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Effect of dietary lipids on macrophage function, stress susceptibility and disease resistance in Atlantic salmon (*Salmo salar*)

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Abstract

As the supply of marine fish oil is becoming a limiting factor in the production of Atlantic salmon (*Salmo salar*), new diets and alternative sources of energy are being tested. Plant oils are natural potential candidates to replace fish oil, but the different levels of essential polyunsaturated fatty acids may influence the health and growth of salmon. In this study, we have investigated the resistance to transport stress and bacterial infection, phagocytic activity in head kidney macrophages and eicosanoid metabolism in salmon fed three different diets. In high-energy fishmeal based diets, 50% and 100% of the supplementary fish oil (FO) was replaced with soybean oil (SO). The three dietary groups were fed for 950 day-degrees at 5 °C (27 weeks) and 12 °C (11 weeks) before challenging the fish with *Aeromonas salmonicida*, analyzing the lipid composition of head kidney and examining macrophage function *in vivo* and *in vitro*. Dietary fatty acids affected the lipid composition of the kidney. The level of eicosanoid precursor's 20:4n-6 and 20:3n-6 were 3 and 7-fold higher in the 100% SO group compared with the FO group. The total fraction of n-3 lipids in kidney was 19% in the SO group, compared to 16% and 12% in the 50% or 100% SO groups, respectively. However, the production of leucotriene B_4 (LTB) and prostaglandin E_2 (PGE) immunoreactive materiel from exogenously added arachidonic acid in head kidney macrophages was only affected by the composite diet (increased) at 5 °C. In addition, the phagocytic activity of kidney macrophages *in vivo* and *in vitro* was not affected by diet. No effect of diet was observed on transport stress or susceptibility to a bacterial infection with *Aeromonas salmonicida*. Atlantic salmon therefore seems to tolerate a diet solely based on soybean oil as lipid source, without any detrimental effects on growth, health and immune functions.

Introduction

The importance of a well balanced diet for good growth and health in cultured fish has been recognized for many years. Traditionally, commercial diets for Atlantic salmon have been based on raw materials similar to the natural food of this species (e.g. herring, capelin). They contain fish oil and therefore are rich in polyunsaturated fatty acids of the n-3 type, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). However fish oils may be a limiting factor for the growth of the industry in the coming years. It is therefore of interest to

evaluate the suitability of other dietary lipid sources, e.g. vegetable oils. The major fatty acids in plant oils are the 18C fatty acids, oleic acid (18:1*n*-1), linoleic (18:2*n*-6) or linolenic acid (18:3n-3), depending on the type of vegetable oil. Changes in the lipid composition from a (n-3) to a (n-6) enriched diet can influence the composition of the cell membranes in the fish (Bell et al. 1991, 1997; Røsjø et al. 1994). This in turn may change the activity of membrane bound enzymes, receptors, ion-channels. Several studies have documented a modulatory role of dietary lipids on immune responses in both experimental animals and humans (Calder 1999; Thies et al. 2001). These effects of lipids on the immune system may be explained by changes in the eicosanoid biosynthesis (Hwang 1989), intracellular signaling pathways (Khan et al. 1995) or lipid regulated transcription factors like PPAR's (Cunard et al. 2002). In addition, changes in membrane lipids affect the biophysical properties of membranes. However, few studies have focused on how the endocytic process in poikilothermic animals such as fish, is affected by changing temperatures or membrane lipids (Løvhøiden et al. 1994; Røsjø et al. 1994; Lappova and Leibush 1995; Rode et al. 1997). However, it has been established that membrane lipids are remodeled upon temperature changes, presumably to maintain membrane fluidity (Hazel 1984, 1988). In one report, degradation of endocytosed protein in isolated char kidney cells was arrested by low temperature, conceivably by inhibition of ligand transfer from early to late endosomes (Dannevig and Berg 1985). This effect has also been observed in rainbow trout liver cells (Kindberg et al. 1991; Løvhøiden et al. 1994).

Changes in the lipid composition of fish membranes may either be induced by temperature changes (homoviscous adaptation) or by diet. Rapid changes in water temperature have been shown to influence immunological function in fishes. Both specific (Bly and Clem 1991) and nonspecific (Ainsworth et al. 1991) defense mechanisms were influenced by a change in temperature. A rapid decrease in temperature suppressed both T- and B-cell function (proliferation and antibody production) in channel catfish (Bly and Clem 1991). Phagocytosis of bacteria in catfish macrophages was also suppressed at low temperature (Ainsworth et al. 1991). Some of these phenomena may be due to altered membrane dynamics in the affected cells.

Macrophages are prime producers of arachidonic acid metabolites like prostaglandins and leukotrienes, which possess modulating activity on the immune system. A number of studies have shown that dietary lipids may influence the production of these important modulators in the immune system of both mammals (Brouard and Pascaud 1990) and fish (Bell et al. 1996a).

In this study, Atlantic salmon were fed with three different diets, one based purely on marine oils (with an n-6/n-3 ratio = 0.2), one diet based purely on soybean oil (with an n-6/n-3 ratio = 4.2) and one diet composed of a 50/50 mixture of these two diets (with an n-6/n-3 ratio = 1.6). The fishes were reared at two different temperatures 5 and 12 °C for 27 and 11 weeks, respectively. When the fish reached an average weight of 300 g, the macrophage function was assessed in experimental fish. Phagocytosis and degradation of protein particles was examined *in vivo* and *in vitro*, eicosanoid production by macrophages was measured *in vitro*. Finally, the effects of transport stress and an experimental challenge test with *A. salmonicida* was performed on the different dietary groups.

Materials and methods

Chemicals

Carrier free $Na^{125}I$ with a specific activity of 644 MBq per μg I was obtained from the Institute for Energiteknikk, Kjeller, Norway. Tyramine cellobiose was kindly donated by Dr. Helge Tolleshaug, Nycomed AS, Oslo. Nycodenz and heparin was obtained from Nycomed, Oslo, Norway. Bovine serum albumin (BSA), trichloroacetic acid (TCA), culture media (Leibowitz-15) and arachidonic acid were obtained from Sigma (St.Louis USA). L-15, streptomycin, penicillin were from Bio Whittaker (Walkersville, USA). Percoll was from Pharmacia, Sweden. Albures® micro spheres (colloidal human serum albumin nanoparticles) were from Solco Nuclear, USA. Prostaglandin E_2 (PGE_2) and leucotriene B_4 (LTB_4) assay kits were obtained from R and D Systems Inc. (Minneapolis, USA).

Fish

A total of 720 Atlantic salmon (*Salmo salar l*) with average initial weight of 113 were randomly allocated to 18 cylindro-conical tanks (0.75 m diameter, app. volume 250 l), supplied with seawater with constant temperature of either 5 or z12 °C. Three dietary groups were randomly assigned to triplicate tanks at each of the two temperatures. The length of the trial was set to give the same number of day-degrees for the fish at each temperature (about 3 months for the 12 °C fish and 7 months for the 5 °C fish).

Diets

The diets, provided by Nutreco Aquaculture Research Center (4 mm extruded pellets) were fish meal based and differed only in the type of added oil, 100% capelin oil (Diet 1), 100% crude soybean oil (Diet 3) or a 50/50 mixture of the two oils (Diet 2). The fatty acid profile of the experimental diets is given in Table 1. A detailed description of the diet production and composition is given by Grisdale-Helland et al. (2002).

Labeling of Albures® particles with ^{125}I

Albures® particles (0.1 mg) were labeled with ^{125}I-tyramine-cellobiose according to the method originally described by Pittman et al. (1983). A specific activity of around 100 cpm/ng protein was obtained by this method. For the degradation studies *in vitro*, Albures particles were labeled directly with ^{125}I according to the method described by Redshaw and Lynch (1974) all radioactivity analyses were made in a Packard Cobra Auto gamma counter with 72% counting efficiency.

Degradation of injected micro spheres in vivo

To determine the phagocytic activity and protein degradation *in vivo*, 15 salmon from each of the three feeding groups were injected with 2 μg (200,000 cpm) TC labeled Albures micro spheres in PBS. 3, 6 and 24 h after injection, the five fish per diet were anaesthetized, weighed and the kidney dissected out. After weighing, the kidney was cut in small pieces and homogenized in a Dounce homogenizer. A sample of the homogenate was precipitated with 10% TCA and the percentage acid soluble radioactivity determined.

Isolation and cultivation of macrophages

Six fish from each dietary group at each temperature were sedated with MS-222, and heparin (1000 IE/kg fish) was injected into the caudal vein to prevent blood coagulation. The head kidney was then removed aseptically, and passed through a 100 μm nylon mesh using a modified L-15 medium containing 100 units/ml penicillin, 0.1 mg/ml streptomycin and 4 mM L-glutamine. For this step of the isolation heparin (40 units/ml) and 2% FCS was also added. Three to four millilitre of the cell suspension was then placed on top of a 37/54% Percoll gradient and centrifuged at 600 g for 40 min at 4 °C. The 37% Percoll solution had been prepared in phosphate buffered saline (PBS; 0,15 M NaCl, 20 mM Na_2HPO_4, pH 7,4), and the 54% Percoll solution in L-15. The band of cells between the Percoll layers was collected and washed (by centrifugation at 400 g for 10 min) at 4 °C in 20 mM PBS containing 2% BSA and heparin (40 units/ml). The cells were then resuspended in L-15 containing 0.1% FCS at a concentration of 2×10^6 cells/ml and seeded in 25 cm^2 Falcon flasks. Four hours after plating the nonadherent cells were removed by two washes in PBS.

Degradation of ^{125} I-Albures® in cultured macrophages

About 1 μg ^{125}I-labelled Albures was added to the 25-cm^2 flasks containing the macrophages. After 0, 3, 6 and 24 h, duplicate 0.5 ml samples were taken from each flask and mixed with 0.1 ml 10% BSA before precipitating with 0.5 ml 10% TCA to determine the level of degradation. After 30 min on ice, the samples were centrifuged at 3000 *g* for 10 min and radioactivity measured in both the supernatant and pellet. Three control flasks without cells were included to measure cell independent degradation. Total cell protein in each flask was also analyzed as described by Bradford (1976).

PGE_2 and LTB_4 analysis

The macrophages were washed in a medium without FCS 12 h after plating, and L-15 medium containing 150 uM arachidonic acid (20:4n-6) was added to the flasks that were then incubated for 24 h. The medium was centrifuged at

Table 1. Fatty acid composition of the diets (% of total fatty acid content)

	100% fish oil Diet 1	50/50 Diet 2	100% soybean oil Diet 3
C14:0	5.7	3.1	0.9
C16:0	14.0	13.2	12.7
C18:0	1.6	2.7	3.7
Saturated not listed	0.4	0.7	0.9
C16:1n-7	7.5	4.2	1.3
C18:1n-7	2.8	2.2	1.6
C18:1n-9	10.3	15.4	20.3
C20:1 (sum isomers)	12.8	6.8	1.1
C22:1 (sum isomers)	15.9	8.4	1.0
C24:1n-9	0.7	0.5	0.3
Monoenes not listed	0.5	0.3	0.0
C18:3n-3	0.8	3.1	5.3
C18:4n-3	3.2	1.8	0.5
C20:4n-3	0.4	0.2	NQ
C20:5n-3 (EPA)	9.0	5.5	2.1
C22:5n-3	0.6	0.4	0.2
C22:6n-3 (DHA)	7.0	4.9	2.8
n-3 not listed	0.3	0.2	0.0
C16:2n-6	0.5	0.3	NQ
C18:2n-6	4.4	25.3	45.4
C20:4n-6	0.2	0.1	NQ
n-6 not listed	0.2	0.0	0.0
Others	1.4	0.6	0.2
Sum saturated	21.6	19.6	18.2
Sum monoenes	50.5	37.9	25.4
Sum n-6	5.3	25.7	45.4
Sum n-3	21.3	16.2	10.9
n-6/n-3 ratio	0.2	1.6	4.2

NQ: not quantified.

3500 rpm (2500 *g*) and 100 μl cell-free medium were immediately analysed for PGE_2 and LTB_4 according to the protocol by R&D Systems (Minneapolis, USA). Shortly, the assay is based on the competitive binding ELISA technique in which PGE_2 or LTB_4 present in the sample competes with a fixed amount of alkaline phosphatase-labelled PGE_2 or LTB_4 for sites on a mouse monoclonal antibody. During the incubation, the mouse antibody binds to the goat anti-mouse antibody coated to the wells of a 96 well microtiterplate. Following washing to remove excess conjugate and unbound sample, a substrate solution is added to the wells. The colour development is stopped and the absorbance read at 405 nm. The intensity of the colour is inversely proportional to the concentration of PGE_2 or LTB_4in the sample.

Lipid extraction and fatty acid analysis

Total lipids were extracted from whole kidney tissue using the method described by Folch et al. The solutions used for lipid extraction contained 2,6-di (tert-butyl)-*p*-cresol (50 mg/l) as an antioxidant, and the lipid extracts were stored under nitrogen in the dark at −50 °C to prevent the oxidation of unsaturated fatty acids. The chloroform phase produced by Folch extraction was dried under nitrogen and dissolved in hexane. The total fatty acid composition of hepatocytes was determined basically as described by Røsjø et al. The methyl esters of fatty acids were separated in a gas chromatograph (Perkin–Elmer Auto system GC equipped with a auto injector, programmable split/split less injector) with a CP Wax 52 column (L = 25 m, ID = 0.25 mm, df = 0.2 μm), flame

ionisation detector and 1022 data system. The carrier gas was He, and the injector and detector temperatures were 280 °C. The oven temperature was raised from 50 to 180 °C at the rate of 10 °C/min, and then raised to 240 °C at the rate of 0.7 °C/min. The relative quantity of each fatty acid present was determined by measuring the area under the peak corresponding to a particular fatty acid.

Determination of protein concentration in the macrophages

When harvesting the cells, culture flasks were placed on ice and scraped with a cell scraper in 2×2 ml ice-cold potassium phosphate buffer (50 mM). The cell suspension was centrifuged at 840 *g* for 5 min and the supernatant removed. The resulting cell pellet was re-suspended in a total volume of 500 μl phosphate buffer. Protein was determined by the method of Bradford (1976).

Transportation stress

The effects of stress caused by transportation were assessed at the end of the feeding period. Fish from the three dietary groups maintained at 12 °C were marked by fin clipping, and then transported by truck from the Akvaforsk facilities in Sunndalsøra to VESO Vikan AkvaVet in Namsos. Groups of 120 fish (average weight 320 *g*) from each dietary treatment were placed in dark plastic tanks with a capacity of 2.4 m^3 (density 16 kg/m^3). The tanks contained seawater at 12 °C. The temperature was monitored at regular intervals during the transportation. Air-diffusers guaranteed a good aeration throughout the transport. The duration of the transport was 12 h. Upon arrival, fish were immediately transferred to a 2 m^3 fibre glass tank and the mortalities collected and recorded.

Challenge test with Aeromonas salmonicida

One week after arrival at VESO Vikan AkvaVet, a pre-challenge test was carried out with some of the transported fish (see above) in order to test the challenge model and to determine the infectious dose required to achieve a percentage of mortalities of 50–70%. The pre-challenge demonstrated that in the present challenge system no secondary mortalities occurred during a 15 days post-challenge observation period due a water born infection set up by the test fish themselves. The challenge dose selected according to the results of the pre-challenge was 10^4 CFU/fish. The actual challenge test was carried out 2 weeks later, according to the methodology described by Nordmo (1997). The fish were anaesthetised and challenged with *Aeromonas salmonicida*(ino 3175/88) by intra-peritoneal injection. Dead fish were removed, weighed and registered daily. Fish were observed for mortalities over a 2-week period. Samples were taken from all the dead fish and analysed bacteriologically to establish specific mortalities due to *A. salmonicida*. The water temperature was kept constant at 10 °C throughout the study. The photoperiod was 12 h light and 12 h darkness.

Statistics

The data were analysed by two-way ANOVA for the factors diet and water temperature. Significant differences between means were analysed using Duncan's test in the software package UNISTAT. The significance levels (p-values) from the statistical tests are presented together with means and standard error of means (SEM) for each variable. The significance level was set at 5%.

Results

Atlantic salmon fed the three different diets grew well and reached final weights of about 320 and 350 *g* in the 12 °C group and 5 °C groups respectively. There was no difference in mortalities that occurred in the groups over the experimental period.

Fatty acid composition of total lipids in head kidney

The fatty acid composition of head kidney was significantly affected by dietary fatty acids (Table 2). Atlantic salmon fed the Diet 1 containing 100% fish oil had only 6% 18:2n-6 in head kidney lipids, while head kidney lipids from fish fed Diets 2 and 3 supplemented with either 50 or 100% soybean oil, contained approximately 16% and 27% 18:2n-6, respectively. The percentage of the desaturation and elongation products from 18:2n-6, the eicosanoid precursors 20:4n-6 and 20:3n-6, were between 3 and 7- fold higher in head

Table 2. Total fatty acid composition of head kidney (mean ± sem)

	5 °C			12 °C		
	Diet 1	Diet 2	Diet 3	Diet 1	Diet 2	Diet 3
C14:0	4.9 ± 0.1^{c}	3.4 ± 0.1^{b}	2.17 ± 0.03^{a}	4.9 ± 0.01^{c}	2.2 ± 0.1^{a}	2.2 ± 0.1^{a}
C16:0	16.7 ± 0.03^{c}	15.6 ± 0.1^{a}	15.5 ± 0.17^{a}	15.5 ± 0.02^{a}	17.7 ± 0.01^{d}	16.2 ± 0.17^{b}
C16:1n-7	6.4 ± 0.25^{c}	4.6 ± 0.02^{b}	2.8 ± 0.05^{a}	6.7 ± 0.01^{c}	4.6 ± 0.01^{b}	2.6 ± 0.2^{a}
C18:0	2.6 ± 0.05^{a}	3.3 ± 0.2^{b}	4.1 ± 0.06^{d}	2.6 ± 0.01a	3.9 ± 0.01^{c}	4.4 ± 0.04^{e}
C18:1n-9	14.8 ± 0.75^{a}	16.4 ± 0.43^{ab}	18.7 ± 0.53^{c}	15.1 ± 0.02^{a}	16.85 ± 0.00^{b}	17.8 ± 0.31^{bc}
C18:1n-7	3.99 ± 0.18^{c}	2.9 ± 0.00^{ab}	2.5 ± 0.03^{a}	3.8 ± 0.00^{c}	3.1 ± 0.00^{b}	2.6 ± 0.15^{a}
C18:2n-6	6.0 ± 0.65^{a}	17.6 ± 0.53^{b}	27.4 ± 1.53^{c}	5.8 ± 0.00^{a}	15.4 ± 0.01^{b}	26.7 ± 0.51^{c}
C18:3n-3	1.0 ± 0.1	N.D.	N.D.	N.D.	N.D.	N.D.
C18:4n-3	1.9 ± 0.10^{d}	1.4 ± 0.01^{c}	0.9 ± 0.04^{ab}	2.1 ± 0.0^{e}	1.1 ± 0.00^{b}	0.9 ± 0.02^{a}
C20:1n-9	8.8 ± 0.01^{d}	6.5 ± 0.00^{c}	3.4 ± 0.12^{a}	9.9 ± 0.00^{e}	5.7 ± 0.00^{b}	3.2 ± 0.08^{a}
C20:2n-6	0.4 ± 0.02^{a}	1.1 ± 0.00^{b}	1.6 ± 0.04^{c}	0.4 ± 0.00^{a}	1.1 ± 0.00^{b}	1.5 ± 0.07^{c}
C20:3n-6	0.2 ± 0.01^{a}	0.6 ± 0.00^{a}	1.4 ± 0.28^{b}	N.D.	0.6 ± 0.00^{a}	1.2 ± 0.15^{b}
C20:4n-6	0.4 ± 0.04^{b}	0.5 ± 0.04^{b}	0.9 ± 0.08^{d}	0.3 ± 0.00^{a}	0.6 ± 0.00^{c}	0.9 ± 0.02^{d}
C20:4n-3	0.8 ± 0.03^{d}	0.9 ± 0.04^{d}	0.5 ± 0.02^{a}	0.8 ± 0.00^{d}	0.7 ± 0.02^{c}	0.6 ± 0.00^{b}
C20:5n-3 (EPA)	5.5 ± 0.24^{c}	4.3 ± 0.20^{b}	2.8 ± 0.23^{a}	5.5 ± 0.01^{c}	4.4 ± 0.02^{b}	3.0 ± 0.14^{a}
C22:1n-11	8.7 ± 0.35^{c}	5.7 ± 0.03^{b}	3.0 ± 0.33^{a}	10.3 ± 0.01^{d}	5.6 ± 0.03^{b}	2.4 ± 0.27^{a}
C22:6n-3 (DHA)	9.8 ± 1.21^{b}	8.5 ± 0.55^{ab}	7.2 ± 0.8^{ab}	8.2 ± 0.00^{b}	9.1 ± 0.00^{ab}	7.6 ± 0.14^{a}
Sum n-6	6.9 ± 1.21^{a}	19.7 ± 0.45^{b}	31.3 ± 1.7^{c}	6.4 ± 0.00^{a}	17.7 ± 0.00^{b}	30.3 ± 0.68^{c}
Sum n-3	19.6 ± 1.56^{b}	16.0 ± 0.1^{ab}	12.4 ± 1.26^{a}	19.1 ± 0.01^{ab}	16.5 ± 0.03^{ab}	12.8 ± 0.1^{a}

^{ab}Values marked with different superscripts are significantly different.

kidney from fish fed the 100% soybean oil diet than in cells from fish fed the pure fish oil diet.

Percentages of the long chain n-3 fatty acids 20:5 and 22:6 decreased from approximately 15% in head kidney from fish fed pure fish oil diet to 10% in head kidney from salmon fed the 100% soybean oil diet. There were no substantial differences in the fatty acid composition of head kidney from fish kept at 5 or 12 °C.

Uptake and degradation of Albures micro spheres in vivo

When labelled Albures microspheres were injected intravenously into salmon, more than 50% of the injected dose was recovered in the kidney (results not shown). Statistical analysis of the data from the different dietary groups held at 5 and 12 degrees did not reveal any difference in percentage uptake of the micro spheres when analysed at the different time points after injection. The fatty acid composition of the diet did therefore not seem to affect the phagocytic capacity of the kidney cells under the experimental conditions used here.

The degradation of phagocytosed microspheres in salmon head kidney was appreciable more sensitive to temperature than the uptake of particles. At 24 h after injection, the degradation was 13.7 ± 0.6 and 10.2 ± 0.7% of total uptake in the 12 and 5 °C groups (all diets) respectively. There was no significant difference between the three dietary groups (Figure 1a and b).

Phagocytosis and degradation of microspheres in vitro

To test macrophage function, head kidney macrophages were isolated from six fish sampled from each dietary group maintained either at 5 or 12 °C and were cultured at their respective temperatures. After 4 h in culture the cells were washed and labelled microspheres added. Figure 2a and b shows the kinetics of protein degradation at 5 and 12 °C, respectively. There was no significant effect of diet on this activity at either 5 or 12 °C. However, by combining the data from all dietary groups, an effect of temperature on this activity could be observed. When the average rate of degradation was calculated by linear regression, it was found that at 5 °C, 0.26 μg Albures/mg protein/h was degraded (r^2 = 0.58), whereas at 12 °C the corresponding value was 0.42 (r^2 = 0.89).

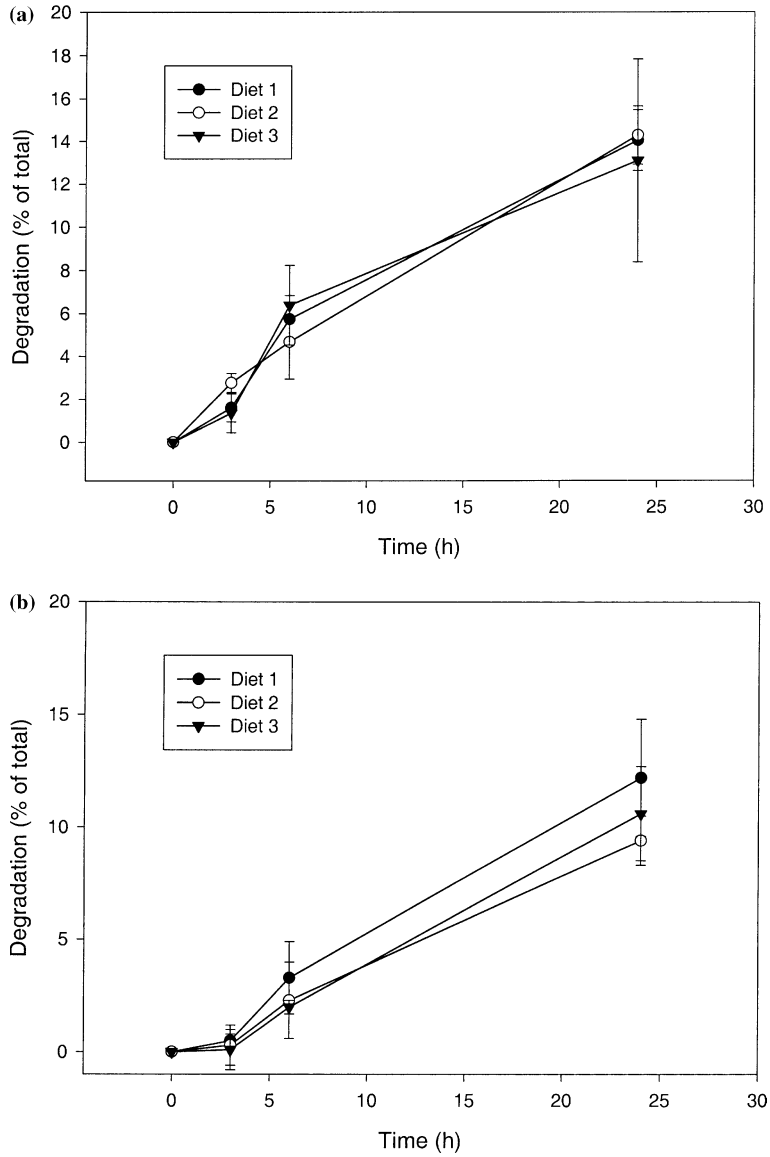

Figure 1. Degradation of albumin micro particles by salmon head kidney *in vivo*. Twenty salmon from each dietary group were injected with ^{125}I -labeled micro particles and five fish were sampled at each time interval. The radioactivity content of kidney was analysed by acid precipitation and centrifugation. The experiment was performed at 12 °C (a) and 5 °C (b). The plot shows mean value of 5 samples ±SD (percentage acid soluble radioactivity).

Eicosanoid production by head kidney macrophages

To test the effect of dietary lipids on eicosanoid metabolism in salmon kidney macrophages, the cultured cells were incubated with arachidonic acid for 12 h and then assayed for production of LTB_4 and PGE_2. These products served as markers of the lipoxygenase and cyclooxygenase pathways, respectively. As seen in Figure 3a, the highest lipoxygenase activity was observed in cells from fish fed Diet 2 (50% capelin oil/50% soybean oil). At 5 °C the increase in immunoreactive material was about 80%, and this was the only significant increase (Two way ANOVA $p < 0.05$) observed. PGE_2production (Figure 3b) was also apparently stimulated in the head kidney macrophages from

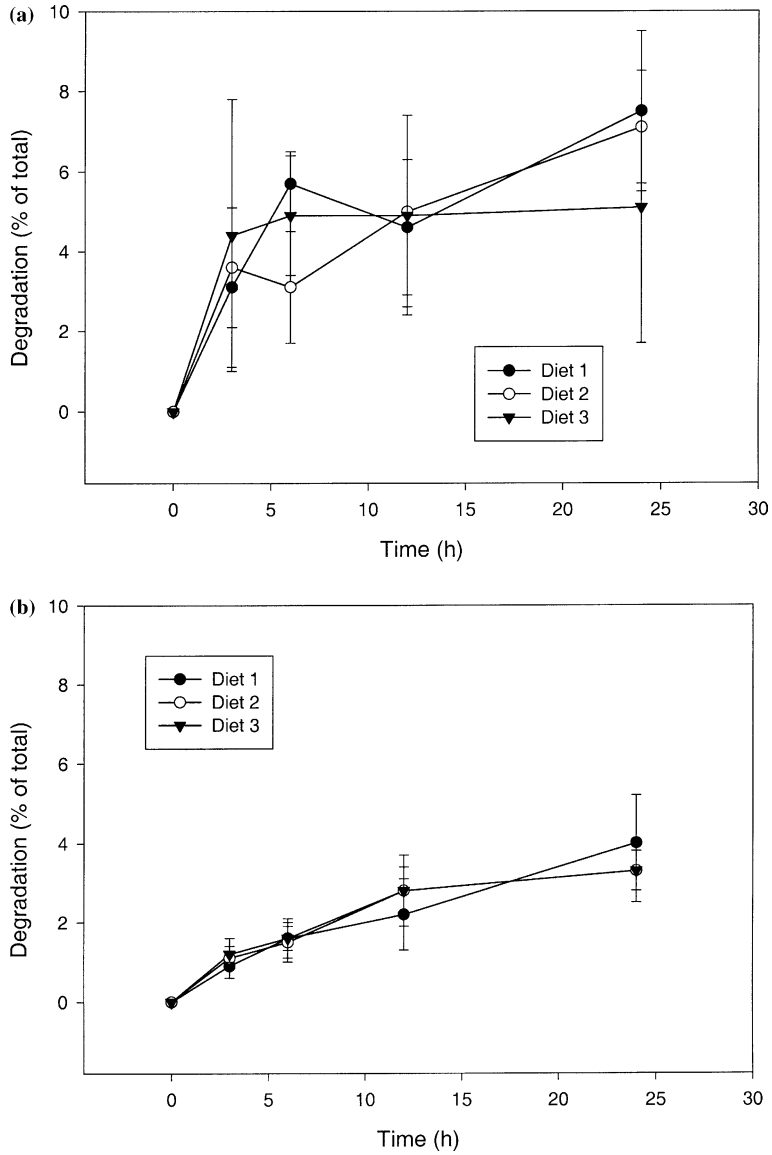

Figure 2. Degradation of albumin micro particles in salmon head kidney macrophages *in vitro*. Cells were isolated from five fish in each dietary group at 12 °C (a) and 5 °C (b) and cultured for 3 h before addition of ^{125}I-labelled micro particles to the culture medium. A sample from the medium was analysed for acid soluble radioactivity at intervals. Values are mean ± SD (n = 5).

the same dietary group (Diet 2) but the difference was not statistically significant ($p = 0.051$). There were no significant differences in eicosanoid production between group 1 and 3.

Transportation stress

To test the ability of the fish in the different dietary groups to withstand stress from transportation, 120 fish from each of the groups raised at 12 °C were transported by truck from the Akvaforsk facilities at Sunndalsøra to the VESO Vikan AkvaVet (Namsos), where the challenge test was performed. The journey lasted for 12 h. No mortality was observed during transport in any of the experimental groups. Upon arrival all the fish were placed in 2 m^3 fibre glass tank and followed up for 2 weeks before the challenge test was

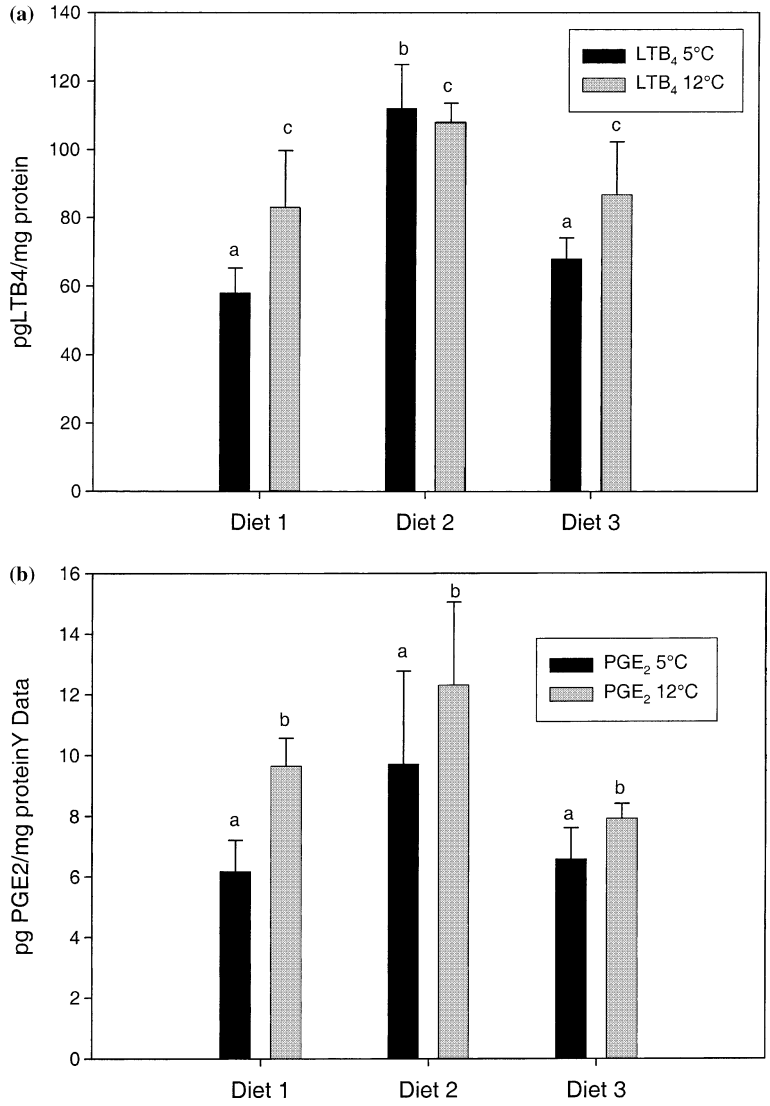

Figure 3. Production of leucotriene B_4 (a) and prostaglandin E_2 (b) in cultured salmon head kidney macrophages. Salmon head kidney macrophages were cultured overnight washed and then incubated for another 24 h in the presence of 150 μM arachidonic acid (20:4n-6). The culture medium was sampled and analysed for eicosanoids as described. Different letters above columns denotes significant difference ($p < 0.05$) (within same temperature).

carried out. During this 15 days period, 1, 4 and 0 fish died in the groups fed diet1, diet 2 and diet 3, respectively.

Challenge test with Aeromonas salmonicida

The fish were intraperitoneally injected with a virulent suspension of *A. salmonicida*. Mortalities began to occur 6 days after the experimental infection in all three experimental groups. Mortality was followed up for 15 days after waterborne challenge. The cumulative mortality in the groups fed diets 1, 2 and 3 was 59.3%, 47% and 58.0%, respectively (Figure 4). These differences were not statistically significant.

Discussion

Numerous studies have reported various effects of dietary lipids on immune functions in both mammals (Hummell 1993; Calder 1999; Thies

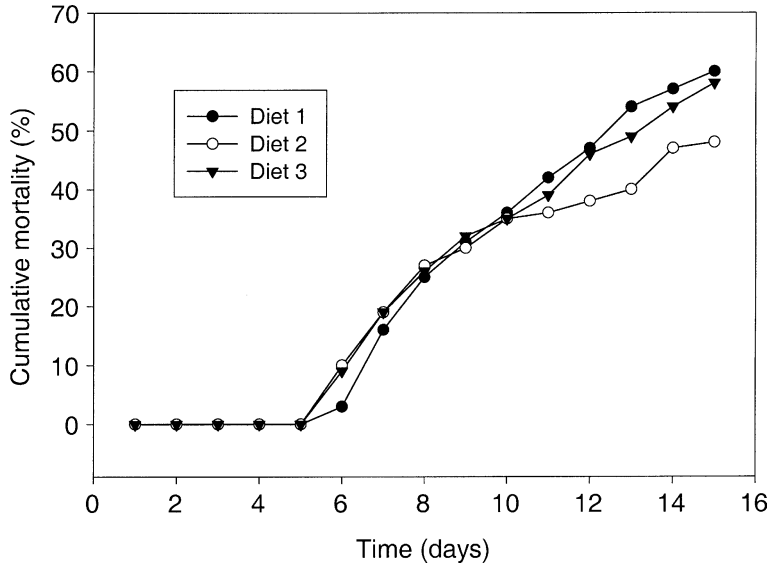

Figure 4. Cumulative mortality in Atlantic salmon challenged with *A. salmonicida*. Fish from the tree dietary groups were challenged with i.p. injection of 10^4 CFU/fish of a virulent suspension of *A. salmonicida*. Mortality was observed for 2 weeks. Dead fish were removed and analysed for cause of death. (Diet 1: n = 119, diet 2: n = 116, diet 3: n = 120).

et al. 2001) and fish (Blazer et al. 1989; Wise et al. 1993; Bell et al. 1996a). Thompson et al. (1996) reported that replacing fish oil with sunflower oil in diets of Atlantic salmon might result in less resistance to infection. There are however, contradictory results in this field as some report effects (Calder 1998), whereas other studies fail to document any effect of dietary lipids on immune parameters *in vivo* or *in vitro* (Yaqoob 1998; Yaqoob et al. 2000). Some of these contradictory findings may be explained by variations in the vitamin E levels in the various diets.

In a previous report by our group, Atlantic salmon fed diets with n-6/n-3 ratios varying from 0.2 to 4.2, tripled their weights during a 3 months trial and showed no mortality or histological pathologies in heart tissue (Grisdale-Helland et al. 2002). This is in accordance with previous studies feeding different levels of vegetable oils to this species (Bell et al. 1996a, b). However, to evaluate possible subclinical effects on non-specific immune functions such as eicosanoid secretion and phagocytosis we have analysed these processes *in vivo* and *in vitro*. The effect of dietary lipids on the fatty acid composition of kidney tissue was profound with the n-6/n-3 ratio increasing from 0.35 in the fish fed the fish oil diet to 2.5 in the fish fed the soybean oil diet at 5 °C. Given the role of membrane lipids in membrane traffic and phagocytosis (Mayorga et al. 1993; Lennartz 1999), it is interesting to observe that even after dramatic changes of dietary lipids, the basic functions of the innate immune system were maintained. Release of AA from membrane phospholipids by the action of phospholipase A_2(PLA_2) has been demonstrated to be essential in Fc-receptor mediated phagocytosis (Lennartz and Brown 1991). AA seems to be necessary for fusion of electron lucent vesicles with the plasma membrane underlaying the particle to be phagocytosed (Karimi and Lennartz 1995). This provides new membrane for pseudopod extension and engulfment of the particle. When this process is inhibited by PLA_2 inhibitors like bromoenol lactone (BEL), total rescue of enzyme activity can be obtained by the addition of AA to the medium. This shows that AA is the main mediator of this membrane fusion step. In our study, even though a 3-fold increase was observed in the percentage of AA in head kidney from salmon fed the 100% soybean oil diet than compared to head kidney from fish fed the fish oil diet, no affect was observed on the phagocytic activity in the fish. Aggregated serum albumin particles were used as probes for phagocytosis, and we have previously demonstrated that these particles are mainly bound by the scavenger

receptor class A on salmonid macrophages (Froystad et al. 1998).

To test the possibility of changes in eicosanoid metabolism after the dietary treatment, the activity of lipoxygenase and cyclooxygenase were estimated by analysis of leucotriene B_4 and prostaglandin E_2, respectively. Such effects have been demonstrated in other species (Ringbom et al. 2001; Watkins et al. 2003). We would therefore expect to see changes in the production of these species from an exogenously added substrate like AA, if the expression of these enzymes were affected by dietary treatment. In this study, feeding the fish a mixture of fish oil with 50% soybean oil resulted in an 80% increase in the production of LTB_4 immunoreactive material (ELISA detectable) from exogenously added AA. This pattern is in agreement with dietary studies on rodents where high n-3 diets decrease eicosanoid formation, whereas high n-6 diets tend to increase the production of both PG's and LT's in macrophages (Leslie et al. 1985; Lokesh and Kinsella 1987; German et al. 1988). Previous studies on eicosanoid metabolism in salmon have also demonstrated an inhibitory effect of fish oil compared to plant oil (Bell et al. 1996a, b). However, macrophages from fish fed the pure soy oil diet showed reduced production of LTB_4 compared to macrophages from fish fed the 50% fish oil/50% soybean oil diet. An inhibited eicosanoid production at high levels of linoleic is also in agreement with a previous study by Galli et al. (1981). These authors showed that excess linoleic acid suppresses eicosanoid production by inhibiting the cyclooxygenase reaction. The desaturation and elongation product from linoleic acid, dihomo-γ-linoleic acid (20:3n-6) competes with AA for cyclooxygenase, resulting in a suppression of prostaglandin formation derived from 20:4n-6. Fatty acid 20:3n-6 does not normally accumulate in animals in significant amounts; however head kidney cells from fish fed the 100% soybean oil diet showed a 7-fold increase in the percentage of 20:3n-6, compared to cells from the fish oil group. 20:3n-6 gives rise to both prostaglandins PGE_1 and 15-OH-DGLA. 15-OH-DGLA is shown to be a powerful inhibitor of 5-lipoxygenase and therefore of the production of LTB_4 Miller et al. (1988).

Proper nutrition plays an important role in maintaining normal growth and health of cultured fish. A variety of nutritional strategies may influence fish health, including adjustment of specific nutrient levels in the diet, manipulation of nutritional condition through feeding regimes, and administration of non-nutrient immunostimulants in the diet. Research with several fish species, including some marine and diadromous species such as salmonids, has established that immunocompetence and disease resistance can be compromised by deficiencies of various nutrients, especially certain vitamins and minerals (Wise et al. 1993; Bell et al. 2000). Thus, adequate levels both macro and micronutrients must be supplied in prepared diets to support optimal growth and production efficiency of aquaculture species. In this study, we could not observe any detrimental effect on fish growth or health after feeding salmon on soybean oil as the main lipid source. Also after the challenge test with *A. salmonicida*, no differences in survival could be detected. This is in contradiction to previous studies showing that a vegetable oil diet can have a detrimental effect on disease resistance in Atlantic salmon (Thompson et al. 1996). In this study sunflower oil were used as the source of n-6 rich diet, and this led to higher mortalities when the fish were challenged with *A. salmonicida*. The explanation for these differences is not clear.

In summary, we found no detrimental effects of vegetable oil on the general growth and health of Atlantic salmon over a 3 month growth period (100–300 g weight). However, the feeding period employed here represents only a part of the growth period for farmed salmon. Recent studies have shown that Atlantic salmon can tolerate a diet solely based on vegetable oil as lipid source through the whole saltwater period (1 year) (Bell et al. (2003, 2004). These raw materials will therefore most probably constitute a important component in the production of high quality commercial feed for the salmon farming industry.

References

Ainsworth, A.J., Dexiang, C., Waterstrat, P.R. and Greenway, T. 1991. Effect of temperature on the immune system of channel catfish (*Ictalurus punctatus*)-I. Leucocyte distribution and phagocyte function in the anterior kidney at 10 °C. Comp. Biochem. Physiol. A 4: 907–912.

Bell, J.G., Ashton, I., Secombes, C.J., Weitzel, B.R., Dick, J.R. and Sargent, J.R. 1996a. Dietary lipid affects phospholipid fatty acid compositions, eicosanoid production and immune

function in Atlantic salmon (*Salmo salar*). Prostaglandins Leukot. Essent. Fatty Acids 54: 173–182.

Bell, J.G., Farndale, B.M., Dick, J.R. and Sargent, J.R. 1996b. Modification of membrane fatty acid composition, eicosanoid production, and phospholipase A activity in Atlantic salmon (*Salmo salar*) gill and kidney by dietary lipid. Lipids 31(11): 1163–1171 .

Bell, J.G., Henderson, R.J., Tocher, D.R. and Sargent, J.R. 2004. Replacement of dietary fish oil with increasing levels of linseed oil: Modification of flesh fatty acid compositions in Atlantic salmon (*Salmo salar*) using a fish oil finishing diet. Lipids 39(3): 223–232.

Bell, J.G., McEvoy, J., Tocher, D.R. and Sargent, J.R. 2000. Depletion of alpha-tocopherol and astaxanthin in Atlantic salmon (*Salmo salar*) affects autoxidative defense and fatty acid metabolism. J. Nutr. 130(7): 1800–1808.

Bell, J.G., McVicar, A.H., Park, M.T. and Sargent, J.R. 1991. High dietary linoleic acid affects the fatty acid compositions of individual phospholipids from tissues of Atlantic salmon (*Salmo salar*): Association with stress susceptibility and cardiac lesion. J. Nutr. 121: 1163–1172.

Bell, J.G., Tocher, D.R., Farndale, B.M., Cox, D.I., McKinney, R.W. and Sargent, J.R. 1997. The effect of dietary lipid on polyunsaturated fatty acid metabolism in Atlantic salmon (*Salmo salar*) undergoing Parr- Smolt transformation. Lipids 32(5): 515–525.

Bell, J.G., Tocher, D.R., Henderson, R.J., Dick, J.R. and Crampton, V.O. 2003. Altered fatty acid compositions in atlantic salmon (*Salmo salar*) fed diets containing linseed and rapeseed oils can be partially restored by a subsequent fish oil finishing diet. J. Nutr. 133(9): 2793–2801.

Blazer, V.S., Ankley, G.T. and Finco-Kent, D. 1989. Dietary influences on disease resistance factors in channel catfish. Dev. Comp. Immunol. 13(1): 43–48.

Bly, J.E. and Clem, L.W. 1991. Temperature-mediated processes in teleost immunity: *In vitro* immunosuppression induced by *in vivo* low temperature in channel catfish. Vet. Immunol. Immunopathol. 28: 365–377.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Brouard, C. and Pascaud, M. 1990. Effects of moderate dietary supplementations with n-3 fatty acids on macrophage and lymphocyte phospholipids and macrophage eicosanoid synthesis in the rat. Biochim. Biophys. Acta 1047(1): 19–28.

Calder, P.C. 1999. Dietary fatty acids and the immune system. Lipids 34: 137–140.

Calder, P.C. 1998. Immunoregulatory and anti-inflammatory effects of n-3 polyunsaturated fatty acids. Braz. J. Med. Biol. Res. 31(4): 467–490.

Cunard, R., Ricote, M., DiCampli, D., Archer, D.C., Kahn, D.A., Glass, C.K. and Kelly, C.J. 2002. Regulation of cytokine expression by ligands of peroxisome proliferator activated receptors. J. Immunol. 168(6): 2795–2802.

Dannevig, B.H. and Berg, T. 1985. *In vitro* degradation of endocytosed protein in pronephros cells of the char (*Salmo alpinus* L.). The effects of temperature and inhibitors. Dev. Comp. Immunol. 9: 231–240.

Frøystad, M.K., Rode, M., Berg, T. and Gjoen, T. 1998. A role for scavenger receptors in phagocytosis of protein-coated particles in rainbow trout head kidney macrophages. Dev. Comp. Immunol. 22(5–6): 533–549.

Galli, C., Agradi, E., Petroni, A. and Tremoli, E. 1981. Differential effects of dietary fatty acids on the accumulation of arachidonic acid and its metabolic conversion through the cyclooxygenase and lipoxygenase in platelets and vascular tissue. Lipids 16(3): 165–172.

German, J.B., Lokesh, B. and Kinsella, J.E. 1988. The effect of dietary fish oils on eicosanoid biosynthesis in peritoneal macrophages is influenced by both dietary N-6 polyunsaturated fats and total dietary fat. Prostaglandins Leukot. Essent. Fatty Acids 34(1): 137–45.

Grisdale-Helland, B., Ruyter, B., Rosenlund, G., Obach, A., Helland, S.J., Sandberg, M.G., Standal, H. and Roesjoe, C. 2002. Influence of high contents of dietary soybean oil on growth, feed utilization, tissue fatty acid composition, heart histology and standard oxygen consumption of Atlantic salmon (*Salmo salar*) raised at two temperatures. Aquaculture 207: 3–4.

Hazel, J.R. 1984. Effects of temperature on the structure and metabolism of cell membranes in fish. Am. J. Physiol. 246: 460–470.

Hazel, J.R. 1988. Advances in membrane fluidity-physiological regulation of membrane fluidity. pp. 149–188. Edited by R.C. Aloia, C.C. Curtain, and L.M. Gordon. Alan R. Liss, New York.

Hummell, D.S. 1993. Dietary lipids and immune function [Review]. Prog. Food Nutr. Sci. 17(4): 287–329.

Hwang, D. 1989. Essential fatty acids and immune response. Faseb. J. 3(9): 2052–2061.

Karimi, K. and Lennartz, M.R. 1995. Protein kinase C activation precedes arachidonic acid release during IgG-mediated phagocytosis. J. Immunol. 155(12): 5786–5794.

Khan, W.A., Blobe, G.C. and Hannun, Y.A. 1995. Arachidonic acid and free fatty acids as second messengers and the role of protein kinase C. Cell Signal 7(3): 171–184.

Kindberg, G.M., Dannevig, B.H., Andersen, K.J. and Berg, T. 1991. Intracellular transport of ovalbumin after *in vivo* endocytosis in rainbow trout liver. Fish Physiol. Biochem. 9: 113–121.

Lappova, Y.L. and Leibush, B.N. 1995. Receptor-mediated endocytosis of insulin in lower vertebrates: Internalization and intracellular processing of ^{125}I- insulin in isolated hepatocytes of lamprey and frog. Gen. Comp. Endocrinol. 100: 1–9.

Lennartz, M.R. 1999. Phospholipases and phagocytosis: The role of phospholipid-derived second messengers in phagocytosis. Int. J. Biochem. Cell Biol. 31(3–4): 415–430.

Lennartz, M.R. and Brown, E.J. 1991. Arachidonic acid is essential for IgG Fc receptor-mediated phagocytosis by human monocytes. J. Immunol. 147(2): 621–626.

Leslie, C.A., Gonnerman, W.A., Ullman, M.D., Hayes, K.C., Franzblau, C. and Cathcart, E.S. 1985. Dietary fish oil modulates macrophage fatty acids and decreases arthritis susceptibility in mice. J. Exp. Med. 162(4): 1336–1349.

Lokesh, B.R. and Kinsella, J.E. 1987. Modulation of prostaglandin synthesis in mouse peritoneal macrophages by enrichment of lipids with either eicosapentaenoic or docosahexaenoic acids *in vitro*. Immunobiology 175(5): 406–419.

Løvhøiden, N., Gjøen, T. and Berg, T. 1994. The effect of temperature on intracellular transport of asialoglycoproteins in rainbow trout liver. J. Fish Biol. 45: 75–86.

Mayorga, L.S., Colombo, M.I., Lennartz, M., Brown, E.J., Rahman, K.H., Weiss, R., Lennon, P.J. and Stahl, P.D. 1993. Inhibition of endosome fusion by phospholipase A2

(PLA2) inhibitors points to a role for PLA2 in endocytosis. Proc. Natl. Acad. Sci. USA 90: 10255–10259.

Miller, R.F., Purvis, A.W., Lefferts, P.L. and Snapper, J.R. 1988. Meclofenamate blocks the pulmonary arterial vasopressor effects of leukotriene B4 in awake sheep. Prostaglandins 36(5): 601–606.

Nordmo, R. 1997. Strengths and weaknesses of different challenge methods. Dev. Biol. Stand. 90: 303–309.

Pittman, R.C., Carew, T.E., Glass, C.K., Green, S.R., Taylor, C.A. Jr and Attie, A.D. 1983. A radioiodinated, intracellularly trapped ligand for determining the sites of plasma protein degradation *in vivo*. Biochem. J. 212: 791–800.

Redshaw, M.R. and Lynch, S.S. 1974. An improved method for the preparation of iodinated antigens for radioimmunoassay. J. Endocrinol. 60: 527–528.

Ringbom, T., Huss, U., Stenholm, A., Flock, S., Skattebol, L., Perera, P. and Bohlin, L. 2001. Cox-2 inhibitory effects of naturally occurring and modified fatty acids. J. Nat. Prod. 64(6): 745–749.

Rode, M., Berg, T. and Gjøen, T. 1997. Effect of temperature on endocytosis and intracellular transport in the cell line SHK-1 derived from salmon head kidney. Comp. Biochem. Physiol. 117(4): 531–537.

Røsjø, C., Berg, T., Manum, K., Gjøen, T., Magnusson, S. and Thomassen, M.S. 1994. Effects of temperature and dietary n-3 and n-6 fatty acids on endocytic processes in isolated rainbow trout (Oncorhynchus mykiss) hepatocytes. Fish Physiol. Biochem. 13: 119–132.

Thies, F., Miles, E.A., Nebe-von-Caron, G., Powell, J.R., Hurst, T.L., Newsholme, E.A. and Calder, P.C. 2001. Influence of dietary supplementation with long-chain n-3 or n-6 polyunsaturated fatty acids on blood inflammatory cell populations and functions and on plasma soluble adhesion molecules in healthy adults. Lipids 36(11): 1183–1193.

Thompson, K.D., Tatner, M.F. and Henderson, R.J. 1996. Effects of dietary (n-3) and (n-6) polyunsaturated fatty acid ratio on the immune response of Atlantic salmon, *Salmo salar* L. Aquacult. Nutr. 2(1): 21–32.

Watkins, B.A., Li, Y., Lippman, H.E. and Feng, S. 2003. Modulatory effect of omega-3 polyunsaturated fatty acids on osteoblast function and bone metabolism. Prostaglandins Leukot. Essent. Fatty Acids 68(6): 387–398.

Wise, D.J., Tomasso, J.R., Schwedler, T.E., Blazer, V.S. and Gatlin, D.M. III 1993. Title Effect of Vitamin E on the immune response of channel catfish to *Edwardsiella ictaluri*. J. Aquat. Anim. Health 5(3): 183–188.

Yaqoob, P. 1998. Monounsaturated fats and immune function. Braz. J. Med. Biol. Res. 31(4): 453–465.

Yaqoob, P., Pala, H.S., Cortina-Borja, M., Newsholme, E.A. and Calder, P.C. 2000. Encapsulated fish oil enriched in alpha-tocopherol alters plasma phospholipid and mononuclear cell fatty acid compositions but not mononuclear cell functions. Eur. J. Clin. Invest. 30(3): 260–274.