Lipids (2008) 43:813–827
DOI 10.1007/s11745-008-3208-z

ORIGINAL ARTICLE

Dietary n-3 HUFA Affects Mitochondrial Fatty Acid β-Oxidation Capacity and Susceptibility to Oxidative Stress in Atlantic Salmon

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Received: 19 March 2008 / Accepted: 3 June 2008 / Published online: 10 July 2008

Abstract Atlantic salmon (*Salmo salar*) (90 g) were fed four different diets for 21 weeks (final weight 344 g). The levels of n-3 highly unsaturated fatty acids (HUFA) ranged from 11% of the total fatty acids (FA) in the low n-3 diet to 21% in the intermediate n-3 diet, to 55 and 58% in the high n-3 diets. The high n-3 diets were enriched with either docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA). Increasing dietary levels of n-3 HUFA led to increasing percentages (from 31 to 52%) of these FA in liver lipids. The group fed the highest level of DHA had higher expressions of peroxisome proliferator-activated receptor (PPAR) β and the FA β-oxidation genes acyl-CoA oxidase (ACO) and carnitine palmitoyltransferase (CPT)-II, compared to the low n-3 groups. The high n-3 groups had reduced activity of mitochondrial cytochrome c oxidase and β-oxidation capacity, together with increased activities of superoxide dismutase (SOD) and caspase-3 activities. In the group fed the highest level of n-3 HUFA, decreased percentages of major phospholipids (PL) in the mitochondrial and microsomal membranes of the liver were also apparent. The percentage of mitochondrial cardiolipin (Ptd_2Gro) was 3.1 in the highest n-3 group compared to 6.6 in the intermediate group. These data clearly show an increased incidence of oxidative stress in the liver of fish fed the high n-3 diets.

Keywords Lipid metabolism · Lipid peroxidation · Gene expression · Fish oil · n-3 fatty acids · n-6 fatty acids

Abbreviations

ACO	Acyl-CoA oxidase
BSA	Bovine serum albumin
CoA	Coenzyme A
CPT	Carnitine palmitoyltransferase
DHA	Docosahexaenoic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
EF1-α_β	Elongation factor 1-α beta isoform
EGTA	Ethylene glycol tetra-acetic acid
EPA	Eicosapentanoic acid
FA	Fatty acid
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FO	Fish oil
HPTLC	High-performance thin-layer chromatography
HUFA	Highly unsaturated fatty acid
L-15	Leibowitz-15 medium
NAD	Nicotinamide adenine dinucleotide
PBS	Phosphate-buffered saline
PL	Phospholipid
PPAR	Peroxisome proliferator-activated receptor
PtdCho	Phosphatidyl-choline
PtdEtn	Phosphatidyl-ethanolamine
Ptd_2Gro	Cardiolipin
PtdIns	Phosphatidyl-inositol
RO	Rapeseed oil
ROS	Reactive oxygen species

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SOD Superoxide dismutase
TAG Triacylglycerol

Introduction

Atlantic salmon (*Salmo salar*) have traditionally been fed diets rich in fish oil (FO) containing relatively high levels of the essential highly unsaturated fatty acids (HUFA) eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). Dietary n-3 HUFA play a prominent role in fish nutrition, providing essential FA that are vital constituents of cell membranes, being necessary for the normal membrane structure and function [1]. However, recent trends in the fish industry include replacing these FO in the diet by more available vegetable oils, having lower n-3 HUFA levels. This change in dietary regimes has resulted in a major research focus on how this reduction in the essential FA affect FA metabolism. The tissues capacity of β-oxidation may be affected by changes in dietary FA, as demonstrated by decreased liver β-oxidation capacity when salmon were fed dietary vegetable oils [2–4]. In addition, both high dietary EPA and an increase in the n-3/n-6 ratio of FA in the diet induce mitochondrial proliferation [5–8]. Furthermore, Jordal et al. [9] found that dietary rapeseed oil (RO), which has a lower level of n-3 HUFA, induced a significant down-regulation of several mitochondrial genes in Atlantic salmon liver. In addition, salmonids fed a diet scarce in n-3 FA generally have higher activities of Δ5- and Δ6-desaturases than fish fed an n-3 FA rich diet [10–13]. They also express higher mRNA levels of the gene for Δ5-desaturase [9, 14].

There has been much focus on the effects of reduced levels of n-3 HUFA, however, less is known about the effects of n-3 HUFA levels above those normally obtained when salmon are fed commercial cod liver oil diets. The South American FO for instance, is generally a rich source of these n-3 HUFA. The HUFA are highly susceptible to peroxidation due to their high number of double bonds [15]. Peroxidation products may influence the HUFA in membrane phospholipids (PL), and thereby impair cell and organelle membrane structure and functions [1]. HUFA taken into the body are mostly delivered to liver cells in mammals [16]. The liver is thus one of the principal targets of HUFA peroxidative effects. Lipid peroxidation generally results in a decrease in membrane fluidity, an increase in the permeability of the membrane, and inactivation of membrane-bound enzymes [17, 18]. These effects can eventually lead to a complete loss of membrane integrity and cause apoptosis. The proteins cytochrome c and cytochrome c oxidase located in the mitochondrial membrane of healthy mammalian cells, function in the respiratory chain interacting with the membrane PL cardiolipin (Ptd_2Gro) [19]. Decline in cytochrome c oxidase activity can be ascribed to a loss in Ptd_2Gro content [20]. Further, both cytochrome c and Ptd_2Gro decrease in mitochondria has been shown as an initial step in the pathway of apoptosis in mammals [21, 22]. Whether these mechanisms are the same in fish or not remains to be studied.

The present study had two primary goals; firstly to study how the mitochondrial and peroxisomal FA β-oxidation and its regulatory genes were affected by increasing levels of n-3 HUFA in the diet. Secondly to determine whether increasing dietary levels of n-3 HUFA affect the composition of membrane PL—both in cells and their organelles, cell morphology, intracellular oxidative stress and apoptosis.

Materials and Methods

Materials

Metacain (MS-222) was obtained from Norsk Medisinaldepot (Oslo, Norway). The radioactive isotope [1-^{14}C]-palmitoyl coenzyme A, (40–60 mCi/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). Sodium chloride, sulfuric acid, perchloric acid, diethyl ether, methyl acetate, hexane, copper acetate, phosphoric acid and benzene were purchased from Merck (Darmstadt, Germany). Ethylene glycol tetra-acetic acid (EGTA) was bought from AppliChem (Darmstadt, Germany). Cell flasks and cell scrapers were obtained from Nalge Nunc International (Rochester, NY, USA). Glutaraldehyde, Epon resin, copper grids, uranyl acetate, osmium tetroxide, sodium cacodylate and lead citrate were supplied by Electron Microscopy Science (Fort Washington, PA, USA). RNeasy Mini Kit and QIAshredder columns were purchased from Qiagen (Valencia, CA, USA). The TaqMan® Gold RT-PCR kit and the ABI Prism 7000 sequence detection system were bought from Applied Biosystems (Foster City, CA, USA). Insta gel II plus was obtained from Packard Instruments (Downers Grove, IL, USA). Chloroform, methanol and acetic acid were obtained from VWR International (West Chester, PA, USA). Total protein kit, cytochrome c oxidase (EC 1.9.3.1) assay kit, acid phosphatase (EC 3.1.3.2) assay kit, Leibowitz-15 medium (L-15), phosphate-buffered saline (PBS), fetal bovine serum (FBS), bovine serum albumin (BSA), sucrose, ethylene diamine tetra-acetic acid (EDTA), antibiotics, HEPES, L-glutamine, collagenase type 1 (EC 3.4.24.3), trypsin, sodium bicarbonate solution, trypan blue, laminin, potassium ferrocyanide, NaOH, nicotinamide adenine dinucleotide (NAD), dithiothreitol (DTT), flavin adenine dinucleotide (FAD), Triton-X 100, L-Carnitine, Coenzyme

A (CoA), Imidazole buffer, Palmitoyl CoA, Tris base, Peroxidase type II (EC 1.11.1.7), HCl, 2,2-dimethoxypropane, 2',7'-dichlorofluorescein and silica gel 60 glass-backed TLC plates were all supplied by Sigma-Aldrich (St. Louis, MO, USA). Primers for the real-time PCR analysis were ordered from Invitrogen Ltd (Paisley, UK). The superoxide dismutase (SOD, EC 1.15.1.1) assay kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA) and the Caspase-3 (EC 3.4.22.56) colorimetric assay kit was from R&D Systems, Inc. (Minneapolis, MN, USA). Hydrogen peroxide and titanium oxysulfate were obtained from Riedel-de Haën (Seelze, Germany). *O*-Nitrophenyl acetate was purchased from MP Biomedicals (Solon, OH, USA). Ethanol and isopropanol were obtained from Arcus (Oslo, Norway). 2',7'-dichlorofluorescine was bought from Eastman Kodak Company (Rochester, NY, USA).

Feeding Trial and Sampling

The feeding trial was carried out at Nofima's research station, Sunndalsøra, Norway. Four diets with approximately 50% (of dry matter, by weight) crude protein and 23% (of dry matter, by weight) crude lipid contained 13.5% (g/100 g feed) oils with increasing levels of n-3 HUFA (Table 1). The diets contained 11, 21, 55 and 58% n-3 HUFA (of total FA), and they were named low n-3 diet, intermediate n-3 diet, high n-3 DHA diet and high n-3 EPA diet, respectively. The high n-3 EPA diet and high n-3 DHA diet were both characterized by high percentages of n-3 HUFA and low percentages of n-6 FA (Table 2). In contrast, the low n-3 diet contained almost four times more n-6 FA than the intermediate n-3 diet, and 2.5 times more n-6 FA than the high n-3 EPA and high n-3 DHA diets. Four groups of post-smolt Atlantic salmon (*Salmo salar*), with an initial average weight of 90 g were distributed into three cylinder-conical tanks (flow through system, 0.85 m diameter) per diet, and fed one of the four diets for 21 weeks. The fish were not fasted prior to the final sampling. The average fish weight was 344 g at the end of the growth period, with no significant differences in the growth (total weight) between the dietary groups.

Isolation of Hepatocytes

Five fish per diet (randomly taken from the three tanks) were used for the isolation of hepatocytes at the end of the growth period. The fish were anaesthetized with metacain (MS-222) and their individual weights and lengths determined. The abdominal cavity was exposed and the vena portae cannulated. The liver was perfused following a two-step collagenase procedure developed by Seglen [23] and modified by Dannevig and Berg [24], in order to isolate hepatocytes. The hepatocytes were isolated after collagenase digestion by gentle shaking of the digested liver in L-15 medium. The suspension of parenchymal cells was filtered through a 100 μm mesh nylon filter. Hepatocytes

Table 1 Formulation and chemical composition of the diets

	Diets			
	Low n-3	Intermediate n-3	High n-3 DHA	High n-3 EPA
Formulation (% of total)				
Fish meal, LT	67.9	67.9	67.9	67.9
Rapeseed oil	13.5			
Fish oil		13.5		
DHA[a]			13.5	
EPA[b]				13.5
Wheat	17.1	17.1	17.1	17.1
Vitamin premix[c]	1.0	1.0	1.0	1.0
Mineral premix[c]	0.4	0.4	0.4	0.4
Yttrium oxide[d]	0.01	0.01	0.01	0.01
Carophyll Pink[e] (8%)	0.064	0.064	0.064	0.064
Chemical composition				
Dry matter (%)	92.2	92.3	92.2	93.0
Percentage of dry matter				
Protein	54.6	55.4	54.4	55.1
Fat	23.0	22.5	23.3	22.7
Ash	9.9	9.5	9.7	9.5
Energy (MJ/kg)	23.9	23.8	23.8	23.6

The EPA-enriched oil used in the high n-3 EPA diet, the DHA-enriched oil used in the high n-3 DHA diet and the rapeseed oil used in the low n-3 diet were added 150 ppm butylated hydroxytoluene (BHT) before coated onto the feed

[a] Incromega DHA 500TG SR, Croda Chemicals Europe Ltd., Goole, England

[b] Incromega EPA 500TG SR, Croda Chemicals Europe Ltd., Goole, England

[c] As described by Mundheim et al. [58]

[d] Inert marker, Y_2O_3, Sigma

[e] Hoffman-LaRoche, Basel, Switzerland

Table 2 Fatty acid compositions of diets

Fatty acids (% of total)	Diet			
	Low n-3	Intermediate n-3	High n-3 DHA	High n-3 EPA
14:0	1.99	6.88	2.22	2.09
16:0	7.58	12.87	5.36	4.91
18:0	1.53	1.46	1.50	0.62
20:0	0.44	3.66	0.39	4.57
22:0	0.30	0.07	ND	0.07
24:0	0.08	0.03	0.47	0.12
Σ Saturated[a]	12.19	25.85	10.27	12.68
16:1n-7	1.23	4.26	1.37	1.27
18:1n-7	2.77	1.67	1.14	0.88
18:1n-9	40.22	7.84	5.26	3.73
20:1n-9	4.27	9.96	5.18	3.65
22:1n-11	0.19	15.76	6.59	5.28
Σ Monounsaturated[b]	49.87	43.07	23.83	18.37
18:2n-6	14.09	2.70	1.61	2.20
18:3n-3	7.27	1.71	0.91	1.02
18:3n-6	ND	0.11	0.52	0.31
20:3n-6	0.19	0.40	1.34	2.35
20:4n-3	0.45	1.04	0.67	0.65
20:4n-6	0.06	ND	0.32	0.12
20:5n-3	4.95	7.12	8.68	42.87
22:5n-3	0.29	0.76	3.27	1.58
22:5n-6	2.56	0.39	0.53	1.29
22:6n-3	4.96	12.18	41.73	12.36
Σ Polyunsaturated[c]	36.14	28.14	63.23	66.63
Σ n-3	18.79	23.34	56.13	59.38
Σ n-6	17.05	4.50	6.61	6.80
∑ n-3 HUFA	11.37	21.32	54.79	57.92
EPA + DHA	9.91	19.30	50.41	55.23

nd not detected

[a] Includes 15:0, 17:0, 19:0

[b] Includes 14:1n-5, 16:1n-9, 17:1n-7, 18:1n-11, 20:1n-7, 22:1n-7, 22:1n-9, 24:1n-9

[c] Includes 16:2n-3, 16:2n-6, 16:3n-4, 18:3n-4, 18:4n-3, 20:2n-6, 20:3n-3, 21:5n-3, 22:4n-6

were washed three times in L-15 medium and sedimented by spinning for 2 min at $50\times g$. The hepatocytes were resuspended in L-15 culture medium containing 10% FBS, 1% bicarbonate, 1% L-glutamine, 1% penicillin-streptomycin solution and 5 mM HEPES. Cell viability was assessed by staining with Trypan Blue (0.4%). Approximately 1×10^7 hepatocytes were plated onto 25 cm^2 cell flasks coated with laminin, and left to attach overnight at 12 °C. Two hepatocyte flasks per fish were used for transmission electron microscopy and one flask for the isolation of RNA.

Preparation of Hepatocytes for Transmission Electron Microscopy

The cells were washed in PBS and harvested in 1.5 ml of fixative solution (2% glutaraldehyde in 0.1 M cacodylate buffer, pH = 7.4). Then, the cells were washed twice in 0.1 M cacodylate buffer, and post-fixed for 1 h in 0.1 M cacodylate buffer containing 2% osmium tetroxide and 1.5% potassium ferrocyanide. The specimens were stained for 30 min in 1.5% uranyl acetate, dehydrated in a series of ethanol solutions (70, 90, 96 and 100%), and embedded in epon resin. The resin was polymerized at 60 °C for at least 12 h. Ultra-thin sections were prepared and stained with lead citrate. EM specimens were examined using a Philips CM100 transmission electron microscope (80 V) (FEI, Acht, The Netherlands).

Liver Homogenization

A further three fish per diet (one from each tank) were used for liver homogenization. The fish were anaesthetized with metacain (MS-222) and their weights and lengths determined. They were killed by a blow to the head, and the livers were dissected out and weighed. The hepato-somatic indexes (HSI: liver weight/body weight × 100) were 1.00 ± 0.11, 1.06 ± 0.08, 0.88 ± 0.04 and 0.88 ± 0.03

for low n-3, intermediate n-3, high n-3 DHA and high n-3 EPA dietary groups, respectively, with no significant differences between the groups. The tissue was homogenized in a sucrose buffer (0.25 mM sucrose, 15 mM HEPES, 1 mM EDTA and 1 mM EGTA) by using a glass Dounce homogenizer powered by a drill.

Analysis of Fatty Acid Composition

Liver homogenate from three fish in each dietary group (one from each tank) was used for the analysis of FA composition. The total FA profiles in the diets (Table 2) and the livers (Table 4) were determined. Lipids were extracted using the Folch method [25]. The chloroform phase was dried under N_2 and the residual lipid extract was redissolved in benzene, and then transmethylated overnight with 2,2-dimethoxypropane and methanolic HCl at room temperature, as described by Mason and Waller [26] and by Hoshi et al. [27]. The methyl esters of FA thus formed were separated in a gas chromatograph (Hewlett Packard 6890) with a split injector, SGE BPX70 capillary column (length 60 m, internal diameter 0.25 mm and thickness of the film 0.25 μm) flame ionization detector and the results analysed using HP Chem Station software. The carrier gas was helium. The injector and detector temperatures were 280 °C. The oven temperature was raised from 50 °C 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 FA present was determined by measuring the area under the peak corresponding to that FA.

Lipid Class Analysis

The lipid class compositions in different liver fractions (total homogenate, mitochondria and microsomes) were quantified using high-performance thin-layer chromatography (HPTLC). Ten microgram total lipid was applied using an automatic sample applicator (ATS4, CAMAG, Muttenz, Switzerland) onto a 10 × 20 cm HPTLC plate that had been pre-run in hexane/diethyl ether (1:1, by vol.) and activated at 110 °C for 30 min. The plates were developed for the first 5.5 cm in methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol.) to separate PL classes with neutral lipids running at the solvent front [28] using an automatic developing chamber (AMD2, CAMAG, Muttenz, Switzerland). After drying, the plates were developed fully in hexane/diethyl ether/acetic acid (80:20:2, by vol.) to separate neutral lipids and cholesterol. Lipid classes were visualized by charring at 160 °C for 15 min after dipping the plate into a glass tank with 3% copper acetate (w/v) in 8% (v/v) phosphoric acid. The lipid classes were identified by comparison with commercially available standards, and quantified by scanning densitometry using a CAMAG TLC Scanner 3. Scan lines were analysed using an integrator (WinCATS-Planar Chromatography, Version 1.3.3). Further, the weight of each lipid class per gram of tissue was determined by establishing standard equations for each lipid class within a linear area, and a standard mix of all the lipid classes was included on each HPTLC plate to correct for between plate variations.

Subcellular Fractionation

Subcellular fractions were separated by differential centrifugation first described by de Duve [29]. The homogenate was centrifuged at $1{,}000\times g$ for 10 min to separate out the nuclear fraction. The pelleted nucleus was re-homogenized in 1 ml of homogenization buffer. Mitochondria and peroxisomes were then isolated by two differential centrifugation steps for 10 min at $2{,}000\times g$ and $25{,}000\times g$, respectively, in a fixed-angle rotor 70-Ti (Beckman). Each mitochondrial and peroxisomal pellet recovered was resuspended in 1.5 and 1 ml of the buffer, respectively. The remaining supernatant was the microsomal fraction. The different organelles in the fractions were identified by measuring the activities of subcellular marker enzymes in the diluted pellets and supernatant.

The presence of mitochondria was assessed by a Sigma kit based on the method of Storrie and Madden [30]. The amounts of mitochondria were determined by measuring the activity of cytochrome c oxidase. Total cytochrome c oxidase activity was measured by observing the decrease in absorbance at 550 nm of Fe^{2+}-cytochrome c caused by its oxidation to Fe^{3+}-cytochrome c by cytochrome c oxidase in mitochondria (measured at room temperature for 3 min). Further, the cytochrome c oxidase assay allowed us to measure the integrity of the outer mitochondrial membrane, by measuring the cytochrome c oxidase activity in the presence and in the absence of the detergent *n*-dodecyl β-D-maltoside. This detergent allows the maintenance of the cytochrome c oxidase dimer in solution. Absorbance was measured in a GBC UV/VIS 918 spectrophotometer (GBC Scientific Equipment, Victoria, Australia).

The presence of peroxisomes was assessed by the activity of catalase (EC 1.11.1.6), using a method based on that of Baudhuin et al. [31]. The substrate hydrogen peroxide, produced in peroxisomes, is broken down by catalase to oxygen and water. This reaction is stopped by the addition of a saturated solution (0.45%) of titanium oxysulfate in 2 N sulfuric acid. Titanium oxysulfate reacts with the amount of hydrogen peroxide that remains to give a yellow solution of peroxy titanium sulfate. The amount of the product was measured spectrophotometrically at 405 nm in a Victor 3 1420 Multilabel counter spectrophotometer (PerkinElmer, CT, USA).

Acid phosphatase is an acid hydrolase that normally resides in lysosomes. This enzyme was assayed as described by Bergmeyer [32], using an acid phosphatase assay kit to identify the lysosomes. The measurement was based on the hydrolysis of 4-nitrophenyl phosphate by acid phosphatase. The samples were mixed with 4-nitrophenyl phosphate and citrate buffer solution (0.09 M, pH 4.8), and incubated for 20 min at room temperature. The reaction was stopped by NaOH (0.5 N). The color formed was measured spectrophotometrically at 405 nm in a Victor 3 1420 Multilabel counter spectrophotometer (PerkinElmer, CT, USA).

Esterase (EC 3.1.1.1) was first assayed by Beaufay et al. [33]. Esterase is a marker enzyme for microsomes, and catalyzes the production of o-nitrophenol from o-nitrophenyl acetate. To 2.7 ml of medium containing 20 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 0.1% Triton X-100 and 0.25 ml of sample were added. The changes in absorbance were measured at 420 nm for 3 min in a GBC UV/VIS 918 spectrophotometer (GBC Scientific Equipment, VIC, Australia).

Beta-Oxidation Assay

Mitochondrial β-oxidation was measured in freshly isolated mitochondria by the method of Lazarow [34]. This assay determines the amounts of acid-soluble products in mitochondrial fractions. Acid-insoluble ^{14}C-palmitoyl-CoA is converted to acid soluble ^{14}C-acetyl-CoA. Incubation media contained 50 mM Tris–HCl (pH 8.0), 20 mM NAD, 0.33 M DTT, 1.5% BSA, 10 mM CoA, 1 mM FAD, 5 mM palmitoyl-CoA and ^{14}C-palmitoyl-CoA (60 Ci/mol, 20 μCi/ml), 0.25 M sucrose and 1 mM L-carnitine. The samples were incubated for 30 min at room temperature. Reactions were stopped by adding ice-cold 6% perchloric acid. A zero-time control was used in which perchloric acid was added to the reaction mixture before the addition of organelle. This control established the acid stability of ^{14}C-palmitoyl-CoA, as virtually all unchanged substrate was precipitated by acid. The radioactivity in 500 μl of the supernatant was determined by liquid scintillation spectrometry.

Acyl-CoA Oxidase Assay

Acyl-CoA oxidase (ACO, EC 1.3.3.6) activity was assayed in peroxisomes by determining the rate at which hydrogen peroxide was produced, coupled to the oxidation of 2',7'-dichlorofluorescine, essentially as described by Small et al. [35]. The oxidation of 2',7'-dichlorofluorescine by hydrogen peroxide to 2',7'-dichlorofluorescein was followed spectrophotometrically at 502 nm in a GBC UV/VIS 918 spectrophotometer (GBC Scientific Equipment). The reaction mixture contained 0.1 M Tris-HCl (pH 8.5), 0.05 M 2',7'-dichlorofluorescine, 50 μM horseradish peroxidase type II, 0.015 mM FAD, 60 mg/ml BSA and 0.02% Triton-X 100, and was started with 60 μM palmitoyl-CoA. All concentrations are given as final values. The reaction mixture contained 30–85 μg of protein in a total volume of 1 ml at 20 °C. The ACO activity was calculated as total activity per gram liver.

Protein Measurements

Protein concentrations were determined using a total protein kit (Micro Lowry/Peterson's modification) based on the method of Lowry [36] and modified by Peterson [37]. Standards (BSA) were prepared by diluting 400 μg/ml of BSA in water. Sodium chloride (final concentration of 0.1 M) was added in order to reduce ampholyte interference. Proteins were precipitated by adding 0.1% trichloroacetic acid in the presence of 0.15% deoxycholate. Color was measured at 500 nm in a Wallac 1420 VICTOR^{3TM} Multilabel counter spectrophotometer (PerkinElmer, CT, USA).

Superoxide Dismutase Assay

The SOD catalyzes the reduction of superoxide to oxygen and hydrogen peroxide. A cell that has a high level of SOD has probably increased production of this enzyme in order to deal with high levels of superoxide. The kit uses a tetrazolium salt to detect superoxide radicals generated by xantine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to achieve 50% dismutation of the superoxide radical. Color was measured at 405 nm in a Titertek Multiskan PLUS MKII (Labsystems, Helsinki, Finland) plate reader.

Caspase-3 Assay

Caspases are a group of cysteine proteases that exist as proenzymes, becoming activated during the cascade of events associated with apoptosis. The caspase-3 colorimetric assay kit detects the increased enzymatic activities of the caspase-3 classes of proteases in apoptotic cells by a colorimetric reaction. Cells are first lysed to release their intracellular contents. The cell lysate can then be tested for protease activity by the addition of a caspase-specific peptide that conjugates to a color reporter molecule, *p*-nitroanaline. The cleavage of the peptide by the caspase releases the chromophore *p*-nitroanaline, which can be quantified spectrophotometrically. Color was measured at 405 nm in a Titertek Multiskan PLUS MKII (Labsystems, Helsinki, Finland) plate reader.

RNA Isolation

Total RNA was isolated from hepatocytes from three fish in each dietary group using an RNeasy® Mini Kit. Samples were first lysed in RNeasy lysis buffer and then homogenized using QIAshredder columns. Ethanol was added to the lysate to provide ideal binding conditions. The lysate was then loaded onto the RNeasy silica-gel membrane. The RNA bound to the membrane, while all contaminants were efficiently washed away. Pure, concentrated RNA was eluted in water. Residual amounts of remaining DNA were removed using an RNase-Free DNase set during the RNeasy procedure. The total RNA concentrations were determined by spectrophotometry.

Sequence Information, Primer Design and Real-Time PCR

The designs of the PCR primers and TaqManMGB probes were based on published sequences from Atlantic salmon or comparative species. The forward and reverse primers and probes are listed in Table 3. RT-PCR efficiency was monitored using two-fold dilution curves of RNA. Four concentrations were used for the two-fold dilution curve (starting at 250 ng total RNA). For analysis of gene expression (separate 96 well plates) three parallels were used at a total RNA concentration of 125 ng (± 5%). The following conditions were used for the amplification of cDNA: 2 min at 50 °C, followed by denaturation for 10 min at 95 °C, followed by 50 cycles of 15 s at 95 °C and finally 1 min at the annealing temperature listed in Table 3. Thermal cycling and fluorescence detection was done using an ABI Prism 7000 sequence detection system.

Data Analysis and Statistical Analysis of Gene Expression Data

Each assay was tested on different samples from the same plate to determine how reproducible the results were. QGene was used to normalize and calculate relative expression data [38]. QGene takes into account the PCR efficacy, calculated on the basis of two-fold dilution curves, and obtains, in this way, normalized expression data. The gene expression levels were normalized against a reference gene. Two different reference genes, β-actin and elongation factor 1-alpha beta isoform (EF1-α_β) [39], were measured for all dietary treatments. Results based on the use of β-actin as reference gene had a higher variation between replicates of hepatocytes, and thus EF1-α_β was used for the final calculations of relative gene expression in hepatocytes. Gene expressions from fish in the intermediate n-3 dietary group were set to 1, and the expression of each target gene for the experimental groups low n-3, high n-3 EPA and high n-3 DHA was expressed relative to this. Significant differences ($P \leq 0.05$) in normalized gene expression levels between dietary treatments were detected by one-way analysis of variance (ANOVA) (Statistica, version 6.1; Statsoft, Tulsa, USA).

Statistical Analysis

All data, except for gene expression data, were subjected to one-way ANOVA for the factor "diet", and differences were ranked by Duncan's multiple range test. We used the software package UNISTAT (London, England). The significance level was set to $P \leq 0.05$.

Results

Fatty Acid Composition

The percentages of n-3 HUFA in the fish livers increased with increasing level of n-3 HUFA in the diet, while the percentages of n-6 FA decreased (Table 4). The main contributor to n-3 FA in the intermediate n-3 and high n-3 DHA dietary groups was 22:6n-3, while 20:5n-3 and 22:5n-3 also contributed in the high n-3 EPA dietary group. In contrast, the livers from fish fed the low n-3 diet contained more than twice as much n-6 FA as those from the other three dietary groups, with 18:2n-6 being the dominating n-6 FA. In addition, the low n-3 group contained approximately 20% 18:1n-9, whereas the intermediate n-3, high n-3 EPA and high n-3 DHA dietary groups all contained less than 8% of this FA. The percentage of 22:6n-3 in the liver lipids was the same in fish fed the low n-3 diet as in the fish fed the high n-3 EPA diet, despite a threefold higher 22:6n-3 level in the latter diet. The percentage of the elongation product 22:5n-3 was significantly higher in livers from the high n-3 EPA group.

Lipid Classes

There were between 33 and 53% polar lipids in the total liver homogenate, and between 47 and 66% neutral lipids (Table 5). TAG was the major lipid class followed by phosphatidyl-choline (PtdCho) and then cholesterol and phosphatidyl-ethanolamine (PtdEtn) in the total liver homogenate. The fraction of polar lipids was significantly lower in the high n-3 EPA group than in the other groups, and the fraction of neutral lipids higher. Ptd_2Gro was present almost exclusively in mitochondria. Fish from the high n-3 EPA dietary group contained less Ptd_2Gro than fish from the intermediate n-3 group. The amount of PtdCho in microsomes was slightly higher than in the mitochondria. Phosphatidyl-inositol (PtdIns) was present in

Table 3 TaqMan assays. All sequences are presented as 5′–3′

Gene	Accession number	Direction	Primer sequence	MGB probe	Annealing temperature	Amplicon size
CPTI	AM230810	F	CTTTGGGAAGGGCCTGATC	AGTGTCGTACCAGCCC	60	121
		R	CATGGACGCCTCGTACGTTA			
CPTII	BG934647	F	TGCTCAGCTAGCGTTCCATATG	AGGCAGTATGGGCAGACA	54	75
		R	AGTGCTGCAGGACTCGTATGTG			
ACO	DQ364432	F	CACTGCCAGGTGTGGTGGTA	AGGACCCAAGTTTG	54	92
		R	GGAATTGTACGTTCTCCAATTTCA			
ACAD	CB511813	F	GCCAAGTACTGGGCGTCTGA	AGTACCCCATCGCCAAG	54	127
		R	TGGGCTGGATACGGGAATC			
PPARβ	AJ416953	F	AAGTCTCATGACGAAGAGGTTGTG	AGCAGGAGCAAGAGG	58	71
		R	GTGGCTGTTACTATTCCCCTTCTC			
PPARγ	AJ416952, AJ416951, AJ292963, AJ292962	F	GCCTCAGGCTTCCACTATGG	CTGCAAGGGATTCTTC	60	78
		R	CAGTTTTAACCGCACGGTTCTG			
D5D	AF478472	F	GGAACCACAAACTGCACAAGT	CAGAGGCACCCTTTAGGTG	60	83
		R	GTGCTGGAAGTGACGATGGT			
D6D	AY458652	F	GGGATTTAATCCATCGCATATTAACT	TGTGAACAGAGATAGTTTCCCCAGACGTTTG	60	87
		R	CGTCACAACAAAATACAGCATCTG			
β-actin	BG933897	F	CCAAAGCCAACAGGGAGAAG	TGACCCAGATCATGTTT	60	91
		R	AGGGACAACACTGCCTGGAT			
EF1-α_β	BG933853	F	TGCCCCTCCAGGATGTCTAC	CCAATACCGCCGATTTT	60	59
		R	CACGGCCCACAGGTACTG			

CPTI, II carnitin palmitoyl transferase I, II, *ACO* acyl-CoA oxidase, *ACAD* acyl-CoA dehydrogenase, *PPARβ, γ* peroxisome proliferating activating receptor β, γ, *D5D* Δ5-desaturase, *D6D* Δ6-desaturase, *EF1-α_β* elongation factor 1-α_β, *F* forward, *R* reverse

Table 4 FA compositions of the livers

Fatty acids (% of total)	Low n-3	Intermediate n-3	High n-3 DHA	High n-3 EPA
14:0	1.3 ± 0.06[b]	2.5 ± 0.19[a]	1.7 ± 0.08[b]	1.8 ± 0.29[b]
16:0	11.9 ± 0.92[ab]	14.8 ± 0.84[a]	12.3 ± 0.96[ab]	10.8 ± 0.99[b]
18:0	4.6 ± 0.04[b]	4.1 ± 0.15[a]	5.0 ± 0.14[b]	4.8 ± 0.16[b]
∑ Saturated[a]	21.6 ± 0.59	22.2 ± 1.04	21.4 ± 1.23	19.4 ± 1.67
16:1 n-7	0.9 ± 0.15[b]	1.8 ± 0.27[a]	1.1 ± 0.08[ab]	1.5 ± 0.27[ab]
18:1 n-7	1.9 ± 0.08[a]	1.8 ± 0.09[a]	1.5 ± 0.06[b]	1.4 ± 0.06[b]
18:1 n-9	21.1 ± 0.88[b]	8.4 ± 1.25[a]	7.7 ± 0.45[a]	7.5 ± 0.15[a]
18:1 n-11	1.2 ± 0.13[b]	2.4 ± 0.17[a]	1.6 ± 0.28[b]	1.6 ± 0.10[b]
20:1 n-9	2.7 ± 0.03[ab]	3.3 ± 0.30[a]	2.7 ± 0.29[ab]	2.2 ± 0.26[b]
22:1 n-7	0.8 ± 0.04[ab]	1.3 ± 0.17[a]	0.7 ± 0.11[b]	2.6 ± 0.32[c]
22:1 n-11	0.5 ± 0.07[b]	1.4 ± 0.10[a]	1.2 ± 0.22[ab]	1.5 ± 0.33[a]
∑ Monounsaturated[b]	30.4 ± 1.10[b]	22.3 ± 2.29[a]	18.6 ± 1.51[a]	20.5 ± 0.64[a]
18:2 n-6	5.6 ± 0.32[b]	1.6 ± 0.09[a]	1.5 ± 0.04[a]	1.7 ± 0.03[a]
18:3 n-3	1.3 ± 0.11[b]	0.5 ± 0.02[a]	0.2 ± 0.05[a]	0.5 ± 0.12[a]
20:2 n-6	2.9 ± 1.57	1.7 ± 1.22	0.7 ± 0.03	0.4 ± 0.04
20:3 n-6	2.4 ± 0.11[c]	0.3 ± 0.05[ab]	0.1 ± 0.02[b]	0.4 ± 0.04[a]
20:4 n-6	2.6 ± 0.23[b]	1.9 ± 0.08[a]	3.6 ± 0.19[c]	3.0 ± 0.04[b]
20:5 n-3	4.3 ± 0.28[b]	6.0 ± 0.67[a]	5.1 ± 0.47[ab]	18.6 ± 0.47[c]
22:4 n-6	ND	ND	1.3 ± 0.03	ND
22:5 n-3	1.4 ± 0.10[a]	2.6 ± 0.56[a]	3.1 ± 0.22[a]	9.3 ± 1.60[b]
22:6 n-3	25.4 ± 1.77[b]	34.7 ± 1.10[a]	41.7 ± 1.27[c]	23.4 ± 0.89[b]
∑ Polyunsaturated[c]	46.0 ± 0.70[a]	49.9 ± 1.22[a]	58.2 ± 0.77[b]	58.3 ± 2.11[b]
∑ n-3	32.4 ± 1.85[b]	44.3 ± 2.02[a]	50.9 ± 0.63[c]	52.5 ± 2.03[c]
∑ n-6	13.6 ± 1.20[b]	5.6 ± 1.15[a]	7.3 ± 0.14[a]	5.8 ± 0.08[a]
EPA + DHA	29.8 ± 1.86[b]	40.7 ± 1.77[a]	46.7 ± 0.83[c]	42.0 ± 1.25[ac]

The FA composition was measured in homogenate of the livers from fish fed four different diets. The quantity of each fatty acid is given as the percentage of total fatty acids. The values given are means ± SEM ($n = 3$). Different letters indicate significant differences ($P \leq 0.05$) between the different dietary treatments within each lipid class

nd not detected

[a] Includes: 12:0, 17:0, 22:0

[b] Includes: 14:1n-5, 16:1n-5, 16:1n-9, 17:1n-7, 20:1n-7, 20:1n-11, 22:1n-7, 22:1n-9, 24:1n-9

[c] Includes: 16:2n-6, 18:3n-4, 18:3n-6, 18:4n-3, 20:3n-3, 20:4n-3

all tissue fractions, but the highest percentage was found in microsomes.

Fatty Acid β-Oxidation

The β-oxidation capacity in the high n-3 EPA and high n-3 DHA dietary groups were significantly lower than the capacity in the intermediate n-3 and low n-3 dietary groups (Fig. 1). There were no significant differences between the intermediate n-3 and the low n-3 groups. There were no significant differences in the total liver ACO activity between the dietary groups. However, a tendency toward higher ACO activity in the high n-3 DHA group was evident (Fig. 2).

Gene Expression

The relative expressions of genes involved in lipid metabolism are shown in Fig. 3. Fish fed the low n-3 diet had significantly higher expression of both Δ5-desaturase and Δ6-desaturase genes than fish fed the high n-3 DHA diet. The relative expressions of genes involved in peroxisomal β-oxidation (ACO), mitochondrial β-oxidation [carnitine palmitoyltransferase (CPT) II, acyl-CoA dehydrogenase (ACAD)] and the transcription factor PPARβ were all up-regulated in livers from fish fed the high n-3 DHA diet. These genes showed the lowest expression levels in livers from fish fed the lowest n-3 HUFA diet.

Oxidative Stress Markers

Cytochrome c oxidase measurements showed that its activity was greatly reduced in the mitochondrial membranes, by a factor of 2–3, in the high n-3 EPA and high n-3 DHA dietary groups than in the group with the lowest level of n-3 HUFA (Fig. 4). Caspase-3 activity, an apoptosis marker, was also significantly different between the dietary groups (Fig. 5a). Caspase activity was significantly higher in the high n-3 EPA group than in all other groups, increasing by 100–200%. The activity of SOD, as well, was significantly different between the dietary groups (Fig. 5b). The activities were lowest in fish fed low n-3 and intermediate n-3 diets, and significantly higher in fish from the two high n-3 groups. EM pictures of representative cells from the low n-3 HUFA dietary group (6a), and the high n-3 EPA group (6b) are shown in Fig. 6. Cells from fish given high n-3 EPA diet had an abnormal morphology. The nuclear chromatin in these

Table 5 Lipid class distribution in liver organelle fractions

	Mitochondria				Microsomes				Total homogenate			
	Low n-3	Intermediate n-3	High n-3 DHA	High n-3 EPA	Low n-3	Intermediate n-3	High n-3 DHA	High n-3 EPA	Low n-3	Intermediate n-3	High n-3 DHA	High n-3 EPA
Percentage distribution												
LPC	2.3 ± 0.24^{a}	1.9 ± 0.26^{a}	2.4 ± 0.29^{a}	1.1 ± 0.09^{b}	0.0 ± 0.0	0.7 ± 0.69	0.0 ± 0.0	0.7 ± 0.67	0.0 ± 0.0^{a}	0.5 ± 0.52^{a}	2.1 ± 0.28^{b}	0.4 ± 0.41^{a}
SM	4.4 ± 1.55	4.7 ± 1.19	3.4 ± 0.39	3.8 ± 2.10	6.8 ± 0.89	5.7 ± 0.89	5.4 ± 0.07	6.0 ± 0.51	3.5 ± 0.28	4.1 ± 0.87	4.5 ± 0.24	2.9 ± 0.39
PtdCho	22.5 ± 1.02^{ab}	28.1 ± 2.51^{a}	23.5 ± 2.89^{ab}	16.1 ± 2.82^{b}	29.0 ± 2.24	29.3 ± 1.36	29.6 ± 3.38	23.0 ± 0.94	25.0 ± 1.29^{a}	26.9 ± 1.85^{a}	23.3 ± 1.65^{a}	16.1 ± 2.72^{b}
PtdSer	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}	1.0 ± 0.50^{b}	0.0 ± 0.0^{a}	2.3 ± 0.17^{a}	2.0 ± 0.14^{a}	1.8 ± 0.17^{ab}	1.4 ± 0.16^{b}	1.4 ± 0.11^{ab}	1.7 ± 0.13^{a}	1.6 ± 0.08^{ab}	1.0 ± 0.30^{b}
PtdIns	4.1 ± 0.32^{ab}	4.3 ± 0.77^{ab}	5.2 ± 0.57^{b}	2.3 ± 0.59^{a}	6.6 ± 0.69	6.1 ± 0.85	6.1 ± 0.48	4.6 ± 0.18	5.3 ± 0.21^{a}	5.7 ± 0.78^{a}	4.9 ± 0.46^{ab}	3.6 ± 0.39^{b}
Ptd_2Gro	5.1 ± 0.31^{ab}	6.6 ± 0.96^{a}	5.1 ± 0.63^{ab}	3.1 ± 0.61^{b}	0.0 ± 0.0^{b}	0.6 ± 0.36^{a}	0.0 ± 0.0^{b}	0.0 ± 0.0^{b}	1.8 ± 0.18	1.7 ± 0.18	1.8 ± 0.18	1.4 ± 0.21
PtdEtn	11.7 ± 0.12^{ab}	14.6 ± 0.99^{a}	11.2 ± 1.23^{ab}	8.9 ± 1.46^{b}	11.5 ± 1.03^{ab}	12.6 ± 0.16^{a}	9.6 ± 1.02^{c}	8.7 ± 0.25^{bc}	10.9 ± 0.62^{a}	11.8 ± 0.72^{a}	9.7 ± 0.62^{ab}	7.5 ± 1.34^{b}
DAG	1.5 ± 0.10^{a}	1.3 ± 0.23^{a}	1.8 ± 0.18^{ab}	2.5 ± 0.39^{b}	1.5 ± 0.12^{ab}	1.7 ± 0.13^{a}	1.6 ± 0.13^{a}	1.1 ± 0.13^{b}	1.6 ± 0.04^{b}	2.1 ± 0.20^{a}	1.8 ± 0.06^{ab}	1.8 ± 0.05^{ab}
CHOL	7.5 ± 0.27	8.9 ± 1.03	9.0 ± 0.97	5.7 ± 1.35	11.6 ± 1.05^{ab}	13.5 ± 1.77^{a}	11.8 ± 1.00^{ab}	8.9 ± 0.49^{b}	10.8 ± 0.88^{ab}	13.3 ± 0.47^{a}	11.1 ± 0.72^{ab}	8.4 ± 1.53^{b}
FFA	16.4 ± 0.34^{a}	13.6 ± 1.65^{ab}	11.0 ± 0.82^{b}	13.4 ± 0.33^{ab}	6.4 ± 0.47	8.6 ± 1.25	7.7 ± 0.67	6.4 ± 0.05	14.0 ± 0.25	14.0 ± 1.27	11.2 ± 1.29	13.4 ± 0.51
TAG	20.5 ± 1.52^{a}	13.3 ± 2.87^{a}	24.6 ± 7.26^{ab}	41.3 ± 7.20^{b}	24.0 ± 5.67^{ab}	18.0 ± 3.97^{a}	26.3 ± 6.08^{ab}	39.2 ± 1.52^{b}	25.7 ± 3.12^{ab}	17.7 ± 4.10^{a}	27.1 ± 5.49^{ab}	42.7 ± 7.29^{b}
$\sum$ PL	54.1 ± 1.10^{ab}	62.9 ± 5.74^{a}	53.6 ± 5.98^{ab}	37.1 ± 5.96^{b}	56.5 ± 4.17^{ab}	58.3 ± 3.92^{a}	52.5 ± 4.83^{ab}	44.3 ± 0.96^{b}	47.9 ± 2.12^{a}	53.0 ± 3.31^{a}	48.9 ± 3.61^{a}	33.7 ± 5.39^{b}
$\sum$ NL	45.9 ± 1.10^{ab}	37.1 ± 5.74^{a}	46.4 ± 5.98^{ab}	62.9 ± 5.96^{b}	43.5 ± 4.17^{ab}	41.7 ± 3.92^{a}	47.5 ± 4.83^{ab}	55.7 ± 0.96^{b}	52.1 ± 2.12^{a}	47.0 ± 3.31^{a}	51.1 ± 3.61^{a}	66.3 ± 5.39^{b}

The lipid class composition was measured in total homogenate, microsomes and mitochondria in liver from fish fed four different diets. The quantity of each lipid class is given as the percentage of total lipids. The values given are means ± SEM ($n = 3$). Different letters indicate significant differences ($P \leq 0.05$) between the different dietary treatments within each lipid class, within each fraction

LPC lyso-phosphatidyl-choline, *SM* sphingomyelin, *PtdCho* phosphatidyl-choline, *PtdSer* phosphatidyl-serine, *PtdIns* phosphatidyl-inositol, *Ptd_2Gro* cardiolipin, *PtdEtn* phosphatidyl-ethanolamine, *DAG* diacyl-glycerol, *CHOL* cholesterol, *FFA* free fatty acids, *TAG* triacylglycerol, *PL* polar lipids, *NL* neutral lipids

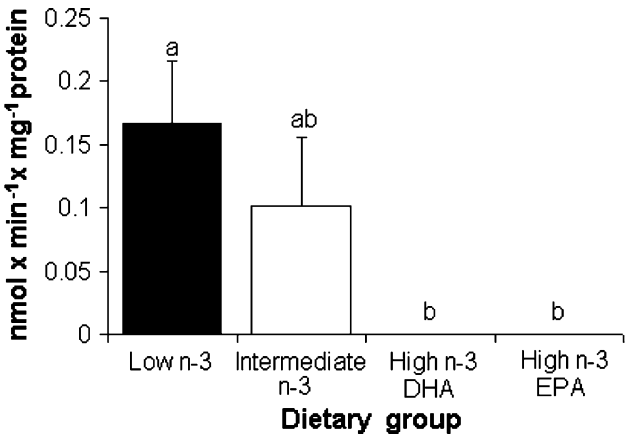

Fig. 1 Mitochondrial β-oxidation activity. β-oxidation was measured in the mitochondrial fraction. Data are means ± SEM ($n = 3$). *Different letters* indicate significant differences ($P \leq 0.05$)

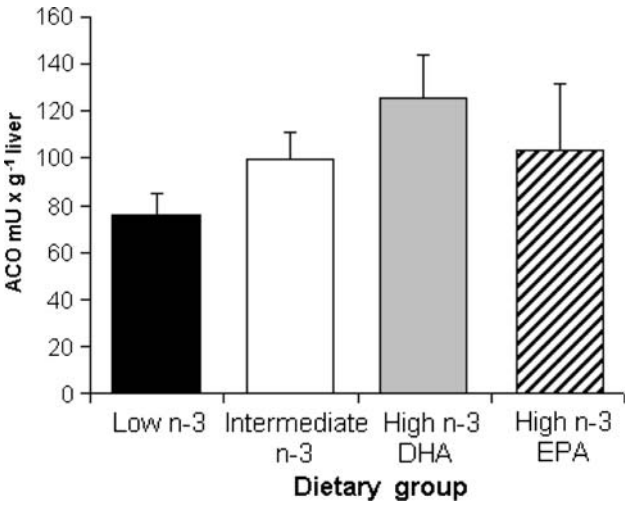

Fig. 2 ACO enzyme activity. ACO activity was measured in the peroxisomal fractions, and is displayed here as the total activity per gram liver tissue. Data are means ± SEM ($n = 3$). *Different letters* indicate significant differences ($P \leq 0.05$)

cells was condensed, and the cellular contents seemed to be released.

Discussion

Feeding Atlantic salmon diets having major differences in the n-3 HUFA content affected the FA composition of the liver. N-3 HUFA in the liver increased with increasing dietary level of these FA. However, even though the level of 22:6n-3 in the low n-3 diet was less than half of the 22:6n-3 level in the high n-3 EPA diet, the percentages of 22:6n-3 recovered in the liver lipids were the same in these two dietary groups. One explanation for this relatively low percentage of 22:6n-3 recovered in liver of fish fed the high n-3 EPA diet may be explained by a lower capacity to produce 22:6n-3 than in those fed a diet with low levels of n-3 HUFA. In addition, the livers of the fish fed the high n-3 EPA diet had significantly higher percentages of 22:5n-3, indicating that the further conversion of 22:5n-3 to 22:6n-3, which involves a Δ6-desaturation step, is inhibited. These findings are further supported by the lower expression of genes for both Δ5-desaturase and Δ6-desaturase in the liver tissue from fish fed the two high-level n-3 HUFA diets. It has previously been shown that Atlantic salmon fed diets containing plant oils have significantly higher desaturation enzyme activity [10–13] and higher expressions of hepatic Δ5-desaturase and Δ6-desaturase genes than those fed FO diets [9, 14]. An alternative explanation to the relatively high 22:6n-3 level in the tissue of fish fed low n-3 diet could be selective utilization of shorter chain, less unsaturated FA, and a more highly conservation of 22:6n-3 in the liver PL of fish fed low n-3 HUFA levels. This is in agreement with what was found in fish fed an essential FA free diet [40]. We have recently shown that feeding salmon diets rich in EPA increases the number of mitochondria in the liver [8]. We know that the shorter chained FA are mainly oxidized within mitochondria [41], and that the longer chained FA, like 22:6n-3, are very poor substrates for mitochondrial FA β-oxidation [42]. The finding that several mitochondrial and peroxisomal genes, involved in FA β-oxidation, were up-regulated in fish fed high levels of dietary 22:6n-3 may then support the last explanation. An unexpected result was therefore that the mitochondrial β-oxidation capacities were dramatically lower in isolated liver mitochondria from fish fed the diets with the high levels of n-3 HUFA, than they were in liver mitochondria from fish fed low n-3 and intermediate n-3 diets. Though not well studied in fish, the mitochondrial membrane is found to be a very sensitive target for lipid peroxidation in mammals [43]. The low FA β-oxidation capacity in our study, seen in livers of fish fed the high n-3 HUFA diets may be the result of an extensive lipid peroxidation of the mitochondrial membranes. This assumption is further supported by the loss of cytochrome c oxidase activity and mitochondrial membrane PL found in livers of fish fed high levels on n-3 HUFA. The higher SOD levels found in both high n-3 EPA and high n-3 DHA dietary groups compared to the two groups with lower level of n-3 HUFA further support the theory that the mitochondrial function has been damaged due to oxidative stress. The decreased percentages of PL, especially Ptd_2Gro, in the high n-3 EPA mitochondrial membranes may also be consistent with a higher level of apoptosis in this group. It has been suggested in mammalian studies that Ptd_2Gro is required for the activity of cytochrome c oxidase [19], and that the Ptd_2Gro content in mitochondria decreases during

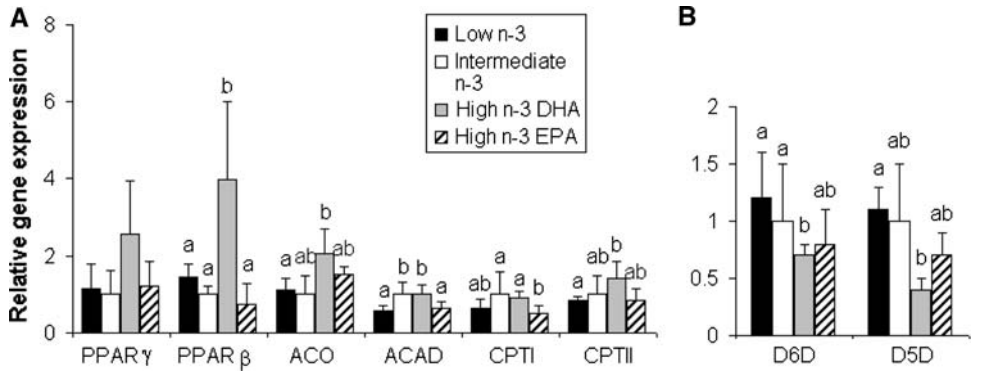

Fig. 3 Relative gene expressions of lipid-related genes. **3a** Gene expressions were measured using isolated mRNA from hepatocytes. Genes involved in gene regulation (PPARs), peroxisomal β-oxidation (ACO) and mitochondrial β-oxidation (ACAD, CPTI, CPTII) were measured. **3b** Δ6-desaturase and Δ5-desaturase activity. Data are presented as means $\pm$ SD ($n = 3$). *Different letters* indicate significant differences between dietary groups within each gene. Significance level is set at $P \leq 0.05$. *PPAR* peroxisome proliferator-activated receptor, *ACO* acyl-CoA oxidase, *ACAD* acyl-CoA dehydrogenase, *CPT I, II* carnitine palmitoyltransferase I, II, *D6D* delta 6 desaturase, *D5D* delta 5 desaturase

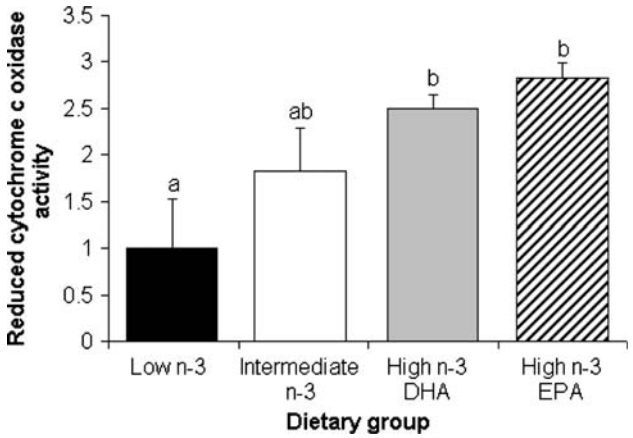

Fig. 4 Cytochrome c oxidase activity. The activity of cytochrome c oxidase was measured in mitochondrial fractions with and without dodecyl. The difference in activity between preparations with and without dodecyl measured in the low n-3 HUFA group was set to 1, and the differences in the other dietary groups are expressed relative to this. Data are means $\pm$ SEM ($n = 3$). *Different letters* indicate significant differences ($P \leq 0.05$)

apoptosis [44]. Oxidative stress in mitochondria is known to mediate the apoptotic signalling pathway in mammals [45], and caspases, in particular caspase-3, play a central role. The activity of caspase-3 increased in the high n-3 EPA dietary group. These results are further confirmed by our EM pictures showing abnormal morphology of hepatocytes from fish fed the high n-3 EPA diet. Nuclear chromatin was more highly condensed in these cells, and cellular contents were released [46]. Although not studied in such detail, similar results were found in grass carp (*Ctenopharyngodon idella*), which show decreased growth performance, increased blood lipid peroxidation, altered lipoprotein synthesis and impaired FA β-oxidation capacity when fed a diet with high lipid level, especially high HUFA levels [47, 48]. These results led us to yet another possible explanation for the lower level of 22:6n-3 found in the high n-3 EPA dietary group relative to the level found in the low n-3 HUFA group. The cause could be increased breakdown of 22:6n-3 due to peroxidation.

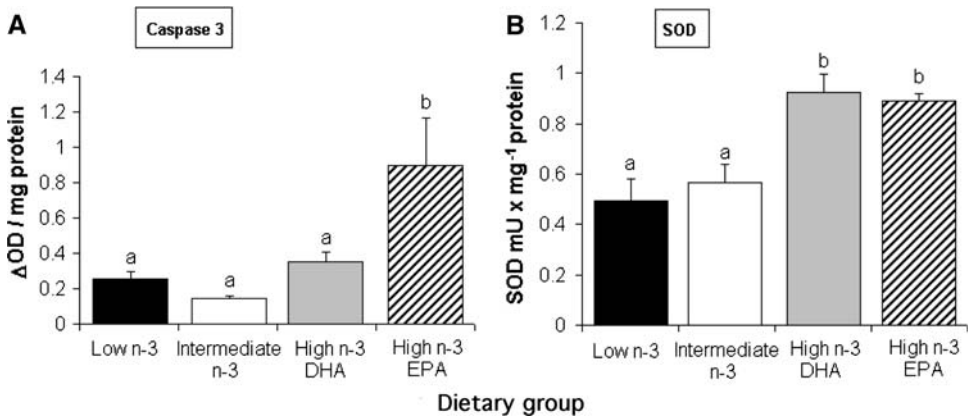

Fig. 5 Enzyme activities. Caspase-3 (5a) and SOD (5b) activity was measured in total homogenates. Data are means $\pm$ SEM ($n = 3$). *Different letters* indicate significant differences ($P \leq 0.05$)

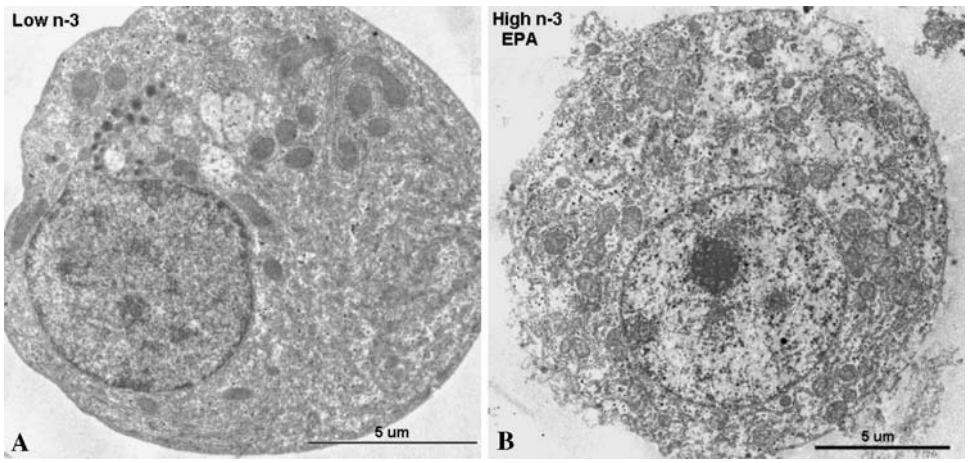

Fig. 6 Electron micrographs of hepatocytes. The images shown are representative cells for fish fed the low n-3 diet (**a**) and fish fed the high n-3 EPA diet (**b**). *Bars* 5 μm

In the present study we fed fish with diets containing high levels (55% of total FA) of n-3 HUFA, and these high values of n-3 rich lipids clearly led to oxidative stress in the liver. In our study, cases of damage were apparent despite of added antioxidants (160 ppm Vitamin E and 150 ppm BHT). This show the importance of protecting high n-3 HUFA feed with proper antioxidants and proper storage in order to minimize the peroxidation of FAs both prior to feeding and in fish after feeding. Stephan et al. [49] demonstrated that a FO-enriched diet fed to turbot (*Scophthalmus maximus*), increased the susceptibility to FA peroxidation. They found that increased levels of dietary and tissue HUFA require increased dietary supplementation with antioxidants to prevent the occurrence of oxidative damage. A correlation between increased dietary HUFA and antioxidant requirement has been found in other fish species as well [50–52], including Atlantic salmon [53]. More research is needed in order to increase the knowledge about suitable antioxidants and concentrations of these in fish diets, in order to protect FA from oxidative damages both in the feed and in the fish.

In this study, we have shown that the expressions of genes encoding proteins involved in lipid metabolism differ between the low n-3 HUFA group and the groups with the highest n-3 HUFA levels. We do not know whether it is peroxidative damage due to the increased level of HUFA, or the HUFA themselves that are involved in the regulation of these genes. Some studies have suggested that the inhibitory effect of HUFA on lipogenic genes is linked to cytotoxic effects, due to the peroxidative mechanism [54, 55]. On the other hand, other studies have shown that lipid-related genes are altered due to the direct transcriptional effect of HUFA themselves, not to peroxidative damage [56, 57].

In conclusion, we have shown that the expressions of several genes related to hepatic lipid metabolism in Atlantic salmon change in response to changes in dietary HUFA. These genes include those for Δ5-desaturase and Δ6-desaturase and several genes whose gene products are involved in mitochondrial functions such as FA *β*-oxidation. The observed increased production of SOD and caspases, and loss of cytochrome c oxidase and Ptd_2Gro in the membranes of fish fed high levels of n-3 HUFA may be a result of increased dietary HUFA levels in the membranes, and subsequent increased oxidative breakdown of FA rich in double bonds. Clearly, the presence of lipids vulnerable to oxidation may be involved in oxidation processes that can have toxic consequences for fish, whether they arise from dietary input or from deficiencies in essential antioxidant nutrients. These results reveal new elements in favor of greater sensitivity of n-3 FAs to peroxidation in fish. Nevertheless, it is important to appreciate that n-3 FA have both adverse and beneficial effects, to adequately evaluate the consequences of membrane enrichment with these lipids.

Acknowledgments We wish to thank Inger Ø. Kristiansen and Målfrid T. Bjerke (Nofima) for skillful technical assistance. This work was carried out with support from the Norwegian Research Council.

References

1. Sargent JR, Tocher DR, Bell JG (2002) The lipids. In: Halver JE, Hardy RW (eds) Fish nutrition. Academic Press, New York, pp 181–257
2. Stubhaug I, Froyland L, Torstensen BE (2005) Beta-Oxidation capacity of red and white muscle and liver in Atlantic salmon (*Salmo salar* L.)—effects of increasing dietary rapeseed oil and olive oil to replace capelin oil. Lipids 40:39–47
3. Stubhaug I, Lie O, Torstensen BE (2006) Beta-oxidation capacity in liver increases during parr-smolt transformation of Atlantic salmon fed vegetable oil and fish oil. J Fish Biol 69:504–517
4. Stubhaug I, Lie O, Torstensen BE (2007) Fatty acid productive value and beta-oxidation capacity in Atlantic salmon (*Salmo salar* L.) fed on different lipid sources along the whole growth period. Aquacult Nutr 13:145–155
5. Vegusdal A, Gjoen T, Berge RK, Thomassen MS, Ruyter B (2005) Effect of 18:1n–9, 20:5n–3, and 22:6n–3 on lipid accumulation and secretion by Atlantic salmon hepatocytes. Lipids 40:477–486

6. Willumsen N, Vaagenes H, Lie O, Rustan AC, Berge RK (1996) Eicosapentaenoic acid, but not docosahexaenoic acid, increases mitochondrial fatty acid oxidation and upregulates 2,4-dienoyl-CoA reductase gene expression in rats. Lipids 31:579–592
7. Vamecq J, Vallee L, de la Porte PL, Fontaine M, de Craemer D, van den Branden C, Lafont H, Grataroli R, Nalbone G (1993) Effect of various n-3/n-6 fatty acid ratio contents of high fat diets on rat liver and heart peroxisomal and mitochondrial [beta]-oxidation. Biochim Biophys Acta 1170:151–156
8. Kjaer MA, Vegusdal A, Gjoen T, Rustan AC, Todorcevic M, Ruyter B (2008) Effect of rapeseed oil and dietary n-3 fatty acids on triacylglycerol synthesis and secretion in Atlantic salmon hepatocytes. Biochim Biophys Acta 1781:112–122
9. Jordal AE, Torstensen BE, Tsoi S, Tocher DR, Lall SP, Douglas SE (2005) Dietary rapeseed oil affects the expression of genes involved in hepatic lipid metabolism in Atlantic salmon (Salmo salar L.). J Nutr 135:2355–2361
10. Bell JG, McEvoy J, Tocher DR, McGhee F, Campbell PJ, Sargent JR (2001) Replacement of fish oil with rapeseed oil in diets of Atlantic salmon (Salmo salar) affects tissue lipid compositions and hepatocyte fatty acid metabolism. J Nutr 131:1535–1543
11. Moya-Falcon C, Thomassen MS, Jakobsen JV, Ruyter B (2005) Effects of dietary supplementation of rapeseed oil on metabolism of [1–14C]18:1n–9, [1–14C]20:3n-6, and [1–14C]20:4n-3 in Atlantic salmon hepatocytes. Lipids 40:709–717
12. Ruyter B, Thomassen MS (1999) Metabolism of n-3 and n-6 fatty acids in Atlantic salmon liver: stimulation by essential fatty acid deficiency. Lipids 34:1167–1176
13. Tocher DR, Bell JG, Dick JR, Crampton VO (2003) Effects of dietary vegetable oil on Atlantic salmon hepatocyte fatty acid desaturation and liver fatty acid compositions. Lipids 38:723–732
14. Zheng X, Tocher DR, Dickson CA, Bell JG, Teale AJ (2004) Effects of diets containing vegetable oil on expression of genes involved in highly unsaturated fatty acid biosynthesis in liver of Atlantic salmon (*Salmo salar*). Aquaculture 236:467–483
15. Gray J (1978) Measurement of lipid oxidation: a review. J Am Oil Chem Soc 55:539–546
16. Nilsson A, Hjelte L, Strandvik B (1992) Incorporation of dietary [14C]arachidonic acid and [3H]eicosapentaenoic acid into tissue lipids during absorption of a fish oil emulsion. J Lipid Res 33:1295–1305
17. Winston GW, Di Giulio RT (1991) Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat Toxicol 19:137–161
18. Halliwell B, Gutteridge J (1999) Free radicals in biology and medicine, 3 edn. Oxford University Press, Oxford
19. Schlame M, Rua D, Greenberg ML (2000) The biosynthesis and functional role of cardiolipin. Prog Lipid Res 39:257–288
20. Paradies G, Petrosillo G, Pistolese M, Di Venosa N, Serena D, Ruggiero FM (1999) Lipid peroxidation and alterations to oxidative metabolism in mitochondria isolated from rat heart subjected to ischemia and reperfusion. Free Radical Bio Med 27:42–50
21. Ostrander DB, Sparagna GC, Amoscato AA, McMillin JB, Dowhan W (2001) Decreased cardiolipin synthesis corresponds with cytochrome c release in palmitate-induced cardiomyocyte apoptosis. J Biol Chem 276:38061–38067
22. Petrosillo G, Ruggiero FM, Pistolese M, Paradies G (2001) Reactive oxygen species generated from the mitochondrial electron transport chain induce cytochrome c dissociation from beef-heart submitochondrial particles via cardiolipin peroxidation. Possible role in the apoptosis. FEBS Lett 509:435–438
23. Seglen PO (1976) Preparation of isolated rat liver cells. Methods Cell Biol 13:29–83
24. Dannevig BH, Berg T (1985) Endocytosis of galactose-terminated glycoproteins by isolated liver cells of the rainbow trout (Salmo gairdneri). Comp Biochem Physiol B 82:683–688
25. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497–509
26. Mason ME, Waller GR (1964) Dimethoxypropane induced transesterification of fats + oils in preparation of methyl esters for gas chromatographic analysis. Anal Chem 36:583–586
27. Hoshi M, Williams M, Kishimoto Y (1973) Esterification of fatty acids at room temperature by chloroform-methanolic HCl-cupric acetate. J Lipid Res 14:599–601
28. Vitiello F, Zanetta JP (1978) Thin-layer chromatography of phospholipids. J Chromatogr 166:637–640
29. De Duve C, Pressman BC, Gianetto R, Wattiaux R, Appelmans F (1955) Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. Biochem J 60:604–617
30. Storrie B, Madden EA (1990) Isolation of subcellular organelles. Methods Enzymol 182:203–225
31. Baudhuin P, Beaufay H, Rahman-Li Y, Sellinger OZ, Wattiaux R, Jacques P, De DC (1964) Tissue fractionation studies. 17. Intracellular distribution of monoamine oxidase, aspartate aminotransferase, alanine aminotransferase, D-amino acid oxidase and catalase in rat-liver tissue. Biochem J 92:179–184
32. Bergmeyer HU, Gawehn K, Grassl M (1974) Methods of enzymatic analysis, 2nd edn. Academic Press, Inc., New York, pp 495–496
33. Beaufay H, Amar-Costesec A, Feytmans E, Thines-Sempoux D, Wibo M, Robbi M, Berthet J (1974) Analytical study of microsomes and isolated subcellular membranes from rat liver: I. Biochemical methods. J Cell Biol 61:188–200
34. Lazarow PB (1981) Assay of peroxisomal beta-oxidation of fatty acids. Methods Enzymol 72:315–319
35. Small GM, Burdett K, Connock MJ (1985) A sensitive spectrophotometric assay for peroxisomal acyl-CoA oxidase. Biochem J 227:205–210
36. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
37. Peterson GL (1977) A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem 83:346–356
38. Simon P (2003) Q-Gene: processing quantitative real-time RT-PCR data. Bioinformatics 19:1439–1440
39. Olsvik P, Lie K, Jordal AE, Nilsen T, Hordvik I (2005) Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. BMC Mol Biol 6:21–29
40. Ruyter B, Rosjo C, Einen O, Thomassen MS (2000) Essential fatty acids in Atlantic salmon: time course of changes in fatty acid composition of liver, blood and carcass induced by a diet deficient in n-3 and n-6 fatty acids. Aquacult Nutr 6:109–117
41. Mannaerts GP, Debeer LJ, Thomas J, De Schepper PJ (1979) Mitochondrial and peroxisomal fatty acid oxidation in liver homogenates and isolated hepatocytes from control and clofibrate-treated rats. J Biol Chem 254:4585–4595
42. Madsen L, Froyland L, Dyroy E, Helland K, Berge RK (1998) Docosahexaenoic and eicosapentaenoic acids are differently metabolized in rat liver during mitochondria and peroxisome proliferation. J Lipid Res 39:583–593
43. Vladimirov YA, Olenev VI, Suslova TB, Cheremisina ZP (1980) Lipid peroxidation in mitochondrial membrane. Adv Lipid Res 17:173–249
44. Nomura K, Imai H, Koumura T, Kobayashi T, Nakagawa Y (2000) Mitochondrial phospholipid hydroperoxide glutathione peroxidase inhibits the release of cytochrome c from mitochondria by suppressing the peroxidation of cardiolipin in hypoglycaemia-induced apoptosis. Biochem J 351:183–193
45. Shimizu S, Eguchi Y, Kamiike W, Waguri S, Uchiyama Y, Matsuda H, Tsujimoto Y (1996) Retardation of chemical

hypoxia-induced necrotic cell death by Bcl-2 and ICE inhibitors: possible involvement of common mediators in apoptotic and necrotic signal transductions. Oncogene 12:2045–2050
46. Otsuki Y, Li Z, Shibata MA (2003) Apoptotic detection methods—from morphology to gene. Prog Histochem Cytochem 38:275–339
47. Du ZY, Clouet P, Zheng WH, Degrace P, Tian LX, Liu YJ (2006) Biochemical hepatic alterations and body lipid composition in the herbivorous grass carp (Ctenopharyngodon idella) fed high-fat diets. Br J Nutr 95:905–915
48. Du ZY, Clouet P, Huang LM, Degrace P, Zheng WH, He JG, Tian LX, Liu YJ (2008) Utilization of different dietary lipid sources at high level in herbivorous grass carp (Ctenopharyngodon idella): mechanism related to hepatic fatty acid oxidation. Aquacult Nutr 14:77–92
49. Stephan G, Guillaume J, Lamour F (1995) Lipid peroxidation in turbot (Scophthalmus maximus) tissue: effect of dietary vitamin E and dietary n-6 or n-3 polyunsaturated fatty acids. Aquaculture 130:251–268
50. Puangkaew J, Kiron V, Satoh S, Watanabe T (2005) Antioxidant defense of rainbow trout (Oncorhynchus mykiss) in relation to dietary n-3 highly unsaturated fatty acids and vitamin E contents. Comp Biochem Physiol C 140:187–196
51. Roem AJ, Kohler CC, Stickney RR (1990) Vitamin E requirements of the blue tilapia, *Oreochromis aureus* (Steindachner), in relation to dietary lipid level. Aquaculture 87:155–164
52. Watanabe T, Takeuchi T, Wada M, Uehara R (1981) The relationship between dietary-lipid levels and alpha-tocopherol requirement of rainbow-trout. Bull Jpn Soc Sci Fish 47:1463–1471
53. Waagboe R, Sandnes K, Sandvin A, Lie O (1991) Feeding three levels of *N*-3 polyunsaturated fatty acids at two levels of vitamin E to Atlantic salmon (*Salmo salar*). Growth and chemical composition, Fiskeridir Skr (Ernaering) vol 4, no 1, pp 51–63, 51–63
54. Foretz M, Foufelle F, Ferré P (1999) Polyunsaturated fatty acids inhibit fatty acid synthase and spot-14-protein gene expression in cultured rat hepatocytes by a peroxidative mechanism. Biochem J 341:371–376
55. Mikkelsen L, Hansen HS, Grunnet N, Dich J (1993) Inhibition of fatty acid synthesis in rat hepatocytes by exogenous polyunsaturated fatty acids is caused by lipid peroxidation. Biochim Biophys Acta 1166:99–104
56. Eder K, Kirchgessner M (1998) The effect of dietary vitamin E supply and a moderately oxidized oil on activities of hepatic lipogenic enzymes in rats. Lipids 33:277–283
57. Kim H, Choi S, Lee HJ, Lee JH, Choi H (2003) Suppression of fatty acid synthase by dietary polyunsaturated fatty acids is mediated by fat itself, not by peroxidative mechanism. J Biochem Mol Biol 36:258–264
58. Mundheim H, Aksnes A, Hope B (2004) Growth, feed efficiency and digestibility in salmon (Salmo salar L.) fed different dietary proportions of vegetable protein sources in combination with two fish meal qualities. Aquaculture 237:315–331