remove any precipitate. The supernatants were extracted using octadecyl silyl (ODS, C18)
13 "Sep-Pak" mini-columns (Millipore) as described in detail by Bell *et al.*²⁶. C18 "Sep-Pak"
14 mini columns were pre-washed with 5 ml methanol and 10 ml of distilled water, plasma
15 samples were charged on the mini-column, washed with a further 10 ml of distilled water and
16 the eicosanoids eluted in 5 ml of ethyl acetate. Samples were dried under nitrogen and
17 redissolved in immunoassay buffer. Quantification of prostaglandin E was performed using
18 enzyme immunoassay (EIA) kits, according to the manufacturers protocol (SPI-Bio, Massy,
19 France).

20

21 *Measurement of cellular immune parameters*

22 Eight fish per dietary treatment were sampled after 64 weeks feeding the experimental diets.
23 Fish were anaesthetised with a lethal dose of tricaine methanesulphonate (MS-222, Sigma,
24 UK). Blood samples were collected in heparinised vacuum tubes (vacutainer Becton Dickinson
25 Vacutainer System, Oxford, UK) from the caudal vein.

26

27 *Preparation of peripheral blood leucocytes*

28 Peripheral blood leucocytes (PBL) were isolated from blood from three fish per dietary
29 treatment using the lymphocyte separation medium, Histopaque[®] (Sigma, UK) and density
30 gradient centrifugation. One ml of blood was diluted with 4 ml of L-15 medium and 3 ml of
31 the diluted blood was layered onto 4 ml of Histopaque[®] and centrifuged at 400 x g for 45
32 minutes. The leucocyte band was collected using a Pasteur pipette and stored in 1 ml of
33 chloroform:methanol (2:1 v/v) at -20°C until required for lipid extraction. If erythrocyte
34 contamination of PBL was considered to be excessive (> 2%) then the PBL fraction was

1 centrifugation again on 4 ml of fresh Histopaque®.

2

3 *Haematology*

4 Blood was used immediately for haematological studies. Haematocrit values were obtained
5 using heparinised micro-haematocrit tubes and centrifuging at 12,000 x g for 4 min
6 (Microcentrifuge MH2, Sarstedt Ltd). Total erythrocyte and total leukocyte counts (including
7 thrombocytes) were made using phosphate buffered saline (PBS) for dilution and an improved
8 Neubauer haemocytometer (Hawksley, UK).

9

10 *Serum lysozyme activity*

11 An aliquot of blood was allowed to clot at 4°C overnight. Serum was separated by
12 centrifugation at 4000 x g for 15 min and stored at -20°C until analysis. Serum lysozyme
13 activity was assayed by a turbidimetric assay which measures the lytic activity of the seabass
14 serum against *Micrococcus lysodeikticus*.^{27,28} A suspension of 190 µl of bacteria (*Micrococcus*
15 *lysodeikticus*, Sigma, UK) and 10 µl of serum sample was measured spectrophotometrically at
16 540nm in five replicate wells per serum sample after 1 and 5 min at 25°C, using a Dynatech
17 MRX 1.2 ELISA reader (Dynatech Laboratories Limited, West Sussex, UK). The bacterial
18 suspension (0.2 mg/ml) was prepared in sodium phosphate buffer (0.04 M, pH 5.8). The results
19 are given as units (U)/ml/min (1U = the amount of sample causing a decrease in absorbance of
20 0.001min⁻¹).

21

22 *Macrophage respiratory burst activity*

23 The reduction of nitroblue tetrazolium salt (NBT) to formazan by oxygen radicals produced by
24 head kidney macrophages during respiratory burst activity was measured
25 spectrophotometrically as described by Chung & Secombes²⁹. Isolation and culture of head
26 kidney macrophages were performed as described by Secombes³⁰ however instead of placing
27 the cell suspensions on Percoll to isolate the macrophages, 200 µl of each kidney suspension
28 was added directly to four replicate wells of a 96 well microtitre plate. Plates were sealed and
29 incubated for 3 h before washing them gently three times to remove non-adherent cells. Two
30 hundred µl of L-15, containing 10 % foetal bovine serum, was added to all wells and cultures
31 were incubated at 18° C for 2 to 3 days, after which the respiratory burst activity of the
32 macrophages was determined by incubating the cells with 100 µl of NBT (1mg/ml)/phorbol
33 myristate acetate (PMA, 1 µg/ml). This was added to three of the four wells and incubated at
34 18-20°C for 40 min. The assay was developed as described by the authors using a microplate

1 reader as described above to read the absorbance at 620 nm. The remaining well was used to
2 determine the numbers of macrophages attached to the plate for individual kidney samples³⁰.
3 The results were expressed as “macrophage activity” by calculating the mean optical density
4 for each of the triplicate cultures and dividing the mean OD by the number of cells/well to
5 obtain an OD per 10⁵ cells and multiplying by 100.

6

7 *Histological examination of fish tissues*

8 Samples were collected at 64 weeks to identify any effects of dietary treatment on the
9 histology of the heart, liver or intestine. Samples of proximal, mid and distal intestine were
10 collected from 6 fish from each dietary treatment, in addition to the heart and liver, for
11 histopathological examination. Sections were fixed in 10 % buffered formalin at the time of
12 dissection, embedded in paraffin wax and 5 µm sections were cut and stained with
13 haematoxylin and eosin. Processed sections were examined “blind” to eliminate bias in
14 interpretation. Stained sections of heart were assessed for signs of endocarditis and
15 pericarditis. Liver sections were assessed on fat content, any indication of inflammation in the
16 tissue, the degree of peri-vascular cuffing (PVC) and finally the presence of single cell
17 necrosis (SCN). Intestinal sections were examined on the integrity of the intestinal mucosa,
18 the appearance of the submucosa and lamina propria and the presence of any inflammatory
19 response.

20

21 *Statistical analysis*

22 Results are reported as means ± SD (n=3) unless otherwise stated. All statistical analyses were
23 performed using the statistical computer package, Prism 4.0, GraphPad Software, Inc., San
24 Diego, California, USA. The significance of treatment effects on biometry and growth rates,
25 liver and leucocyte fatty acid compositions, haematology, serum lysozyme activity and
26 macrophage respiratory burst activity were determined by one-way ANOVA followed by
27 Tukey’s multiple comparison test where appropriate. Percentage data and data which were
28 identified as non-homogeneous (Bartlett’s test) were subjected to either arcsine, square root or
29 log transformation before analysis. Differences were reported as significant at $p < 0.05$ ³¹.
30 Immune parameter results are reported as means ± SD (n=8).

31

32 **Results**

33 There were no significant differences in the total length of fish between dietary treatments, but
34 fish fed Diet B showed significantly lower values for total live mass and liver mass than fish

kdt1 2/3/07 14:36

Deleted: the number, size and variability of the absorptive vacuoles in the mucosal enterocytes.

1 fed Diets A (control) and C. Fish fed Diets A (control) and B presented significantly lower
2 values for flesh dry mass (%) at the end of the 64 week feeding trial (Table 3).

3 The total lipid fatty acid compositions of livers from sea bass following 64 weeks of
4 feeding the experimental diets are shown in Table 4. Total saturated fatty acids (primarily
5 16:0) were identical in all treatments. Total monounsaturated fatty acids, primarily oleic acid
6 (OA), were significantly higher in liver total lipids from fish fed Diet C, followed by fish fed
7 Diets B and A (control) due to the higher inclusion of OA in the VO blends. The proportion of
8 linoleic acid (LA; 18:2*n*-6) in total lipid from liver was highest in fish fed the VO diets, due to
9 the high inclusion of LA in RO, LO and PO, and was about 50 % lower in liver of fish fed Diet
10 A (control). However, total *n*-6 PUFA values were not significantly different for all treatments.
11 In contrast, arachidonic acid (20:4*n*-6, ARA) was highest in liver of control fish (Diet A) and
12 fish fed Diet B, followed by fish fed Diet C. Total *n*-6 HUFA, primarily ARA, was highest in
13 liver from fish that had been fed Diet A (control) followed by fish from treatments B and C
14 that showed identical values. The percentage of linolenic acid (LNA; 18:3*n*-3) in liver total
15 lipids was highest in fish fed Diets B and C (which contained the highest proportions of LO
16 and, in consequence, the highest level of LNA), followed by fish fed diet A (control). Liver
17 total lipid percentages of EPA, DHA and total *n*-3 PUFA were highest in fish fed Diet A
18 (control) due to the highest content of these fatty acids in FO. The level of total PUFA was not
19 significantly different in liver total lipids from all treatments (Table 4).

20 Inclusion of VO in the diets of sea bass modified the fatty acid composition of their PBLs.
21 The fatty acid compositions of PBL from VO-fed fish were different from the corresponding
22 PBL of fish fed FO, with the latter having more monounsaturated fatty acids and higher *n*-6
23 PUFA. PBLs of sea bass maintained on Diets B and C had significantly increased levels of
24 18:0, OA, LA, 20:2*n*-6 and LNA, and significantly reduced amounts of *n*-3 PUFA, ARA, EPA,
25 DHA and others (Table 5). The overall ratio of *n*-3/*n*-6 was significantly reduced in sea bass
26 fed the VO diets.

27 The effect of partial replacement of dietary FO with VO blends on the concentration of
28 plasma PGE₂ in European sea bass after 64 weeks of feeding the diets is shown in Fig. 1a. The
29 highest values were found in plasma of fish fed the control (FO) and C diets, with significantly
30 lower values seen in fish fed diet B (46 % less). The source of dietary lipid did not affect any
31 of the haematological parameters measured. No significant differences were found in
32 haematocrit values (Fig. 1b) or the total number of leukocytes (Fig. 1c) and erythrocytes (Fig.
33 1d) between groups. The production of superoxide anion by head kidney macrophages,
34 measured by the reduction of NBT, is presented in Fig. 1e. It appears that following PMA

1 triggering, the respiratory burst activity was significantly reduced in fish fed the VO based
2 diets. Whether or not this change affects the innate immune response of the fish needs further
3 investigation. It could be that the respiratory burst event takes place earlier or later than seen
4 with macrophages from fish fed the FO diet and that this activity has not been measured at the
5 optimal time for fish fed the VO diets. No effect of dietary VO was observed on sea bass
6 serum lysozyme activity (Fig. 1f). Fish fed the FO diet showed the highest (1452.5 U/ml/min)
7 (but not significantly different) value of lysozyme activity in serum compared to 1351.1
8 U/ml/min found for fish fed the Diet B and 1171.1 U/ml/min for the C diet.

9 Hearts examined from all three dietary groups showed no signs of pathological change. In
10 livers, fat vacuoles were variable in size in many sections with some very large vacuoles
11 present within some hepatocytes and relatively smaller vacuoles in other hepatocytes. Due to
12 the level of vacuolation in some hepatocytes, there was some distortion of the cellular
13 architecture and occasional breakdown of cells. Again, there were no differences between the
14 three dietary groups. Small foci of inflammation were seen in some sections in all three
15 groups, with a slightly higher incidence in sections from the Diet C group. PVC was not a
16 feature in any of the dietary groups examined. With regard to the intestinal sections, mucus
17 levels appeared very similar in all segments and in all dietary groups. Absorptive vacuoles
18 were small and multiple in all sections. In the FO diet these were at relatively low levels in the
19 proximal and mid segments and higher in the distal segments. In fish fed Diet B or Diet C,
20 vacuolation in the proximal segments appeared to be much more pronounced, interestingly less
21 so than in the mid sections. Some cellular infiltration was seen in the lamina propria of one
22 fish in the FO diet (Fig. 2a) and two fish on Diet C (Fig. 2b). Sloughing of the mucosal
23 membrane was not a feature in any of the sections examined. The major difference seen
24 between these groups was the level of absorptive vacuolation in the proximal segment of fish
25 fed Diet B or Diet C, compared with the FO diet.

26

27 Discussion

28 Considerable data has been accumulated on the effects of different dietary lipids on tissue fatty
29 acid compositions of both mammals and fish, although the effects of dietary lipids on fish
30 health and immune function are less well documented. Changes in dietary fatty acid
31 composition have been shown to affect both innate^{32,33,34,35} and adaptive immunity^{16,34,36,37,38},
32 as well as the resistance to infectious diseases^{16,36,37,38,39}. However, the role of *n*-3 and *n*-6 fatty
33 acids in fish immune response is unclear, and reports are not conclusive and are often
34 contradictory.

1 The modulatory process is likely to occur at different cellular levels with the most
2 obvious being a change in cell membrane phospholipid fatty acid composition, affecting the
3 activity of membrane-bound enzymes, receptors and ion channels⁴⁰. In addition, eicosanoids, a
4 group of bioactive derivatives of ARA, EPA and dihomo- γ -linolenic acid (20:3 n -6), which
5 include prostaglandins (PG), thromboxanes, leukotrienes and lipoxins, act to regulate the
6 immune response^{13,41}. Other immune modulatory processes involving fatty acids include
7 changes in intracellular signalling pathways⁴² and direct interactions between fatty acids and
8 nuclear transcription factors in cells of the immune system, such as the peroxisome
9 proliferators activated receptors, that act to regulate immune cell function²⁰.

10 Fish tissues and cell membranes, including phagocytic cells (macrophages,
11 neutrophils), contain relatively high concentrations of n -3 PUFA, and their compositions can
12 be altered by changes in dietary lipid¹⁸. Specific macrophage functions may also be altered by
13 lipids, mainly due to changes in membrane fluidity. If fluidity is altered by fatty acid
14 composition, then potentially several aspects of phagocyte function may be affected including
15 phagocytosis and eicosanoid production. Calder *et al.*⁴³ reported that unsaturated fatty acid
16 incorporation is associated with an increase in the phagocytosis of zymosan particles.

17 Reduction in head kidney macrophage respiratory burst activity has been observed in
18 sea bass and gilthead sea bream fed VO^{9,44}. Sea bass fed 60% RO, LO and olive oil had
19 significantly reduced phagocytic capacity of head kidney macrophage to engulf yeast particles⁹
20 while Montero *et al.*⁴⁴ found reduced macrophage activity in sea bream fed 60% RO. In
21 addition, Sheldon & Blazer³² found that channel catfish macrophage killing activity was
22 positively correlated to the dietary content of n -3 PUFA. They found phagocytosis of live
23 *Edwardsiella ictaluri* by catfish head kidney macrophages was not significantly affected by
24 feeding soybean oil compared to fish fed menhaden oil or beef tallow. However, feeding
25 soybean oil significantly reduced the ability of macrophages to kill engulfed bacteria compared
26 to macrophages from those fed menhaden oil. Macrophages from the latter group also had a
27 significantly higher killing index than macrophages from fish fed soybean oil. Waagbø *et al.*³⁴
28 showed that Atlantic salmon fed diets rich in n -3 PUFA significantly reduced the bacterial
29 killing ability of macrophages at 12°C but not at 18°C indicating that temperature, perhaps
30 related to membrane fluidity, also influences the activity of macrophages. In contrast,
31 Thompson *et al.*¹⁶ found no differences in phagocytosis and bactericidal activities of head
32 kidney macrophages from Atlantic salmon fed diets enriched with either n -3 or n -6 PUFA.

33 In the present study, the concentration of circulating PGE₂ in plasma of sea bass fed the
34 60% VO blend (Diet B) was significantly lower than in fish fed FO. In addition, the fish fed

1 60% VO diets B and C also showed significantly reduced respiratory burst activity which
2 coincided with a reduction in plasma PGE₂ levels. Since the production of PGE₂ was reduced
3 in fish fed VO diets, it may be that the activity or expression of the cyclo-oxygenase enzymes
4 is inhibited by dietary lipid. It is also possible that feeding VO for a long period may reduce
5 the levels of ARA in plasma membranes and, thereby, compromise immune function. In
6 support of the present study, a number of studies also showed a reduction in production of
7 PGE₂ and leukotriene B₄ (LTB₄) by stimulated head kidney macrophages from salmon fed a
8 diet containing LO compared to those fed diets containing sunflower oil or FO^{17,18,45,46}.
9 However, no differences in serum lysozyme activity were found in the present study, which
10 was also reported in other studies with fish fed VO^{18,44}.

11 Montero *et al.*⁴⁴ found that seabream fed a FO diet had higher numbers of circulating
12 erythrocytes compared to fish fed 60% LO or soybean oil diets, which may be related to a
13 higher oxygen requirement due to higher peroxisomal β -oxidation activity induced by the VO
14 diets³⁷. Leray *et al.*⁴⁷ found that the fatty acid composition of erythrocyte membrane
15 phospholipids from trout can be profoundly altered by dietary oils. Trout fed highly saturated
16 coconut oil showed increased *n*-9 fatty acids in their phospholipids, and, consequently, their
17 erythrocytes had a more shrunken appearance than fish fed FO. Perhaps the high levels of
18 saturates in the diets caused reduced haematocrit levels, linked to a shrunken erythrocyte
19 morphology causing a lower packed erythrocyte volume.

20 The lipid composition of monocytes, macrophages, lymphocytes and polymorpho
21 nuclear cells (PMN's) reflect the fatty acid composition of dietary lipids in mammals^{11,48}.
22 Studies by Waagbø *et al.*³⁵ Farndale *et al.*⁴⁹ and Montero *et al.*⁴⁴ reported that dietary oil
23 determines the fatty acid profile of macrophages and immune cells in cod, sea bass and sea
24 bream. Montero *et al.*⁴⁴ reported selective incorporation of certain fatty acids into head kidney
25 macrophages of seabream. DHA was found to be preferentially incorporated and retained in
26 this cell type. Generally, fish fed with a VO-containing diet had increased levels of oleic acid,
27 LA, LNA and total *n*-6 PUFA in both their liver and their PBLs and decreased levels of EPA,
28 DHA, total *n*-3 PUFA and a lower *n*-3/*n*-6 ratio than fish fed a FO diet. Fish fed a FO diet
29 showed the highest *n*-3 HUFA in immune cells in the present study and in previous
30 studies^{35,44,49}. Evidence suggests that changing the fatty acid composition of immune cells can
31 influence immune function by changing the physiology of the cell membrane but perhaps more
32 importantly by influencing the production of modulatory prostaglandins and leukotrienes²⁰.
33 The production of eicosanoids is influenced, in part, by the availability of precursor fatty acids
34 and, in particular, the EPA/ARA ratio. In a previous study with Atlantic salmon fed single VO

1 3-fold differences in the EPA/ARA ratio of immune cells were recorded¹⁸ while in the present
2 study with VO blends the difference in the EPA/ARA ratio, between the three dietary
3 treatments, was only 13%. Perhaps the minor changes to the EPA/ARA ratio in the present
4 study can partly explain lack of effect observed in innate immune function.

5 The overall histological appearance of sea bass sampled from all of the dietary
6 treatments was normal with very few differences observed between the groups. The only
7 difference was in the levels of absorptive vacuoles present in the proximal intestine. Sea bass
8 fed the VO diets showed elevated numbers of absorptive vacuoles compared to fish fed the FO
9 diet. The presence of increased absorptive vacuoles tends to suggest an “active” mucosa,
10 however with increased mucosal vacuolation this could, in turn, leave the intestinal mucosal
11 membrane more vulnerable to sloughing and breakdown. **However, the vacuolization was**
12 **minor and was still regarded as being within normal ranges for Atlantic salmon.**

13 Results of this study suggest that potential exists for replacing FO with a blend of VO
14 in the feeds of farmed sea bass without compromising growth, non-specific immune function
15 and overall histological appearance. It is important to establish that alternative dietary lipids to
16 FO are not only supplied in the correct quantities and balance for optimal growth and feed
17 conversion, but can maintain optimal immune function and not increase susceptibility to
18 infectious pathogens. This study suggests that normal immune function can be more
19 successfully attained if dietary FO is replaced by a blend of VO, **that provides a more**
20 **physiologically balanced fatty acid composition**, in comparison to **replacement with** a single
21 VO^{9,17,18,44}.

23 Acknowledgements

24 This work was supported by contract n° QLRT-2000-30058 of the project “Researching
25 alternatives to fish oils in aquaculture, RAFOA” of the European Commission, Directorate
26 General Fisheries as part of the Fifth Framework Programme “Quality of Life & Management
27 of Living Resources”. Joanne Good was supported in her doctoral studies by Ewos Innovation
28 AS, Norway.

30 References

- 31 1. Pike IH & Barlow SM (2002) Impact of fish farming on fish stocks, Aquaculture
32 and Environment Symposium, 7th Bordeaux Aquaculture, Bordeaux Exhibition
33 Centre, France, Book of Abstracts, p 6-7.

- 1 2. Tacon AGJ (2004) Use of fish meal and fish oil in aquaculture: a global
2 perspective. *Aquatic Resources, Culture & Development* **1**, 3-14.
- 3 3. Kaushik SJ, Coves D, Dutto G & Blanc, D (2004) Almost total replacement of fish
4 meal by plant protein sources in the diet of a marine teleost, the European sea
5 bass, *Dicentrarchus labrax*. *Aquaculture* **230**, 391-404.
- 6 4. Tacon AGJ (2005) Salmon aquaculture dialogue: Status of information on salmon
7 aquaculture feed and the environment. *Int Aquafeed* **8**, 22-37.
- 8 5. Bell JG, Henderson RJ, Tocher DR & Sargent JR (2004) Replacement of dietary
9 fish oil with increasing levels of linseed oil: modification of flesh fatty acid
10 compositions in Atlantic Salmon (*Salmo salar*) using a fish oil finishing diet.
11 *Lipids* **39**, 223-232.
- 12 6. Torstensen BE, Bell JG, Sargent JR, Rosenlund G, Henderson RJ, Graff IE, Lie Ø
13 & Tocher DR (2005) Tailoring of Atlantic salmon (*Salmo salar* L.) flesh lipid
14 composition and sensory quality by replacing fish oil with a vegetable oil blend. *J*
15 *Agric Food Chem* **53**, 10166-10178.
- 16 7. Caballero MJ, Obach A, Rosenlund G, Montero D, Gisvold M & Izquierdo MS
17 (2002) Impact of different dietary lipid sources on growth, lipid digestibility,
18 tissue fatty acid composition and histology of rainbow trout, *Oncorhynchus*
19 *mykiss*. *Aquaculture* **214**, 253-271.
- 20 8. Izquierdo MS, Obach A, Arantzamendi L, Montero D, Robaina L & Rosenlund G
21 (2003) Dietary lipid sources for seabream and sea bass: growth performance,
22 tissue composition and flesh quality. *Aquacult Nutr* **9**, 397-407.
- 23 9. Mourente G, Good JE & Bell JG (2005) Partial substitution of fish oil with
24 rapeseed, linseed and olive oils in diets for European sea bass (*Dicentrarchus*
25 *labrax* L.) effects on flesh fatty acid composition, plasma prostaglandins E₂ and
26 F_{2α}, immune function and effectiveness of a fish oil finishing diet. *Aquacul Nutr*
27 **11**, 25-40.
- 28 10. Blazer VS (1992) Nutrition and disease resistance in fish. *Ann Rev Fish Dis* **2**,
29 309-323.
- 30 11. Meade CJ & Mertin J (1978) Fatty acids and immunity. *Adv Lipid Res* **16**, 127-
31 165.
- 32 12. Calder PC (2001) Polyunsaturated fatty acids, inflammation and immunity. *Lipids*
33 36, 1007-1024.

- 1 13. Yaqoob P (2004) Fatty acids and the immune system: from basic science to
2 clinical applications. *Proc Nutr Soc* **63**, 89-104.
- 3 14. Clamp AG, Ladha S, Clark DC, Grimble RF & Lund EK (1997) The influence of
4 dietary lipids on the composition and membrane fluidity of rat hepatocyte plasma
5 membrane. *Lipids* **32**, 179-184.
- 6 15. Sargent JR, Tocher DR & Bell JG (2002) The lipids. In *Fish Nutrition*, 3rd ed., pp.
7 181-257 [JE Halver and RW Hardy, editors]. San Diego: Academic Press.
- 8 16. Thompson KD, Henderson RJ & Tatner MF (1995) A comparison of the lipid
9 composition of peripheral blood cells and head kidney leucocytes of Atlantic
10 salmon (*Salmo salar* L.). *Comp Biochem Physiol* (1995) **112B**, 83-92.
- 11 17. Bell JG, Dick JR, McVicar AH, Sargent JR & Thompson KD (1993) Dietary
12 sunflower, linseed and fish oils affect phospholipid fatty acid composition,
13 development of cardiac lesions, phospholipase activity and eicosanoid production
14 in Atlantic salmon (*Salmo salar*), *Prostaglandins Leukot Essent Fatty Acids* **49**,
15 665-673.
- 16 18. Bell JG, Ashton I, Secombes CJ, Weitzel BR, Dick JR & Sargent JR (1996)
17 Dietary lipid affects phospholipid fatty acid compositions, eicosanoid production
18 and immune function in Atlantic Salmon (*Salmo salar*). *Prostagandins Leukot*
19 *Essent Fatty Acids* **54**, 173-182.
- 20 19. De Pablo MA & De Cienfuegos GA (2000) Modulatory effects of dietary lipids on
21 immune system functions. *Immunol Cell Biol* **78**, 31-39.
- 22 20. Calder PC (2006) *n*-3 polyunsaturated fatty acids, inflammation, and inflammatory
23 diseases. *Am J Clin Nutr* **83**, 1505S-15019S.
- 24 21. Wootten, RJ (1990) Ecology of Teleost Fishes. Fish and Fisheries Series 1,
25 Chapman and Hall, Ashburton, UK. 404 pp.
- 26 22. Woyewoda AD, Shaw SJ, Ke PJ & Burns BG (1986) Recommended Laboratory
27 Methods for Assessment of Fish Quality. Canadian Technical Report of Fisheries
28 and Aquatic Sciences No. 1448.
- 29 23. Folch J, Lees LM & Sloane-Stanley GH (1957) A simple method for isolation and
30 purification of total lipids from animal tissues. *J Biol Chem* **226**, 497-509.
- 31 24. Christie WW (1982) Lipid Analyses, 2nd edition, Pergamon Press, Oxford, UK.
- 32
33

- 1 25. Mourente G & Tocher DR (1994) In vivo metabolism of [1-¹⁴C] linolenic acid
2 [18:3(*n*-3)] and [1-¹⁴C] eicosapentaenoic acid [20:5(*n*-3)] in a marine fish: time
3 course of the desaturation/elongation pathway. *Biochim Biophys Acta* **1212**, 109-
4 118.
- 5 26. Bell JG, Tocher DR, Macdonald FM & Sargent JR (1994) Effects of diets rich in
6 linoleic (18:2*n*-6) and α -linolenic (18:3*n*-3) acids on the growth, lipid class and
7 fatty acid compositions and eicosanoid production in juvenile turbot
8 (*Scophthalmus maximus* L), *Fish Physiol Biochem* **13**, 105-118.
- 9 27. Anderson DP & Siwicki AK (1994) Simplified assays for measuring non-specific
10 defense mechanisms in fish *Am Fish Soc Fish Health* section Spec Publ **12**, 26-35.
- 11 28. Rungruangsak-Torrissen K, Wergeland HI, Glette J & Waagbø R (1999) Disease
12 resistance and immune parameters in Atlantic salmon (*Salmo salar* L.) with
13 genetically different trypsin isozymes. *Fish Shellfish Immunol* **9**, 557-568.
- 14 29. Chung S & Secombes CJ (1988) Analysis of events occurring within teleost
15 macrophages during the respiratory burst. *Comp Biochem Physiol* **89B**, 539-544.
- 16 30. Secombes C.J (1990) Isolation of salmonid macrophages and analysis of their
17 killing activity, In *Techniques in Fish Immunology*, p. 137-154 [JS Stolen TC
18 Fletcher DP Anderson BS Robertson WB van Muiswinkel, editors]. New Jersey:
19 SOS Publications.
- 20 31. Zar JH (1984) Biostatistical Analysis, 2nd edition. Prentice-Hall, Englewood
21 Cliffs, NJ, USA.
- 22 32. Sheldon WM & Blazer VS (1991) Influence of dietary lipid and temperature on
23 bactericidal activity of channel catfish macrophages. *J Aquat Animal Health* **3**, 87-
24 93.
- 25 33. Obach A, Quentel C & Laurencin FB (1993) Effects of α -tocopherol and dietary
26 oxidized fish oil on the immune response of sea bass *Dicentrarchus labrax*. *Dis*
27 *Aquat Org* **15**, 175-185.
- 28 34. Waagbø R, Sandnes K, Joergensen J, Engstad R, Glette J & Lie Ø (1993) Health
29 aspects of dietary lipid sources and vitamin E in Atlantic salmon (*Salmo salar*): 2 -
30 spleen and erythrocyte phospholipid fatty acid composition, nonspecific immunity
31 and disease resistance. *Fiskeridir Skr (Ernaering)* **6**, 63-80.

- 1 35. Waagbø R, Hemre GI, Holm JC & Lie Ø (1995) Tissue fatty acid composition,
2 haematology and immunity in adult cod, *Gadus morhua* L., fed three dietary lipid
3 sources, *J Fish Dis* **18**, 615-622.
- 4 36. Erdal JI, Evensen O, Kaustad OK, Lillehaug A, Solbakken R & Thorud K (1991)
5 Relationship between diet and immune response in Atlantic salmon (*Salmo salar*
6 L) after feeding various levels of ascorbic acid and omega-3 fatty acids.
7 *Aquaculture* **98**, 363-379.
- 8 37. Waagbø R, Sandnes K, Lie Ø & Nilsen ER (1993) Health aspects of dietary lipid
9 sources and vitamin E in Atlantic Salmon (*Salmo salar*): 1 - erythrocyte total lipid
10 fatty acid composition, haematology and humoral immune response. *Fiskeridir*
11 *Skr (Ernaering)* **6**, 47-62.
- 12 38. Fracolossi DM & Lovell RT. (1994) Dietary lipid sources influence responses of
13 channel catfish (*Ictalurus punctatus*) to challenge with the pathogen *Edwardsiella*
14 *ictaluri*. *Aquaculture* **119**, 287-298.
- 15 39. Salte R, Thomassen MS & Wold K (1988) Do high levels of dietary
16 polyunsaturated fatty acids (EPA/DHA) prevent disease associated with
17 membrane degeneration in farmed Atlantic salmon at low water temperatures?
18 *Bull Eur Assoc Fish Path* **8**, 63-65.
- 19 40. Theis F, Miles EA, Nebe-von-Caron G, Powell JR, Hurst TL, Newsholme EA &
20 Calder PC (2001) Influence of dietary supplementation with long-chain *n*-3 or *n*-6
21 polyunsaturated fatty acids on blood inflammatory cell populations and functions
22 and on plasma soluble adhesion molecules in healthy adults. *Lipids* **36**, 1183-
23 1193.
- 24 41. Hwang D (1989) Essential fatty acids and immune response. *FASEB Journal*, **3**,
25 2052-2061.
- 26 42. Khan WA, Blobe GC & Hannun YA (1995) Arachidonic acid and free fatty acids
27 as second messengers and the role of protein kinase C. *Cell Signal* **7**, 171-184.
- 28 43. Calder PC, Bond JA, Harvey DJ, Gordon S & Newsholme EA. (1990) Uptake and
29 incorporation of saturated and unsaturated fatty acids into macrophage lipids and
30 their effect upon macrophage adhesion and phagocytosis, *Biochem J* **269**, 807-
31 814.
- 32 44. Montero D, Kalinowski T, Obach A, Robaina A, Tort L, Caballero, MJ &
33 Izquierdo MS (2003) Vegetable lipid sources for gilthead sea bream (*Sparus*
34 *aurata*): effects on fish health. *Aquaculture* **225**, 353-370.

- 1 45. Bell JG, Dick JR & Sargent JR (1993) Effect of diets rich in linoleic or α -linolenic
2 acid on phospholipid fatty Acid composition and eicosanoid production in Atlantic
3 salmon (*Salmo salar*), *Lipids* **28**, 819-826.
- 4 46. Bell JG, Farndale BM, Dick J.R & Sargent JR (1996) Modification of membrane
5 fatty acid composition, eicosanoid production, and phospholipase A activity in
6 Atlantic salmon (*Salmo salar*) gill and kidney by dietary lipid. *Lipids* **31**, 1163-
7 1171.
- 8 47. Leray C, Nonnotte G & Nonnotte L (1986) The effect of dietary lipids on the trout
9 erythrocyte membrane, *Fish Physiol Biochem* **1**, 27-35.
- 10 48. Johnston DV & Marshall LA (1984) Dietary fat, prostaglandins and the immune
11 response, *Prog Food Nutr Sci* **8**, 3-25.
- 12 49. Farndale BM, Bell JG, Bruce MP, Bromage NR, Oyen F, Zanuy S & Sargent JR
13 (1999) Dietary lipid composition affects blood leucocyte fatty acid compositions
14 and plasma eicosanoid concentrations in European sea bass (*Dicentrarchus*
15 *labrax*). *Aquaculture* **179**, 335-350.
- 16
17
18
19
20
21
22
23
24
25
26

1 List of Figures

2 Fig. 1. Effects of feeding diets containing fish oil (FO, diet A), or two 60% vegetable oil
3 blends (60% VO, diets B & C) on: (a) plasma PGE₂ concentration; (b) % haematocrit; (c)
4 total circulating leukocytes; (d) total circulating erythrocytes; (e) head kidney macrophage
5 activity (NBT reduction, measured as absorbance at 620 nm/10⁵ cells x 100); (f) serum
6 lysozyme activity. Values are mean ± SEM, n = 9. Columns assigned a different letter are
7 significantly different (P < 0.05).

8

9 Fig. 2. Histopathology of sea bass fed (a) Fish oil, distal intestine showing slight cellular
10 infiltration in the lamina propria and high levels of absorptive vacuoles (mag. x 175) (b)
11 60% VO diet C, distal intestine showing cellular infiltration but no sloughing of the
12 mucosal folds (mag. x 430).

1 **Table 1.** Formulation and proximate composition of 5mm experimental diets (g/kg feed)

2				
3	Diets	A	B	C
4				
5	Components			
6	Fish meal ^a	400.0	400.0	400.0
7	Maize gluten ^b	262.7	262.7	262.7
8	Wheat ^c	152.3	152.3	152.3
9	Oil	160.0	160.0	160.0
10	Premixes ^d	25.0	25.0	25.0
11				
12	Composition (%) of added oil			
13	Anchovy oil (FO) ^e	100	40	40
14	Rapeseed oil (RO) ^f	0	10	24
15	Linseed oil (LO) ^g	0	35	24
16	Palm oil (PO) ^h	0	15	12
17				
18	Proximate composition (%)			
19	Crude protein	47.8	46.2	47.8
20	Crude lipid	22.2	24.3	21.2
21	Carbohydrate	13.1	13.3	15.4
22	Ash	6.7	6.3	6.2
23	Moisture	10.2	10.9	9.4
24				

25 ^a Scandinavian LT-fish meal (Nordsildmel, Norway).

26 ^b Cargill, Staley, USA

27 ^c Statkorn, Oslo, Norway.

28 ^d Vitamin and mineral premix added exceed NRC (1993) recommendations.

29 ^e Anchovy oil (Denofa, Fredrikstad, Norway) supplemented with 200 ppm BHT.

30 ^f Crude rapeseed oil (Oelmühle Hamburg, Germany) no antioxidant added.

31 ^g Crude E.C.C. linseed oil (N.V. Oliefabriek Lictervelde, Belgium) supplemented with
32 500ppm Ronoxan A (Roche, Basel, Switzerland).

33 ^h Crude palm oil (Denofa, Norway).

1 **Table 2.** Total lipid content (% of dry mass) and fatty acid composition (weight % of total
 2 fatty acids) of the 5 mm experimental diets.

3				
4	Dietary treatments	A	B	C
5				
6	Total lipid (%)	20.2 ± 3.1	21.2 ± 4.3	21.1 ± 2.8
7	Fatty acid			
8	14:0	4.3 ± 0.1 ^a	1.9 ± 0.2 ^b	1.9 ± 0.2 ^b
9	15:0	0.5 ± 0.0	0.3 ± 0.1	0.6 ± 0.0
10	16:0	14.0 ± 0.2 ^a	13.3 ± 0.1 ^b	12.5 ± 0.1 ^c
11	18:0	3.0 ± 0.1 ^a	3.1 ± 0.1 ^a	2.7 ± 0.0 ^b
12	Total saturated ¹	22.9 ± 0.1 ^a	19.5 ± 0.2 ^b	18.6 ± 0.3 ^c
13	16:1 <i>n</i> -9/ <i>n</i> -7	13.6 ± 0.6 ^a	11.4 ± 1.9 ^b	10.2 ± 0.3 ^b
14	18:1 <i>n</i> -9	9.4 ± 0.2 ^c	16.9 ± 0.4 ^b	21.6 ± 0.4 ^a
15	18:1 <i>n</i> -7	2.2 ± 0.1 ^a	1.8 ± 0.0 ^c	2.0 ± 0.0 ^b
16	20:1 <i>n</i> -9	2.3 ± 0.0 ^a	1.9 ± 0.0 ^c	2.0 ± 0.0 ^b
17	22:1 <i>n</i> -11	2.0 ± 0.1 ^a	1.7 ± 0.1 ^b	1.7 ± 0.0 ^b
18	Total monoenes ²	30.7 ± 0.7 ^c	34.6 ± 1.5 ^b	38.1 ± 0.4 ^a
19	18:2 <i>n</i> -6	4.9 ± 0.0 ^c	9.0 ± 0.7 ^b	10.4 ± 0.2 ^a
20	20:4 <i>n</i> -6	0.6 ± 0.0 ^a	0.3 ± 0.0 ^b	0.3 ± 0.0 ^b
21	Total <i>n</i> -6	7.4 ± 0.1 ^c	10.7 ± 0.5 ^b	11.8 ± 0.2 ^a
22	18:3 <i>n</i> -3	1.5 ± 0.0 ^c	12.3 ± 0.7 ^a	10.3 ± 0.3 ^b
23	18:4 <i>n</i> -3	2.0 ± 0.0 ^a	1.1 ± 0.1 ^b	1.0 ± 0.0 ^b
24	20:4 <i>n</i> -3	0.5 ± 0.0 ^a	0.3 ± 0.0 ^b	0.3 ± 0.0 ^b
25	20:5 <i>n</i> -3	9.8 ± 0.1 ^a	5.3 ± 0.3 ^b	5.2 ± 0.1 ^b
26	22:5 <i>n</i> -3	1.2 ± 0.0 ^a	0.7 ± 0.0 ^b	0.6 ± 0.0 ^b
27	22:6 <i>n</i> -3	11.0 ± 0.3 ^a	6.3 ± 0.5 ^b	6.2 ± 0.1 ^b
28	Total <i>n</i> -3	27.9 ± 0.5 ^a	27.0 ± 1.7 ^{ab}	24.5 ± 0.6 ^b
29	Total PUFA ³	35.4 ± 0.6	37.8 ± 2.1	36.3 ± 0.9
30	<i>n</i> -3/ <i>n</i> -6	3.8 ± 0.5 ^a	2.5 ± 0.5 ^b	2.1 ± 0.5 ^b

31
 32 Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. ¹Includes 20:0
 33 and 22:0. ²Includes 18:1*n*-11, 20:1*n*-11, 20:1*n*-7 22:1*n*-9 and 24:1. ³Includes 16:2, 16:3,
 34 16:4, 20:2*n*-6, 20:3*n*-6 and 22:5*n*-6. Values bearing different superscript letter are
 35 significantly different (P<0.05). nd, not detected.

1 **Table 3.** Effect of partial replacement (60 %) of dietary fish oil (FO) with vegetable oils
 2 (rapeseed oil, RO; linseed oil, LO and palm oil, PO) on growth and performance of
 3 European sea bass (*Dicentrarchus labrax*, L.) fed experimental diets for 64 weeks

4	Dietary treatment	Initial	A*	B	C
5					
6					
7	<u>64 weeks</u>				
8	<u>(final sampling point)</u>				
9					
10	Fish length (cm)	7.9 ± 0.5	24.9 ± 1.3	23.2 ± 1.4	24.1 ± 1.4
11	Fish live mass (g)	5.2 ± 1.0	176.2 ± 32.9 ^a	143.2 ± 29.4 ^b	159.8 ± 34.2 ^{ab}
12	HSI ¹	1.4 ± 0.2	2.0 ± 0.3	1.9 ± 0.2	2.1 ± 0.3
13	Flesh dry mass (%)		22.9 ± 1.7 ^b	21.3 ± 1.5 ^b	26.0 ± 1.4 ^a
14	FCR (feed/gain) ²		1.2 ± 0.3	1.6 ± 0.4	1.3 ± 0.3
15	Condition factor (K) ³		1.14 ± 0.2	1.15 ± 0.3	1.14 ± 0.2
16	SGR ⁴		0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.1
17					

18
 19 Data are mean ± SD (n = 90) for fish length and weight and (n = 30) for liver mass, HSI
 20 and flesh mass. Values in the same row assigned different superscript letters are
 21 significantly different (P<0.05). ¹ Hepato Somatic Index. ² FCR = Food Conversion Ratio.
 22 ³ Condition Factor, K = (W/L³)*100. ⁴ Specific Growth Rate (SGR) = (LnW₁-LnW₀) x
 23 100/t. * Control diet where W = weight (g), L = length in cm, W₀ = initial weight in g,
 24 W₁ = final weight in g and t = time in days.

25

1 **Table 4.** Total lipid content (% of dry mass) and total lipid fatty acid composition (weight
2 % of total fatty acids) of liver from European sea bass (*Dicentrarchus labrax*) fed the
3 experimental diets for 64 weeks.

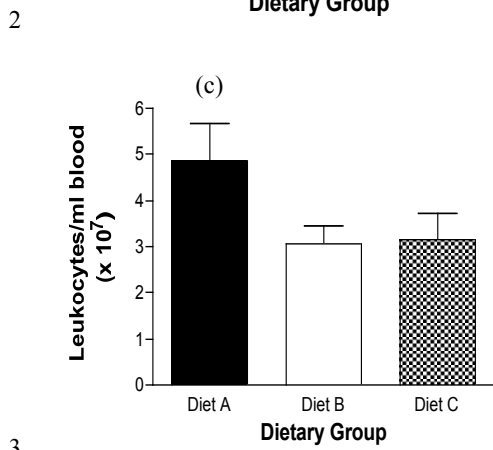
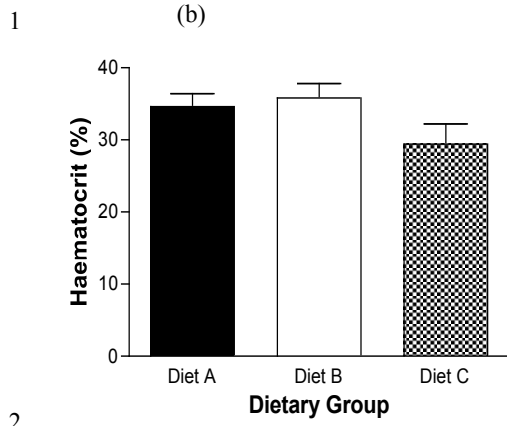
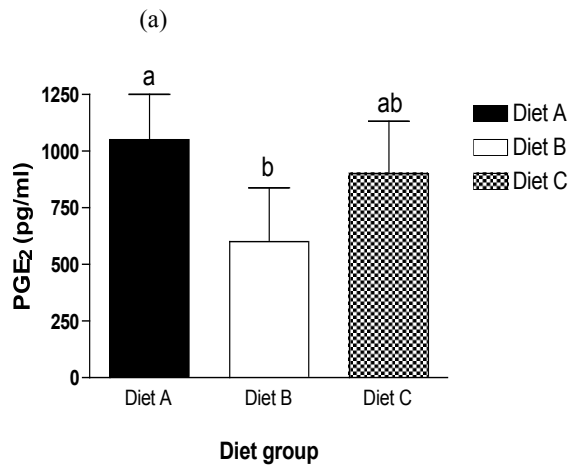
4	Dietary treatments	A*	B	C
5	Total lipid (%)	54.8 ± 7.6	52.9 ± 0.9	54.7 ± 5.8
6	Fatty acid (%)			
7	14:0	1.8 ± 0.3 ^a	1.3 ± 0.2 ^{ab}	1.0 ± 0.1 ^b
8	15:0	0.2 ± 0.0 ^a	0.2 ± 0.0 ^a	0.1 ± 0.0 ^b
9	16:0	16.6 ± 2.4	16.1 ± 2.1	17.3 ± 2.1
10	18:0	3.1 ± 0.7	3.7 ± 0.2	3.4 ± 0.7
11	Total saturated ¹	22.4 ± 3.1	21.8 ± 2.5	22.5 ± 2.9
12	16:1n-9/n-7	8.8 ± 1.0 ^a	6.0 ± 1.0 ^b	6.1 ± 0.2 ^b
13	18:1n-9	28.8 ± 2.0 ^b	34.1 ± 2.2 ^{ab}	39.2 ± 2.3 ^a
14	18:1n-7	3.4 ± 0.4 ^a	2.5 ± 0.2 ^b	2.7 ± 0.2 ^{ab}
15	20:1n-9	2.1 ± 0.1	2.0 ± 0.2	2.1 ± 0.3
16	22:1n-11	0.7 ± 0.0	0.6 ± 0.0	0.5 ± 0.1
17	Total monoenes ²	44.7 ± 1.2 ^b	46.1 ± 1.5 ^b	51.3 ± 1.6 ^a
18	18:2n-6	3.3 ± 0.3 ^b	6.4 ± 1.5 ^a	6.2 ± 1.3 ^a
19	20:4n-6	0.5 ± 0.1 ^a	0.3 ± 0.1 ^{ab}	0.2 ± 0.0 ^b
20	Total n-6	4.9 ± 0.5	7.6 ± 1.4	7.2 ± 1.5
21	18:3n-3	0.8 ± 0.2 ^b	7.5 ± 2.8 ^a	5.1 ± 1.2 ^a
22	18:4n-3	1.0 ± 0.1 ^a	0.9 ± 0.1 ^{ab}	0.7 ± 0.1 ^b
23	20:4n-3	0.5 ± 0.0 ^a	0.3 ± 0.1 ^{ab}	0.2 ± 0.0 ^b
24	20:5n-3	6.2 ± 0.7 ^a	3.2 ± 1.0 ^b	2.5 ± 0.6 ^b
25	22:5n-3	1.2 ± 0.1 ^a	0.6 ± 0.2 ^b	0.4 ± 0.1 ^b
26	22:6n-3	10.2 ± 1.6 ^a	5.4 ± 1.8 ^b	4.2 ± 0.9 ^b
27	Total n-3	21.1 ± 2.5 ^a	19.3 ± 0.5 ^{ab}	13.8 ± 2.9 ^b
28	Total PUFA ³	26.0 ± 2.9	27.0 ± 1.1	21.1 ± 4.4
29	n-3/n-6	4.3 ± 0.8 ^a	2.5 ± 0.7 ^b	1.9 ± 0.9 ^b
30				
31				
32				

33 Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. ¹Includes 20:0 and
34 22:0. ²Includes 18:1n-11, 20:1n-11, 20:1n-7 22:1n-9 and 24:1. ³Includes 16:2, 16:3, 16:4,
35 20:2n-6, 20:3n-6 and 22:5n-6. Values bearing different superscript letter are significantly
36 different (P<0.05). nd, not detected. * Control diet.

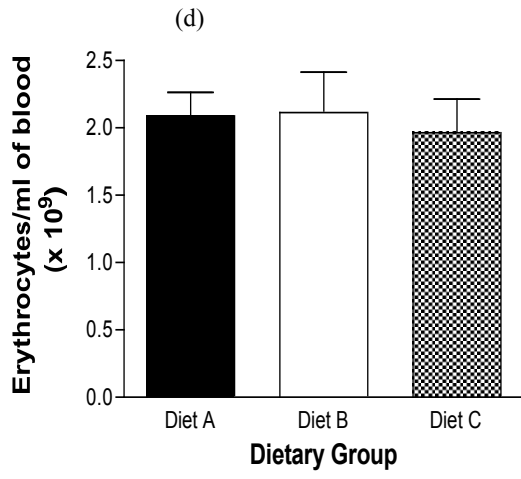
Table 5. Total lipid fatty acid composition (weight % of total fatty acids) of peripheral blood leukocytes from European sea bass (*Dicentrarchus labrax*) fed the experimental diets for 64 weeks.

Dietary treatments/Fatty acid	A*	B	C
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
Total saturated ¹	23.5 ± 1.8	24.0 ± 0.7	23.1 ± 0.7
16:1 $n-9/n-7$	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
22:1 $n-11$	0.9 ± 0.2	1.0 ± 0.3	1.0 ± 0.3
Total monoenes ²	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
20:4 $n-6$	1.2 ± 0.1 ^a	0.7 ± 0.1 ^b	0.7 ± 0.1 ^b
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 ³	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

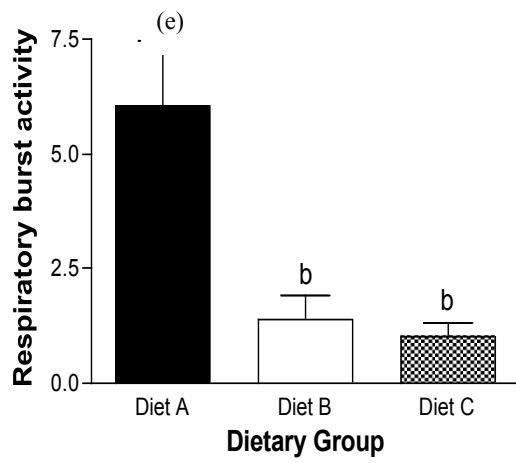
Results are means ± SD (n = 3). An SD of 0.0 implies an SD of < 0.05. ¹Includes 20:0 and 22:0. ²Includes 18:1 $n-11$, 20:1 $n-11$, 20:1 $n-7$ 22:1 $n-9$ and 24:1. ³Includes 16:2, 16:3, 16:4, 20:2 $n-6$, 20:3 $n-6$ and 22:5 $n-6$. Values bearing different superscript letter are significantly different (P<0.05). nd, not detected. * Control diet.



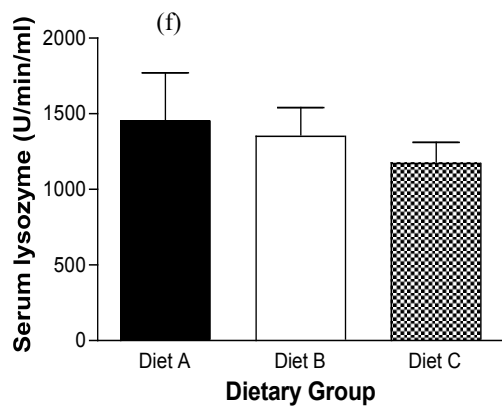
3
4 Fig. 1



1

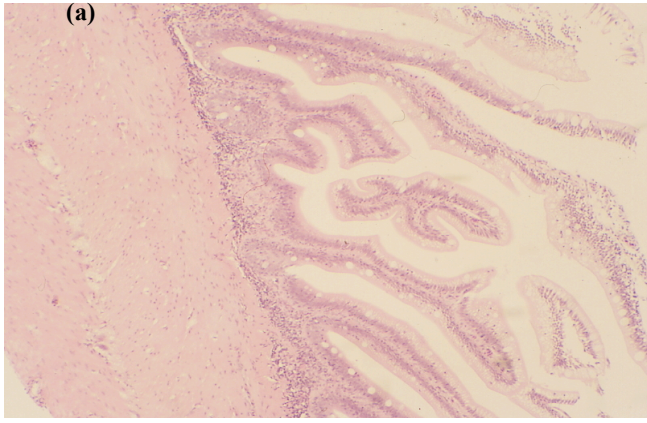


2

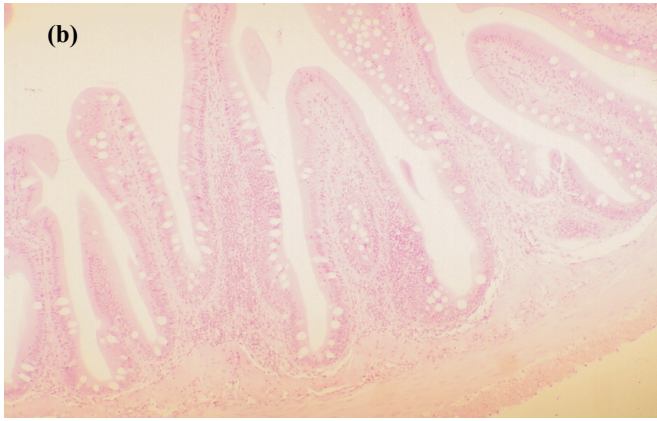


3

4 Fig. 1



1
2



3
4
5
6
7

8 Fig. 2