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ORIGINAL PAPER

Dietary fatty acids affect mitochondrial phospholipid compositions and mitochondrial gene expression of rainbow trout liver at different ages

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Abstract Mitochondria are among the first responders to various stressors that challenge the homeostasis of cells and organisms. Mitochondrial decay is generally associated with impairment in the organelle bioenergetics function and increased oxidative stress, and it appears that deterioration of mitochondrial inner membrane phospholipids (PL), particularly cardiolipin (CL), and accumulation of mitochondrial DNA (mtDNA) mutations are among the main mechanisms involved in this process. In the present study, liver mitochondrial membrane PL compositions, lipid peroxidation, and mtDNA gene expression were analyzed in rainbow trout fed three diets with the same base formulation but with lipid supplied either by fish oil (FO), rapeseed oil (RO), or high DHA oil (DHA) during 6 weeks. Specifically, two feeding trials were performed using fish from the same population of two ages (1 and 3 years), and PL class compositions of liver mitochondria, fatty acid composition of individual PL classes, TBARS content, and mtDNA expression were determined. Dietary fatty acid composition strongly affected mitochondrial membrane composition from trout liver but observed changes did not fully reflect the diet, particularly when it contained high DHA. The changes were PL specific, CL being particularly resistant to changes in DHA. Some significant differences observed in expression of mtDNA with diet may suggest long-term dietary effects in mitochondrial gene expression which could affect electron transport chain function. All the changes were influenced by fish age, which could be related to the different growth rates observed between 1- and 3-year-old trout but that could also indicate age-related changes in the ability to maintain structural homeostasis of mitochondrial membranes.

Keywords Cardiolipin · Diet · Fatty acid · Mitochondria · Rainbow trout · Oxidative stress · Phospholipid

Abbreviations

ANT	Nucleotide translocase
BHT	Butylated hydroxytoluene
cDNA	Complementary DNA
CL	Cardiolipin
COX	Cytochrome c oxidase complex
DHA	Docosahexaenoic acid
E	PCR efficiency
EPA	Eicosapentaenoic acid
ETC	Electron transport chain
FA	Fatty acid
FAME	Fatty acid methyl esters
HPTLC	High performance thin layer chromatography
HUFA	Highly unsaturated fatty acids
LA	Linoleic acid
LC-PUFA	Long-chain polyunsaturated fatty acid
MPH	Membrane pacemaker hypothesis
mtDNA	Mitochondrial DNA
MUFA	Monounsaturated fatty acids
NAC	No-amplification control
ND	NADH-coenzyme Q oxidoreductase complex
NTC	No-template control
PC	Phosphatidylcholine

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PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PIn	Peroxidation index
PL	Phospholipid
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
RO	Rapeseed oil
ROS	Reactive oxygen species
SFA	Saturated fatty acids
FO	Fish oil
SM	Sphingomyelin
RT-PCR	Real-time PCR
TBARS	Thiobarbituric acid reactive substances
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TLC	Thin layer chromatography

Introduction

Membrane lipid composition can be affected by three main factors in fish: temperature, age, and diet. Ectotherm vertebrates are well known to perform cold-induced lipid remodeling in the process of homeoviscous adaptation, and the majority of the cold-active ectotherm species investigated so far respond to cold by either increasing the ratio of unsaturated to saturated fatty acids (FA) or increasing polyunsaturated fatty acids (PUFA) in membranes (Hazel and Williams 1990; Moyes and Ballantyne 2011). Membrane FA composition can also change over the course of an organism's life. In vertebrates and invertebrates with finite growth, PUFA have either been shown to increase or remain constant with age (Hulbert et al. 2006, Hulbert 2007), Finally, It is also known that changes in the FA composition of the diet can drastically modify membrane lipid composition in a wide range of taxa, including fish (Hulbert et al. 2005, Guderley et al. 2008; Martin et al. 2013). These changes affect not only plasma membranes but also those from subcellular organelles, including mitochondria (Rohrbach 2009), altering their physical properties and influencing the activities of membrane enzymes and, thus, cellular metabolic pathways (Barzanti et al. 1994). This would be a crucial process in the life cycle of an organism as it has been observed in a wide number of animal species that membrane composition and lifespan are linked through the regulation of metabolic rate (Hulbert 2008). The membrane pacemaker hypothesis of aging (MPH, also referred to as the homeoviscous-longevity theory of aging) is an extension of the oxidative stress theory of aging that emphasizes variation in the FA composition of membranes as an important influence on lipid peroxidation and consequently on the rate of aging and determination of lifespan. Increased polyunsaturation of cell membrane lipids (high number of double bonds) results in more fluid membranes that can promote higher molecular activity of membrane proteins and, in turn, increase the metabolic activity of cells, tissues, and, consequently, whole animals (Pamplona et al. 2002; Sanz et al. 2006). At the same time, membranes with increased levels of PUFA are more prone to attack by oxidizing agents and will then participate in long, free radical reactions that will propagate oxidative damage throughout the organelle (Hulbert et al. 2005). This hypothesis would explain the huge differences in life span existing among animal species with different levels of unsaturation in their membranes, but could also explain how situations such as thermal or dietary changes in an animal's life cycle, could compromise metabolic activity, oxidative stress, and tissue function.

The key role of FA composition in membrane processes suggests that dietary effects upon membrane lipid structure should be, at least in part, regulated. Both factors, the overall status of the membrane and the specific characteristics of lipid components, must be balanced as they, very likely, participate in maintaining membrane function (Paradies et al. 1992; Zabelinskii et al. 1999; Guderley et al. 2008; Modi and Katyare 2008). It is known that mitochondrial membranes have different compositions of lipids such as phospholipids (PL), glycolipids, and cholesterol compared with plasma membranes (Wiseman 1996), this being related with the role of the organelle in oxygen consumption (Hoch 1992). Mitochondrial inner membrane (MIM) uniquely contains cardiolipin (CL), a key molecule associated with complexes I, III, and IV of the electron transport chain (ETC), F_1F_0ATPase and nucleotide translocase (ANT) (Yamaoka et al. 1988; Paradies et al. 2002). CL has a high content of PUFA which makes it, along with its proximity to the site of ROS production, particularly prone to peroxidation (Paradies et al. 2011). Mitochondrial membranes also contain small amounts of sphingomyelin (SM), another potentially important PL that is present in all cell membranes and has also been suggested as a mediator of aging and determinant of lifespan (Cutler and Mattson 2001). SM not only has membrane-rigidifying properties, which retard the lateral propagation of free radicals (Subbaiah et al. 1999), but also is a precursor of many signaling molecules, some associated with apoptosis (Hannum and Obeid 1997). The particular roles of individual PL classes are associated with characteristic FA compositions that confer specific properties related to membrane fluidity and functions (Zabelinskii et al. 1999). Therefore, not only changes in PL class composition, but also alterations in PL FA compositions would modify their molecular properties and their roles in membrane functions (Shigenaga et al. 1994; Chicco and Sparagna 2007; Crimi and Esposti 2011; Monteiro et al. 2013).

Although lipid peroxidation is quantitatively the main oxidative process inside mitochondria, other organelle

molecules are also attacked and damaged by ROS, including proteins and nucleic acids (Sanz et al. 2006). Mitochondrial DNA (mtDNA) is very much exposed to ROS production as it is located very close to the site of ROS production. Moreover, mtDNA is not extremely condensed and protected by histones, as it is nuclear DNA, and its repair activity is limited (Paradies et al. 2011). It has been suggested that mtDNA is also a primary target of ROS and, as more mutations in critical coding regions accumulate, complexes of the ETC become less efficient or inactive, leading to a decline in mitochondrial function (Paradies et al. 2002). Since mtDNA encodes either polypeptides of ETC or components required for their synthesis, mutations in mtDNA will affect the ETC as a whole. ETC-deficient cells are apoptosis prone and increased cell loss is therefore a likely important consequence of mitochondrial dysfunction in situations of high oxidative stress (Trifunovic and Larsson 2008).

In summary, mitochondria are among the first responders to various stressors that challenge the homeostasis of cells and organisms (Manoli et al. 2007). Mitochondrial decay is generally associated with impairment in mitochondrial bioenergetics function and increased oxidative stress (Paradies et al. 2011), and it seems clear that deterioration of MIM PLs, particularly of CL, and accumulation of mtDNA mutations are mechanisms involved in this process. Changes in the FA composition of the diet modify mitochondrial membrane composition (reviewed in Hulbert et al. 2005) and can alter the organelle function (Clandinin et al. 1985; Barzanti et al. 1994; Guderley et al. 2008; Martin et al. 2013) which can lead to an imbalance in organelle oxidative status. The aim of the present study was to determine the impact of diet lipid composition on mitochondrial membrane composition and mtDNA damage as possible regulators of the processes associated with mitochondrial decay under high oxidative stress situations in fish. Rainbow trout (*Oncorhynchus mykiss*) was used as a vertebrate model because it is a well-studied species, widely reared in Europe, its age can be easily monitored (Almroth et al. 2010) and, along with other species of salmonids, it has been used previously in studies on dietary lipid, oxidative stress, and mitochondrial function (Otto and Moon 1996; Zabelinskii et al. 1999; Kraffe et al. 2007; Østbye et al. 2011). For the present study, fish of two well-differentiated ages (fast growth period and mature period) were used in order to study the influence of fish age and body mass on these mechanisms.

Methods

Experimental fish and sampling

A population of rainbow trout, *O. mykiss*, was maintained from eggs (Howietoun Fishery, Stirling, UK) to 3 years under controlled feeding and rearing conditions throughout in the freshwater aquarium facilities at the Institute of Aquaculture, University of Stirling. Fish were fed twice a day ad libitum with a commercial feed (50 % protein, 20 % fat, Skretting, Northwich, UK) and kept under ambient water temperature conditions before being transferred to experimental thermo-regulated tanks. When fish reached 1 year of age, 240 fish (72.4 ± 12.6 g average body weight) were distributed into six tanks equipped with a thermostat to maintain the temperature at 12 ± 1 °C, kept under a 12L:12D photoperiod and fed, in duplicate, one of three experimental diets consisting of the same base formulation, but with lipid supplied either by fish oil (FO diet), rapeseed oil (RO), or an oil containing high docosahexaenoic acid, 22:6n-3 (DHA) (Table 1). Fish were fed twice a day ad libitum for 6-weeks and then euthanized by exposure to the anesthetic benzocaine hydrochloride (400 mg l^{-1}) for 10 min following the cessation of opercular movement. Livers were collected for analyses. The experiment was repeated identically when the remaining fish (60 fish of 625.1 ± 33.8 g of average body weight; 10/tank) reached 3 years of age. For 1-year-old fish, livers of five animals were pooled to provide sufficient material for the different

Table 1 Feed formulations and analyzed proximate compositions (means, $n = 4$)

	FO	RO	DHA
Ingredients (g per 100 g dry weight)			
Fishmeal[a]	40.0	40.0	40.0
Soya meal[a]	15.0	15.0	15.0
Wheat[b]	10.0	10.0	10.0
Corn gluten[c]	10.0	10.0	10.0
Lysine	0.2	0.2	0.2
Carboxymethyl-cellulose (CMC)	3.06	3.06	3.06
Southern hemisphere fish oil[a]	15.6	0.0	0.0
Rapeseed oil[d]	0.0	15.6	0.0
DHA oil[e]	0.0	0.0	15.6
Choline chloride (40 % w/v)	0.4	0.4	0.4
Premixes[f]	5.74	5.74	5.74
Proximate analysis (% dry matter)			
Dry matter	85.3	82.5	88.3
Crude protein	47.1	46.7	46.9
Crude lipid	24.5	24.5	23.4
Nitrogen-free extract (NFE)	17.1	17.8	18.1
Ash	11.2	10.9	11.6

[a] BioMar Ltd., Grangemouth, UK

[b] Aquatic Feeds, Denmark

[c] MP Biomedicals, LLC

[d] Tesco Ltd., UK

[e] Incromega TG0525. Croda International Plc, East Yorkshire, UK

[f] Vitamins, minerals and antioxidants, University of Stirling, UK

analyses and also to reduce inter-individual variability. For 3-year-old animals, two livers per pool were taken after considering fish size and numbers. A portion of each pool was immediately taken into RNA*later*® (Life technologies, Paisley, UK) following the manufacturer's instructions and stored at −20 °C for molecular analysis, while the remainder of the tissue sample was processed to obtain enriched mitochondrial preparations. The purified mitochondrial isolates were analyzed to determine mitochondrial membrane lipid composition and peroxidation. Fish were treated in accordance with British national ethical requirements established by the UK Government Home Office and guidelines determined by the Animals (Scientific Procedures) Act 1986.

Experimental diets

Practical pelleted diets (2- and 4-mm diameter for 1- and 3-year-old trout, respectively) containing 47 % crude protein and 24 % crude lipid were formulated using the same basal ingredients and varying only in the lipid source used: Southern hemisphere fish oil (FO), rapeseed oil (RO), and Incromega TG0525 (DHA) (Table 1). The fatty acid compositions of the diets are shown in Table 2. The control diet (FO) contained 31.7 % saturated fatty acids (SFA), mainly 16:0, 27.8 % monounsaturated fatty acids (MUFA), 11.7 % of which was 18:1n-9, and 40.6 % PUFA, with 6.8 % as n-6 PUFA and 30.3 % as n-3 PUFA. The FO diet contained a high proportion of the n-3 long-chain polyunsaturated fatty acid (LC-PUFA), eicosapentaenoic acid (20:5n-3, EPA) and DHA, in approximately equal amounts. Diet RO had lower levels of SFA (12.3 %), higher percentages of MUFA (56.0 % with 48.2 % as 18:1n-9), and lower PUFA (31.7 %) with just 2.1 % EPA and 3.6 % DHA. Finally, the DHA diet contained similar proportions of SFA, MUFA, and PUFA to the FO diet but had a lower percentage of EPA (6.6 %) and higher percentage of DHA (22.3 %). The diets were formulated to meet all the known nutritional requirements of salmonid fish (National Research Council 2011).

Table 2 Fatty acid compositions (% of total fatty acids) of experimental diets

	FO	RO	DHA
14:0	7.5	1.3	3.3
16:0	19.9	8.8	18.4
18:0	3.7	2.0	3.9
∑Saturated	31.7	12.3	26.4
16:1n-7	8.8	1.5	5.2
18:1n-9	11.7	48.2	18.5
18:1n-7	3.3	2.6	2.7
20:1n-9	1.6	1.8	2.4
22:1n-11	1.5	1.4	2.0
∑Monounsaturated	27.8	56.0	31.8
18:2n-6	4.7	18.5	5.6
20:3n-6	0.2	0.0	1.6
20:4n-6	1.2	0.0	0.0
22:5n-6	0.3	0.0	1.1
∑n-6 PUFA	6.8	18.5	8.3
18:3n-3	2.2	6.7	1.3
20:5n-3	13.8	2.1	6.6
22:5n-3	1.7	0.2	1.1
22:6n-3	12.1	3.6	22.3
∑n-3 PUFA	30.3	12.9	31.9
∑PUFA	40.6	31.7	41.8
n-3/n-6	4.5	0.7	3.8
PIn	131.7	63.2	204.1

Fatty acids representing less than 1.0 % of total fatty acids are not shown

PIn peroxidation index, *PUFA* polyunsaturated fatty acids

Mitochondria isolation

Approximately, 2 g of liver pate was homogenized in 8 mL ice-cold sucrose buffer (0.4 M phosphate buffer pH 7.4, 0.25 M sucrose, 0.15 M KCl, 40 mM KF, and 1 mM *N*-acetylcysteine) using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK). Homogenates were centrifuged at 600×*g* for 6 min, the pellet discarded (cell/nuclei debris) and the supernatant re-centrifuged at 600 g. Resulting supernatants were then centrifuged at 6,800×*g* for 10 min and the resulting pellet (mitochondrial fraction) was used for lipid extraction. To verify that pellets were highly enriched with mitochondria, a portion was fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer overnight at 4 °C, and then processed as specified by Rajapakse et al. (2001) prior to analysis by transmission electron microscopy (Tecnai™ G2 Spirit BioTWIN, FEI Europe, Eindhoven, The Netherlands).

Lipid extraction and phospholipid class composition

Total lipid contents of livers and diet samples were determined gravimetrically after extraction by homogenization in chloroform/methanol (2:1, v/v) containing 0.01 % butylated hydroxytoluene (BHT) as antioxidant, basically according to Folch et al. (1957). Phospholipid (PL) classes were separated by high-performance thin layer chromatography (HPTLC) using 10 × 10 cm silica gel plates (VWR, Lutterworth, England) and methyl acetate/isopropanol/chloroform/methanol/0.25 % (w/v) KCl (25:25:25:10:9, by vol.) as solvent system (Olsen and Henderson 1989). The lipid classes were visualized by charring at 160 °C for 15 min after spraying with 3 % (w/v) aqueous cupric acetate containing 8 % (v/v) phosphoric acid and quantified

Table 3 Forward and reverse primers used in gene expression studies

Gene name	Forward	Reverse	Amplicon size	Efficiency (%)
COX 3	GTAACATGAGCCCACCACAG	CGACAAAGAAAGTAGAGCCGT	168	92.3
ND1	CAACGTAGCCCAAGAAAGCA	ACTAATTCTGACTCTCCTTCTGTG	128	100.5
ND3	CTATTACCATCACATTATCCGCAG	GAAAGAAGCGTAAGGAGAAGGG	146	96.5
ND4	TGAACTACATCAATCGCCCA	GTTGCTAAATAGAGGTTGGAGG	106	97.2
ND4L	CTCTCAGCCCTTCTATGCCT	AACGCTAGGAGAAGTATCGGG	190	100.5
ND5	CCTATTGCCCTGTATGTAACC	ATGATATAATTCCGACTCCCTCTC	183	92.3
ND6	ACTCCTTAAACTCGTCCACTG	GGGATGCTTGTGGTATTTGCT	183	92.3

by densitometry using a CAMAG-3 TLC scanner (version Firmware 1.14.16) (Henderson and Tocher 1992). Scanned images were recorded automatically and analyzed by computer using winCATS (Planar Chromatography Manager, version 1.2.0).

Phospholipid fatty acid composition

Phospholipid classes were separated by preparative-TLC, using silica gel plates (20 × 20 cm) (VWR) and the solvent system as above. Individual phospholipid classes were identified by comparison with known standards after spraying with 1 % (w/v) 2′,7′-dichlorofluorescein in 97 % (v/v) methanol containing 0.05 % (w/v) BHT, and visualized under UV light (UVGL-58 Minerallight® Lamp, Ultraviolet Prod. Inc., Calif., USA). Silica corresponding to each phospholipid class was scraped from the plate into a test tube and subjected directly (on silica) to acid-catalyzed transmethylation at 50 °C overnight following addition of 2 ml of 1 % (v/v) sulfuric acid in methanol in order to prepare fatty acid methyl esters (FAME) (Christie 2003). FAME were separated and quantified by gas–liquid chromatography (Carlo Erba Vega 8160, Milan, Italy) using a 30 m × 0.32 mm i.d. capillary column (CP Wax 52CB, Chrompak, London, UK) and on-column injection at 50 °C. Hydrogen was used as carrier gas, and temperature programming was from 50 to 150 °C at 40 °C min^{-1} and then to 230 °C at 2.0 °C min^{-1}. Individual methyl esters were identified by comparison with known standards. Data were collected and processed using Chromcard for Windows (version 1.19).

Measurement of thiobarbituric acid reactive substances (TBARS)

Approximately, 1 mg of total lipid extract from liver mitochondria was used for the measurement of TBARS using an adaptation of the protocol of Burk et al. (1980). Briefly, 50 µl of 0.2 % (w/v) BHT in ethanol was added to the sample followed by 0.5 ml of 1 % (w/v) TBA and 0.5 ml 10 % (w/v) TCA, both solutions freshly prepared. The reagents were mixed in a stoppered test tube and heated at 100 °C for 20 min. After cooling, particulate matter was removed from the homogenate by centrifugation at 2,000×*g*, and fluorescence in the supernatant determined in the spectrophotometer at 532 nm against a blank sample. The concentration of TBARS, expressed as ng g of $lipid^{-1}$, was calculated using the absorption coefficient 0.156 μM^{-1} cm^{-1}.

RNA preparation, cDNA synthesis, and quantification

Total RNA was extracted from individual samples using TRI Reagent (Sigma-Aldrich, Dorset, UK) according to manufacturer's instructions with high salt precipitation (Chomczynski and Mackey 1995). RNA quantity, integrity, and purity were assessed by agarose gel electrophoresis and spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, USA). RNA samples were aliquoted and diluted to a final concentration of 200 ng/µl. First-strand complementary DNA (cDNA) was synthesized from 2 µg of RNA using High-Capacity cDNA Reverse Transcription kits (Applied Biosystem, Paisley, UK) and primed with a mixture of Oligo dT and random primers (1:3).

Information about target genes was retrieved from the mitochondrion genome sequence (Genbank accession number DQ288271) and used to design primers for real-time PCR (RT-PCR) with PerlPrimer v1.1.17 (Marshall 2004). Primers were designed to target seven mitochondrial genes: COX3, ND1, ND3, ND4, ND4L, ND5, and ND6 (Table 3). Three housekeeping genes were evaluated as internal reference (elongation factor 1α, glyceraldehyde 3-phosphate dehydrogenase, and β-actin), but they were not stable but variable between diets or age groups and were not used for normalization (data not shown). RT-qPCR reactions were carried out on a Biometra thermal cycler (Gottingen, Germany) using Luminaris Color HiGreen qPCR Master mix (Thermo Scientific, Leicestershire, UK) according to the instructions provided by the manufacturer. To avoid technical variation between runs, all treatment and control samples from both age groups were analyzed on the same plate together with a $\log_{10}$ serial dilution of pooled samples used to calculate PCR efficiency (E). Each reaction

was conducted in a total volume of 20 µl in duplicates. All primer pairs were designed to have an optimal annealing temperature of 60 °C. A melting curve analysis was performed after every amplification program to verify specificity of the target and absence of primer dimers, and a no-template control (NTC) was included with each assay to verify that PCR master mixes were free of contamination. For each assay, E was determined by the equation [$E = 10^{\wedge(-1/\text{slope})}$] (Table 3). As the reference genes tested in this study were not usable for normalization, the relative expression of each gene was calculated using the delta-Ct transformation [$\text{Ratio}_{(\text{test/calibrator})} = E\wedge Ct(\text{calibrator}) - Ct(\text{test})$] and log transformed before statistical analyses. The average of the 1-year-old FO treatment was used as the calibrator.

Indexes and statistical analysis

The LC-PUFA index corresponds to the sum of fatty acids with 20 or more carbons and two or more double bonds. The peroxidation index (PIn) was used as an estimate of PL susceptibility to oxidation and was calculated using the formula: PIn = 0.025 × (percentage of monoenoics) + 1 × (percentage of dienoics) + 2 × (percentage of trienoics) + 4 × (percentage of tetraenoics) + 6 × (percentage of pentaenoics) + 8 × (percentage of hexaenoics) (Witting and Horwitt 1964). Specific growth rate (SGR % day^{-1}): [(ln W_t − ln W_i)/T)] × 100, where W_t = mean final weight, W_i = mean initial weight and T = total experimental days (Jaya-Ram et al. 2008). Results are presented as mean ± SD ($n = 4$ for lipid and TBARS analyses and $n = 6$ for gene expression assays). Data were checked for homogeneity of variances by the Levene's test and percentage data from PL content, PL class composition, and PL FA analyses were arc-sin transformed before further statistical analysis. A two-way analysis of variance (ANOVA) was used to assess the differences among groups based on diet and age. Post-hoc comparisons were made using the Bonferroni t test for multiple comparisons. All statistical analyses were performed using SPSS Statistical Software System version 15.0 (SPSS Inc., Chicago, USA). Differences were regarded as significant when $P < 0.05$.

Results

Fish performance

There were no differences in feed intake between the different dietary groups for either 1- (~1.5 % BW/day) or 3-year-old trout (~0.8 % BW/day). The 1-year-old fish showed a considerably higher SGR (around 1.5) than 3-year-old trout (~0.45) during the feeding trial (Fig. 1). No statistically significant differences among the feeding groups were found for either 1- [$F(2,88) = 0.786$, $P = 0.459$] or 3-year-old trout [$F(2, 56) = 0.116$, $P = 0.891$]. No significant interaction between fish age and diet was observed for SGR ($P = 0.549$).

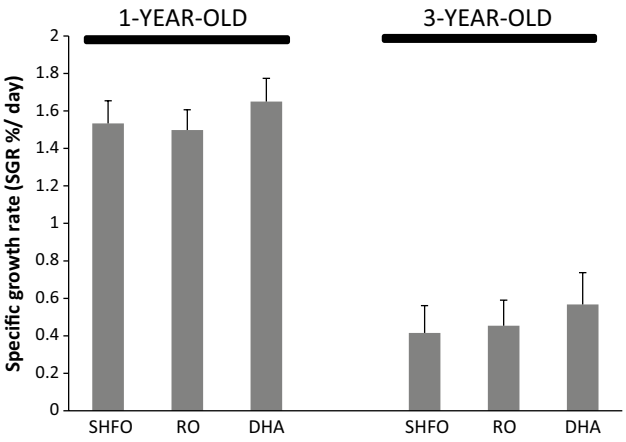

Fig. 1 Specific growth rate (SGR % day^{-1}) of 1- and 3-year-old trout fed the three experimental diets. Results are mean ± SEM ($n = 91$ for 1- and $n = 59$ for 3-year-old fish). No statistical differences among feeding groups for either age group were found

Effects of diet on mitochondrial phospholipid content of trout liver

The phospholipid (PL) content and PL class composition of liver mitochondria from 1- and 3-year-old rainbow trout fed the experimental diets are shown in Fig. 2. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were the most abundant PL species in all the experimental groups, constituting more than 71 % of total PL in 1-year-old trout and more than 68 % in 3-year-old animals. Among the remaining PL classes and focusing only in the control group (FO) in both age groups, the rank order was cardiolipin (CL) (9.5 and 11.4 % for 1- and 3-year-old trout, respectively), followed by phosphatidylinositol (PI) (4.4 and 6.6 %) with lower amounts of phosphatidylserine (PS) and sphingomyelin (SM). Mitochondria from liver of 1-year-old fish fed the FO diet had a different PL composition to that of 3-year-old fish, with significantly more PE ($P = 0.000$), SM ($P = 0.000$) and CL ($P = 0.020$) while the content of PS ($P = 0.027$) and PI ($P = 0.000$) was lower.

Dietary FA composition affected PL content and composition in rainbow trout. In 1-year-old trout, several significant changes were found among experimental groups. The RO group showed a higher content of PC [$F(2.9) = 25.051$, $P = 0.000$], PE [$F(2.9) = 5.050$, $P = 0.020$], and PI [$F(2.9) = 6.173$, $P = 0.010$] and lower proportions of total PL [$F(2.9) = 30.389$, $P = 0.000$], CL [$F(2.9) = 55.566$, $P = 0.000$], and SM [$F(2.9) = 12.062$, $P = 0.001$] compared with the FO and DHA groups. The FO and DHA

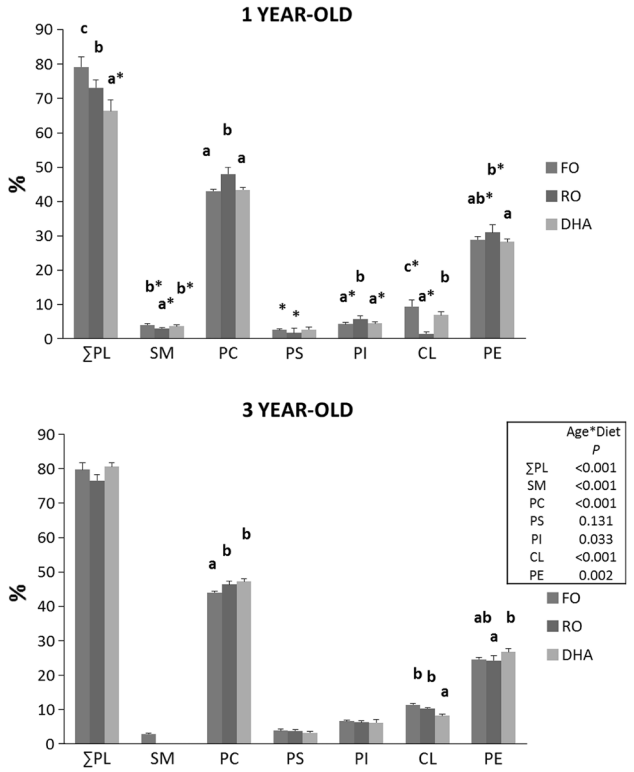

Fig. 2 Phospholipid content (percentage of total lipid weight) and phospholipid class composition (percentage of total phospholipids) of mitochondria isolated from liver of 1- and 3-year-old rainbow trout fed three different diets. Results are mean ± SEM. ($n = 4$). *Different superscript letters* represent differences between feeding groups for each phospholipid class as determined by two-way ANOVA ($P < 0.05$). Table represents P values for interaction Diet and Age for each phospholipid class ($P < 0.05$). *Asterisks* denote significant differences between age groups for each phospholipid class when compared using a Bonferroni test ($P < 0.05$). *CL* cardiolipin, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine, *PI* phosphatidylinositol, *PL* phospholipid, *∑PL* total phospholipids, *PS* phosphatidylserine, *SM* sphingomyelin. SM content found in mitochondria of 3-year-old trout fed the RO and DHA diet was lower than 0.4 %

groups had a similar PL composition with only CL showing a significant difference ($P = 0.009$), being higher in fish fed FO compared to fish fed DHA (Fig. 2). However, the DHA group had the lowest content of total PL. A different situation was found in 3-year-old fish, with fewer differences among the feeding groups. The effect of diet fatty acid composition on mitochondrial PL composition was age-dependent for all classes except PS, (Age*Diet, Fig. 2).

Effects of diet on phospholipid fatty acid compositions of liver mitochondria

Fatty acid compositions of the three main phospholipid classes (PC, PE, and CL) from liver of 1- and 3-year-old rainbow trout are presented in Tables 4, 5 and 6. The fatty acid compositions of the minor PL classes, PI, PS, and SM, are included as Supplementary Tables 1, 2 and 3. The FA profiles of mitochondrial PC, PE, and CL from trout liver were basically similar in both age groups (Tables 4, 5 and 6). Further differences were found in PI (Supplementary Table 1) and, particularly, in PS which showed higher DHA ($P = 0.000$) and PIn ($P = 0.000$) in 1-year-old trout fed the FO diet when compared to 3-year-old fish (Supplementary Table 2). Diet lipid composition significantly affected the FA profiles of individual PL species from 1-year-old fish mitochondria, and these effects were different for each PL class. Mitochondria from 1-year-old fish fed the RO diet showed lower SFA in the main PL classes (around 8 % in PC, $P = 0.000$; 3 % in PE, $P = 0.000$ and CL, $P = 0.030$) and in PS ($P = 0.000$), increased MUFA in PC (around 4 %, $P = 0.0003$), PE (2 %, $P = 0.038$), PS (4 %, $P = 0.001$), and PI (5 %, $P = 0.002$), and decreased MUFA in CL (6 %, $P = 0.000$) when compared with the FO group (Tables 4, 5 and 6, Supplementary Tables 1, 2). There were also significant increased percentages of n-6 PUFA in the three main PL classes, particularly CL ($P = 0.000$), mainly due to increased linoleic acid (18:2n-6, LA, $P = 0.000$) compared to the FO group. There was also higher n-6 PUFA content in mitochondrial PS ($P = 0.002$) and particularly, SM (10 %, $P = 0.000$) from fish fed the RO diet. Regarding n-3 PUFA, mitochondria from 1-year-old fish fed the RO diet had lower percentages of n-3 PUFA in the main PL classes compared to that of the FO group, although this varied quantitatively between the groups. The lowest decrease in n-3 PUFA was observed in PC (around 4 %, $P = 0.012$) due to lower EPA ($P = 0.000$) with no change in DHA ($P = 0.945$), while a higher decrease was obtained in CL (8 %, $P = 0.000$), mainly due to lower DHA content ($P = 0.000$) and very little change in EPA ($P = 0.001$). These changes were reflected in the peroxidation index (PIn) of PC and CL, which was significantly lower in 1-year-old rainbow trout fed the RO diet ($P = 0.003$ and $P = 0.001$, respectively). Mitochondrial PS from fish fed the RO diet had around 26 % less n-3 LC-PUFA than that from fish fed the FO diet ($P = 0.000$) which was reflected in PS PIn ($P = 0.000$). Mitochondria from 1-year-old rainbow trout fed the DHA diet showed fewer significant changes in the main PL classes, particularly CL, where almost no statistical differences were found when compared with the FO group (Table 6). Regarding PC and PE from 1-year-old fish fed the DHA diet, lower SFA in PC (~3 %, $P = 0.000$), lower MUFA in PE (2 %, $P = 0.011$), and higher n-6 PUFA in both (2 and 3 % in PC, $P = 0.001$ and PE, $P = 0.005$, respectively) were found (Tables 4, 5). Total n-3 PUFA was not significantly altered in any of the three PL classes in mitochondria from 1-year-old fish fed the DHA diet ($P = 0.294$, 1.000, and 0.560 for PC, PE, and CL, respectively). In PC and PE, this was mainly due to the fact that EPA content decreased (4 %, $P = 0.000$, and

Table 4 Fatty acid composition (percentage of total fatty acids) of phosphatidylcholine of mitochondria isolated from liver of 1- and 3-year-old rainbow trout fed with one of three experimental diets

Fatty acid	1-year-old			3-year-old			Age*Diet
	FO	RO	DHA	FO	RO	DHA	*P*
14:0	$2.5 \pm 0.1^{b*}$	1.0 ± 0.1^{a}	$1.2 \pm 0.1^{a*}$	2.1 ± 0.4^{c}	1.1 ± 0.1^{a}	1.6 ± 0.1^{b}	0.003
16:0	$23.0 \pm 0.4^{c*}$	18.3 ± 0.7^{a}	20.8 ± 1.3^{b}	19.4 ± 0.3^{a}	18.2 ± 0.6^{a}	21.8 ± 0.8^{b}	<0.001
18:0	4.4 ± 0.3^{b}	3.3 ± 0.3^{a}	$3.7 \pm 0.2^{a*}$	4.9 ± 0.5	3.5 ± 1.1	5.1 ± 0.8	0.166
∑Saturated	$30.6 \pm 0.4^{c*}$	22.9 ± 0.8^{a}	$26.4 \pm 1.4^{b*}$	26.8 ± 0.4^{b}	23.2 ± 1.6^{a}	29.2 ± 0.2^{c}	<0.001
16:1n-7	$2.6 \pm 0.1^{c*}$	0.9 ± 0.1^{a}	1.9 ± 0.2^{b}	2.0 ± 0.3^{b}	1.0 ± 0.1^{a}	2.0 ± 0.1^{b}	<0.001
18:1n-9	6.6 ± 0.3^{a}	$11.5 \pm 0.9^{b*}$	7.2 ± 0.6^{a}	7.4 ± 1.3^{a}	12.9 ± 0.3^{b}	7.6 ± 0.4^{a}	0.359
18:1n-7	1.8 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	3.2 ± 1.2	1.9 ± 0.7	2.3 ± 0.7	0.599
20:1n-9	$0.9 \pm 0.1^{a*}$	2.3 ± 0.3^{b}	$1.0 \pm 0.1^{a*}$	2.0 ± 0.6	2.8 ± 0.7	1.9 ± 0.3	0.277
∑Monounsaturated	12.8 ± 0.5^{a}	16.9 ± 1.4^{b}	$12.2 \pm 1.1^{a*}$	15.5 ± 2.8^{ab}	19.5 ± 1.3^{b}	14.7 ± 1.0^{a}	0.994
18:2n-6	1.1 ± 0.0^{a}	5.4 ± 0.4^{b}	1.2 ± 0.1^{a}	1.7 ± 0.5^{a}	6.0 ± 1.0^{b}	1.4 ± 0.2^{a}	0.523
20:2n-6	0.5 ± 0.0^{a}	$2.1 \pm 0.1^{b*}$	0.6 ± 0.1^{a}	0.6 ± 0.1^{a}	1.6 ± 0.2^{b}	0.6 ± 0.1^{a}	0.001
20:3n-6	0.2 ± 0.0^{a}	1.7 ± 0.2^{b}	0.1 ± 0.0^{a}	0.3 ± 0.0^{a}	1.7 ± 0.3^{b}	0.2 ± 0.0^{a}	0.957
20:4n-6	1.4 ± 0.0^{a}	1.7 ± 0.2^{a}	2.7 ± 0.1^{b}	1.3 ± 0.1^{a}	1.7 ± 0.5^{a}	2.7 ± 0.1^{b}	0.965
22:5n-6	0.4 ± 0.0^{a}	0.5 ± 0.1^{a}	1.4 ± 0.1^{b}	0.3 ± 0.1^{a}	0.4 ± 0.1^{a}	1.4 ± 0.4^{b}	0.558
∑n-6 PUFA	3.9 ± 0.0^{a}	11.6 ± 0.7^{c}	6.2 ± 0.5^{b}	4.3 ± 0.4^{a}	11.7 ± 1.4^{c}	6.6 ± 0.2^{b}	0.927
20:5n-3	10.1 ± 0.3^{c}	$4.8 \pm 0.1^{a*}$	5.7 ± 0.3^{b}	11.6 ± 1.2^{b}	7.0 ± 0.7^{a}	6.8 ± 0.9^{a}	<0.001
22:5n-3	$1.8 \pm 0.1^{b*}$	0.9 ± 0.1^{a}	1.0 ± 0.0^{a}	2.5 ± 0.2^{b}	1.2 ± 0.3^{a}	1.4 ± 0.3^{a}	0.176
22:6n-3	39.7 ± 0.5^{a}	$41.0 \pm 1.2^{a*}$	$47.0 \pm 1.9^{b*}$	38.0 ± 1.7^{ab}	35.1 ± 2.2^{a}	40.0 ± 2.1^{b}	0.024
∑n-3 PUFA	52.3 ± 0.6^{b}	$48.3 \pm 1.3^{a*}$	$54.4 \pm 2.1^{b*}$	53.3 ± 2.9^{c}	45.4 ± 1.9^{a}	49.0 ± 0.8^{b}	0.008
∑PUFA	56.5 ± 0.5^{a}	60.2 ± 0.8^{b}	$61.4 \pm 2.0^{b*}$	57.8 ± 2.6	57.2 ± 2.7	56.1 ± 1.1	0.008
∑n-3 LC-PUFA	51.8 ± 0.5^{b}	46.8 ± 1.2^{a}	$53.4 \pm 2.0^{b*}$	52.8 ± 2.0^{c}	44.0 ± 2.0^{a}	49.1 ± 0.5^{b}	0.009
n-3/n-6	13.3 ± 0.2^{c}	4.1 ± 0.3^{a}	$8.3 \pm 0.1^{b*}$	12.7 ± 2.1^{c}	4.1 ± 0.4^{a}	7.5 ± 0.2^{b}	0.482
PIn	400.3 ± 4.6^{b}	$380.9 \pm 0.1^{a*}$	$442.4 \pm 21.0^{c*}$	405.9 ± 30.4^{b}	347.8 ± 17.3^{a}	402.0 ± 10.3^{b}	0.015

Data expressed as mean ± SD ($n = 4$). Different superscript letters within a row and for each age group represent significant differences between diet treatments as determined by a two-way ANOVA ($P < 0.05$). Asterisks denote statistical differences between 1- and 3-year-old trout for each diet group (FO, RO and DHA) when compared using a Bonferroni test ($P < 0.05$). Right column represents significance values for the interaction between Diet and Age ($P < 0.05$). Fatty acids representing less than 1 % of total fatty acids are not shown

FO fish oil diet group, *RO* rapeseed oil group, *DHA* incromega TG0525 oil group, *LC-PUFA* long-chain polyunsaturated fatty acids, *PIn* peroxidation index, *PUFA* polyunsaturated fatty acids

3 %, $P = 0.048$, respectively), while DHA increased (7 %, $P = 0.000$, and 3 %, $P = 0.035$, respectively). PIn significantly increased in mitochondrial PC ($P = 0.003$) and PE ($P = 0.032$) but not in CL ($P = 1.000$) from 1-year-old trout fed the DHA diet compared with those from fish fed the FO diet. Regarding the minor PL classes, SM showed virtually no differences between DHA and FO groups (S. Table 3) while PI and PS from fish fed the DHA diet showed lower n-3 LC-PUFA, particularly PS (around 20 % less, $P = 0.001$) and PIn ($P = 0.000$) than those from trout fed the FO diet (Supplementary Tables 1, 2).

The effects of diet on mitochondrial PL FA compositions varied between the two age groups as indicated by the significant Age*Diet interactions (Tables 4, 5 and 6, Supplementary Tables 1, 2, and 3). In mitochondrial PC and PE from 3-year-old trout fed the DHA diet, lower DHA levels compared to that of 1-year-old fish were observed (Tables 4, 5). This was reflected in the different impact of diet composition on PIn of mitochondrial PC and PE between the two age groups ($P = 0.015$ and 0.003, respectively). Diet DHA had a similar impact on mitochondrial CL FA composition in both age groups. A similar trend was found in the three minor PL classes. Mitochondrial PI, PS, and SM from 3-year-old fish fed the DHA diet showed fewer changes than those from 1-year-old trout (Supplementary Tables 1, 2, and 3). Regarding the RO diet, CL from 3-year-old trout showed less n-6 PUFA than 1-year-old fish (28.2 vs. 33.2 %, $P = 0.001$). While mitochondrial CL from 1-year-old fish fed the RO diet had around 20 % more n-6 PUFA than that from fish fed the FO diet; mitochondrial CL from 3-year-old animals fed the RO diet had just around 14 % more n-6 PUFA than that from 3-year-old fish fed the FO diet. There were no significant differences in n-6 PUFA levels

Table 5 Fatty acid composition (percentage of total fatty acids) of phosphatidylethanolamine of mitochondria isolated from liver of 1- and 3-year-old rainbow trout fed with one of three experimental diets

Fatty acid	1-year-old			3-year-old			Age*Diet
	FO	RO	DHA	FO	RO	DHA	*P*
16:0	10.1 ± 0.6	9.8 ± 0.5*	9.4 ± 0.6	10.3 ± 1.3^{b}	8.1 ± 0.5^{a}	10.4 ± 0.5^{b}	0.01
18:0	9.3 ± 0.2^{b}*	7.0 ± 0.4^{a}*	8.8 ± 0.8^{b}	7.9 ± 1.1^{b}	5.8 ± 0.3^{a}	9.2 ± 1.0^{b}	0.046
∑Saturated	20.0 ± 0.6^{b}	17.3 ± 0.9^{a}*	18.7 ± 0.7^{b}*	18.8 ± 0.7^{b}	14.4 ± 0.6^{a}	20.2 ± 0.4^{b}	<0.001
16:1n-7	1.1 ± 0.0^{c}	0.3 ± 0.1^{a}	0.6 ± 0.0^{b}	1.0 ± 0.4^{b}	0.3 ± 0.0^{a}	0.7 ± 0.1^{b}	0.416
18:1n-9	8.0 ± 0.5^{a}	12.9 ± 0.4^{b}	8.3 ± 1.1^{a}	8.0 ± 0.6^{a}	13.1 ± 0.5^{b}	7.9 ± 1.0^{a}	0.746
18:1n-7	5.1 ± 0.3^{c}	2.3 ± 0.0^{a}	3.0 ± 0.3^{b}*	5.8 ± 1.3^{b}	2.9 ± 0.3^{a}	5.5 ± 1.0^{b}	0.035
20:1n-9	2.3 ± 0.2^{a}*	3.9 ± 0.2^{b}	2.4 ± 0.4a*	3.6 ± 0.4	3.9 ± 0.3	3.7 ± 0.4	0.004
∑Monounsaturated	17.5 ± 0.8^{b}	19.8 ± 0.4^{c}	14.7 ± 1.8^{a}*	19.1 ± 1.4	20.8 ± 0.3	18.3 ± 1.0	0.128
18:2n-6	2.4 ± 0.1^{a}	5.1 ± 0.6^{b}*	2.6 ± 0.2^{a}	3.0 ± 1.0^{a}	6.5 ± 0.1^{b}	2.8 ± 0.3^{a}	0.122
20:2n-6	1.2 ± 0.2^{a}	2.7 ± 0.1^{b}*	1.3 ± 0.2^{a}	0.9 ± 0.2^{a}	2.1 ± 0.3^{c}	1.3 ± 0.1^{b}	0.016
20:4n-6	2.2 ± 0.1^{a}	2.6 ± 0.3^{a}	3.8 ± 0.2^{b}*	2.9 ± 1.4^{a}	2.6 ± 0.9^{a}	6.4 ± 1.9^{b}	0.046
∑n-6 PUFA	7.3 ± 0.3^{a}	12.5 ± 0.6^{c}	10.0 ± 0.4^{b}*	7.7 ± 1.5^{a}	13.5 ± 1.5^{b}	12.8 ± 1.5^{b}	0.129
20:5n-3	4.9 ± 0.1^{c}*	3.1 ± 0.3^{b}	2.1 ± 0.1^{a}*	9.6 ± 4.1^{b}	4.7 ± 1.0^{a}	7.5 ± 3.7^{ab}	0.229
22:5n-3	1.2 ± 0.2^{b}	0.5 ± 0.1^{a}	0.9 ± 0.2^{b}	1.4 ± 0.3^{b}	0.7 ± 0.1^{a}	1.0 ± 0.1^{ab}	0.653
22:6n-3	47.4 ± 0.4^{b}*	44.9 ± 1.1^{a}	51.8 ± 2.7^{c}*	41.7 ± 5.8	43.4 ± 0.6	38.8 ± 6.0	0.019
∑n-3 PUFA	54.6 ± 0.7^{b}	49.8 ± 1.0^{a}	55.6 ± 2.7^{b}*	54.0 ± 1.5^{b}	50.7 ± 0.8^{ab}	48.3 ± 2.4^{a}	0.001
∑PUFA	62.5 ± 0.5^{a}	63.0 ± 0.8^{a}	66.6 ± 2.3^{b}*	62.1 ± 1.0^{ab}	64.8 ± 0.8^{b}	61.5 ± 1.2^{a}	0.001
∑n-3 LC-PUFA	54.3 ± 0.8^{b}	49.4 ± 1.3^{a}	56.2 ± 2.6^{b}*	53.5 ± 1.5^{b}	49.8 ± 0.7^{ab}	47.9 ± 2.3^{a}	0.001
n-3/n-6	7.6 ± 0.3^{c}	4.0 ± 0.3^{a}	5.7 ± 0.5^{b}*	7.2 ± 1.5^{b}	3.8 ± 0.5^{a}	3.8 ± 0.6^{a}	0.106
PIn	438.3 ± 7.7^{a}	416.1 ± 10.6^{a}	472.5 ± 27.9^{b}*	417.9 ± 17.5	412.3 ± 4.1	406.5 ± 21.4	0.003

Data expressed as mean ± SD ($n = 4$). Different superscript letters within a row and for each age group represent significant differences between diet treatments as determined by a two-way ANOVA ($P < 0.05$). Asterisks denote statistical differences between 1- and 3-year-old trout for each diet group (FO, RO, and DHA) when compared using a Bonferroni test ($P < 0.05$). Right column represents significance values for the interaction between Diet and Age ($P < 0.05$). Fatty acids representing less than 1 % of total fatty acids are not shown

FO fish oil diet group, *RO* rapeseed oil group, *DHA* incromega TG0525 oil group, *LC-PUFA* long-chain polyunsaturated fatty acids, *PIn* peroxidation index, *PUFA* polyunsaturated fatty acids

in the remaining PL classes between the two age groups when fed the RO diet.

Lipid peroxidation

Lipid peroxidation in rainbow trout mitochondria, estimated by measuring the TBARS content, did not show significant differences among feeding groups in 1-year-old fish [$F(2, 9) = 2.626$, $P = 0.097$] (Fig. 3). Some changes, however, were found in 3-year-old trout [$F(2, 9) = 4.852$, $P = 0.019$], with higher lipid peroxidation in the FO group compared to that of the RO group (1164.4 vs. 590.1 ng g^{-1} lipid, respectively). Although 3-year-old fish fed the DHA diet showed lower lipid peroxidation (652.3 ng g^{-1} lipid) than that of the FO group, it was not statistically significant ($P = 0.060$). Regarding age differences, lipid peroxidation was statistically higher in 1-year-old trout fed the DHA diet than in 3-year-old fish fed the same diet ($P = 0.020$) (Fig. 3). No significant differences were found between age groups in fish fed the FO or RO diets. The effect of diet lipid composition on mitochondria lipid peroxidation was not influenced by age as determined by two-way ANOVA ($P = 0.430$).

Mitochondrial gene expression

One-year-old rainbow trout fed the different diets showed some consistent trends in mitochondrial gene expression but these were only statistically significant for ND4 [$F(2, 15) = 4.191$, $P = 0.025$] (Fig. 4). Fish fed the RO diet showed higher expression for almost all the studied genes compared with the FO group, while trout fed the DHA diet had in general lower expression than the FO group. Values for 3-year-old trout were in general more stable between diet groups. When the two age groups were compared, 3-year-old fish showed generally lower gene expression than 1-year-old animals (except for fish fed the DHA diet) (Fig. 4), but statistical significant differences were only found in fish fed the RO diet for COX3 ($P = 0.021$), ND1 ($P = 0.013$), ND4 ($P = 0.018$), and ND5 ($P = 0.022$). The

Table 6 Fatty acid composition (percentage of total fatty acids) of cardiolipin of mitochondria isolated from liver of 1- and 3-year-old rainbow trout fed with one of three experimental diets

Fatty acid	1-year-old			3-year-old			Age*diet
	FO	RO	DHA	FO	RO	DHA	*P*
14:0	$2.3 \pm 0.1^{c*}$	0.9 ± 0.2^{a}	1.2 ± 0.1^{b}	1.8 ± 0.1^{c}	0.9 ± 0.2^{a}	1.3 ± 0.1^{b}	0.001
16:0	8.7 ± 1.1^{b}	$5.7 \pm 1.2^{a*}$	9.9 ± 1.4^{b}	8.3 ± 0.6	8.8 ± 1.9	10.2 ± 1.4	0.053
18:0	1.4 ± 0.3	1.2 ± 0.4	2.5 ± 1.4	1.5 ± 0.2	2.6 ± 1.5	2.4 ± 1.2	0.347
∑Saturated	12.9 ± 1.3^{b}	$8.1 \pm 1.9^{a*}$	14.2 ± 2.7^{b}	12.0 ± 0.8	12.7 ± 3.4	14.5 ± 2.5	0.81
16:1n-7	$7.4 \pm 0.5^{c*}$	$1.4 \pm 0.2^{a*}$	4.4 ± 0.6^{b}	5.7 ± 0.8^{b}	0.0 ± 0.0^{a}	4.7 ± 0.6^{b}	0.016
18:1n-9	6.3 ± 0.1	7.8 ± 0.6	7.0 ± 0.2	6.6 ± 1.0^{a}	9.7 ± 1.5^{b}	7.3 ± 0.4^{a}	0.109
18:1n-7	4.0 ± 0.3^{b}	$1.6 \pm 0.3^{a*}$	3.5 ± 0.2^{b}	4.0 ± 0.6^{b}	2.4 ± 0.3^{a}	4.3 ± 0.9^{b}	0.239
20:1n-9	$1.0 \pm 0.1*$	$1.4 \pm 0.1*$	1.3 ± 0.1	1.7 ± 0.3	2.2 ± 0.2	1.7 ± 0.4	0.36
∑Monounsaturated	19.5 ± 0.2^{b}	$13.5 \pm 1.2^{a*}$	17.3 ± 1.6^{b}	18.5 ± 0.9	16.5 ± 2.7	18.9 ± 0.7	0.056
18:2n-6	$6.5 \pm 0.4^{a*}$	$21.1 \pm 0.9^{b*}$	7.4 ± 1.3^{a}	8.6 ± 0.3^{a}	18.0 ± 1.7^{b}	8.4 ± 1.7^{a}	0.002
20:2n-6	$3.9 \pm 0.3^{a*}$	$6.3 \pm 0.5^{b*}$	5.6 ± 0.8^{b}	2.8 ± 0.2^{a}	4.8 ± 0.9^{b}	4.4 ± 0.8^{b}	0.899
20:3n-6	0.9 ± 0.1^{a}	4.8 ± 0.6^{b}	0.7 ± 0.1^{a}	1.2 ± 0.4^{a}	3.9 ± 0.5^{b}	0.9 ± 0.1^{a}	0.01
20:4n-6	0.8 ± 0.2^{a}	0.7 ± 0.3^{a}	1.7 ± 0.5^{b}	1.0 ± 0.1^{a}	1.2 ± 0.1^{a}	1.8 ± 0.4^{b}	0.616
∑n-6 PUFA	12.9 ± 0.5^{a}	$33.2 \pm 1.0^{b*}$	16.2 ± 1.5^{a}	14.1 ± 0.8^{a}	28.2 ± 2.8^{b}	16.4 ± 1.9^{a}	0.04
18:3n-3	$1.0 \pm 0.1^{a*}$	3.1 ± 0.2^{b}	1.1 ± 0.2^{a}	2.1 ± 0.1^{ab}	2.8 ± 0.4^{b}	1.5 ± 0.6^{a}	0.007
20:4n-3	$1.2 \pm 0.1^{b*}$	1.2 ± 0.2^{b}	0.6 ± 0.1^{a}	2.2 ± 0.2^{b}	1.3 ± 0.3^{a}	1.0 ± 0.1^{a}	0.005
20:5n-3	$1.6 \pm 0.2^{b*}$	$0.6 \pm 0.0^{a*}$	1.0 ± 0.4^{ab}	3.0 ± 0.4^{b}	1.2 ± 0.4^{a}	1.2 ± 0.3^{a}	0.007
22:5n-3	3.5 ± 0.5^{b}	1.6 ± 0.2^{a}	$1.3 \pm 0.3^{a*}$	3.2 ± 0.4^{b}	1.8 ± 0.5^{a}	2.2 ± 0.3^{a}	0.032
22:6n-3	45.6 ± 0.7^{b}	37.6 ± 1.8^{a}	$46.4 \pm 0.8^{b*}$	43.2 ± 1.2^{b}	34.1 ± 3.0^{a}	42.4 ± 2.7^{b}	0.732
∑n-3 PUFA	53.8 ± 1.1^{b}	45.0 ± 2.1^{a}	51.4 ± 0.4^{b}	54.7 ± 1.0^{c}	42.2 ± 4.1^{a}	49.3 ± 2.2^{b}	0.305
Total PUFA	67.7 ± 1.4^{a}	78.4 ± 3.1^{b}	68.5 ± 1.4^{a}	69.5 ± 0.3	70.7 ± 5.5	66.5 ± 1.9	0.021
∑n-3 LC-PUFA	52.7 ± 1.1^{b}	42.6 ± 1.0^{a}	50.2 ± 0.7^{b}	52.1 ± 0.9^{b}	40.5 ± 3.1^{a}	48.6 ± 1.7^{b}	0.58
n-3/n-6	4.2 ± 0.2^{c}	1.4 ± 0.0^{a}	3.2 ± 0.3^{b}	3.9 ± 0.3^{b}	1.6 ± 0.1^{a}	3.2 ± 0.3^{b}	0.279
PIn	421.8 ± 7.0^{b}	376.7 ± 10.6^{a}	$423.1 \pm 2.3^{b*}$	416.1 ± 8.2^{b}	365.5 ± 17.7^{a}	399.4 ± 5.5^{b}	0.464

Data expressed as mean ± SD ($n = 4$). Different superscript letters within a row and for each age group represent significant differences between diet treatments as determined by a two-way ANOVA ($p < 0.05$). Asterisks denote statistical differences between 1- and 3-year-old trout for each diet group (FO, RO and DHA) when compared using a Bonferroni test ($P < 0.05$). Right column represents significance values for the interaction between Diet and Age ($P < 0.05$). Fatty acids representing less than 1 % of total fatty acids are not shown

FO fish oil diet group, *RO* rapeseed oil group, *DHA* incromega TG0525 oil group, *LC-PUFA* long-chain polyunsaturated fatty acids, *PIn* peroxidation index, *PUFA* polyunsaturated fatty acids

interaction between age and diet was only significant for ND4 ($P = 0.037$).

Discussion

Diet fatty acid composition and mitochondrial lipids

Dietary lipid composition markedly influenced PL contents and FA compositions of individual PL classes of mitochondrial membranes from rainbow trout liver, but had generally low impact upon mitochondrial DNA (mtDNA) gene expression. The effects differed in relation to fish age. One-year-old trout fed RO and, mainly, DHA diet showed a significantly lower total PL content (percentage of total lipid) in their liver mitochondrial membranes compared with the FO group. Moreover, the RO group showed significantly higher proportions of PC, PE, and PI and lower percentages of CL and SM, while trout fed the DHA diet only showed a significantly lower CL content compared with the FO group. These changes in the proportions of individual PL classes may alter mitochondrial membrane function by affecting charge distribution across the membrane, membrane permeability properties, catalytic activities, or specific enzymes and ETC function (Daum 1985). Especially interesting was the observed loss of CL considering the critical role that CL plays in the MIM as a regulator of processes related to oxidative phosphorylation and mitochondrial integrity (Paradies et al. 2002). Loss of CL has been shown in a variety of tissues in mammals and fish (Chicco and Sparagna 2007; Almaida-Pagán et al. 2012) and suggested as one of the first signs of damage caused by high

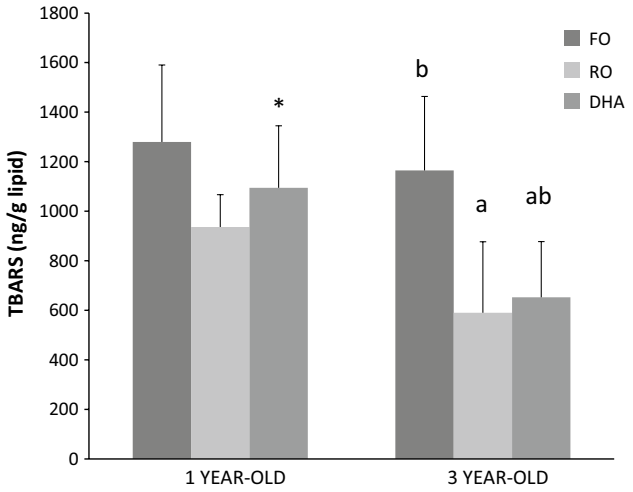

Fig. 3 TBARS contents (ng/g lipid) of liver mitochondria of 1- and 3-year-old rainbow trout fed the three experimental diets. Data expressed as mean ± SEM ($n = 4$). *Letters* represent the existence of statistical differences among feeding treatments for each age group as determined by a two-way ANOVA ($P < 0.05$). *Asterisks* denote significant differences between 1- and 3-year-old trout mitochondria for each feeding group when compared using a Bonferroni test ($P < 0.05$). *P* value for interaction between Age and Diet was 0.430

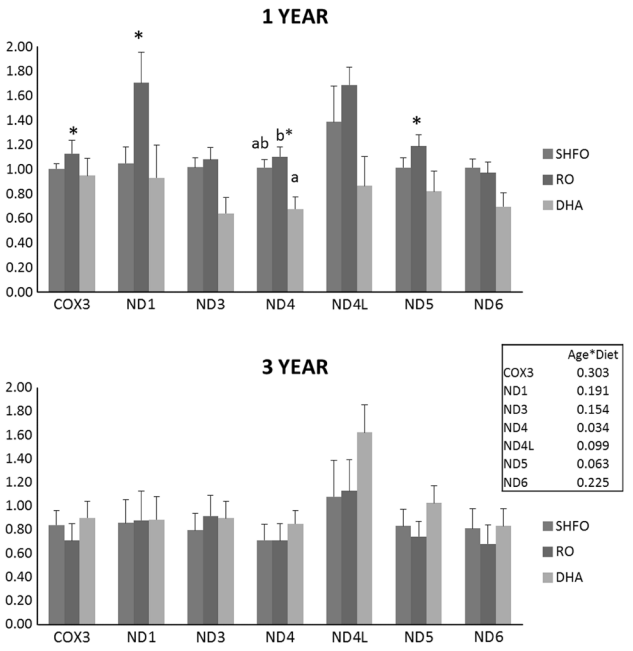

Fig. 4 Relative gene expression of COX3, ND1, ND3, ND4, ND4L, ND5, and ND6 from liver mitochondria of 1- and 3-year-old rainbow trout fed the three experimental diets. Data are expressed as mean ± SEM ($n = 6$). *Asterisks* indicate significant differences between age groups for a given diet group as determined by a Bonferroni test ($P < 0.05$). Table presents significance values for the interaction between Age and Diet for each gene

oxidative stress in mitochondrial membranes. Decreased SM has also been related with oxidative stress since SM can retard the lateral propagation of free radicals through the membrane and it is an important mediator of mitochondrial pathways, including apoptosis (Hannum and Obeid 1997; Subbaiah et al. 1999; Cutler and Mattson 2001).

The FA compositions of individual PL classes from liver mitochondria of 1-year-old trout were strongly affected by the diet lipid composition, but the observed changes did not entirely reflect differences among the diets. These data agree with those showing marked modifications of FA of total mitochondrial PL by diet in rats (Lemieux et al. 2008; Abbott et al. 2010) and fish (Ushio et al. 1997; Robin et al. 2003; Guderley et al. 2008; Østbye et al. 2011; Martin et al. 2013), and indicates the existence of mechanisms regulating FA within mitochondrial PL classes. Moreover, these data point to the importance of FA composition of specific PL classes in mitochondrial membranes. Individual mitochondrial PL classes were affected by the diet in different ways. CL in liver mitochondria from one-year-old fish fed the DHA diet had almost the same FA composition than that from the FO group, while CL from the RO group had around 20 % more n-6 PUFA than that from the FO group, considerably larger than the difference in n-6 PUFA content between the two diets (12 %). The FA compositions of PC and PE reflected more clearly the diet composition although changes in these two PL classes were attenuated compared with the diets. The influence of diet on liver mitochondrial DHA level was generally smaller than expected, considering the large differences between diets, especially between RO and DHA (3.6 vs. 22.3 %). This can be in part explained by preferential retention of DHA in fish tissues when this FA is very low in the diet, as found in Atlantic salmon and other fish species (Bell et al. 2004; National Research Council 2011) and/or increased endogenous synthesis of DHA from 18:3n-3 in rainbow trout when dietary levels of n-3 LC-PUFA are reduced (Tocher et al. 2002). Selective incorporation of FA from the diet and biosynthesis of FA are regulatory mechanisms that have been shown in trout (Guderley et al. 2008). Another explanation may be an increase in the degree of oxidative stress and, particularly, lipid peroxidation that would decrease membrane levels of PUFAs (Hulbert et al. 2007). The observed decrease in mitochondrial total PL content in RO and especially, DHA groups could indicate the existence of high oxidative stress as was found in liver and muscle mitochondria of Atlantic salmon fed diets with a high level of n-3 LC-PUFA (Kjaer et al. 2008; Østbye et al. 2011). These studies also showed a decrease in CL content in mitochondrial membranes that agrees with data in the present study. PL classes

incorporating LC-PUFA would become more susceptible to oxidative attack and would contribute to the observed reduction in total PL. Among the minor PL classes, a noteable decrease in DHA content in mitochondrial PS from fish fed the RO and DHA diets was found which could be also indicating high oxidative stress since PS is the PL with the highest PIn. However, these data were not consistent with lipid peroxidation results from the present study that showed no significant differences among feeding groups.

Diet lipid composition and mitochondrial genes

The expression of mtDNA genes showed significant differences for only ND4 among feeding groups although there was a consistent trend among all the studied genes. One-year-old fish fed the RO diet tended to display higher expression of almost all the analyzed genes compared to the other two groups, while trout fed the DHA diet tended to show lower values. Although only significant for ND4, the consistency of the trend among all the genes could suggests a minor effect of diet FA composition on mtDNA expression which could, over a longer time, influence the functioning of the ETC and mitochondrial respiratory rate as found in rainbow trout red muscle (Guderley et al. 2008). The studied genes belong to a specific region of the mitochondrial genome that has been reported to be especially prone to oxidative attack in several tissues of humans and mice (Cortopassi and Wong 1999; Vu et al. 2000). Genes in this area encode predominantly for subunits of complexes I [NADH-coenzyme Q (CoQ) oxidoreductase] and IV (cytochrome c oxidase) which appear to be particularly prone to age-related decline in activity in several tissues (Shigenaga et al. 1994; Richter 1995; Trifunovic and Larsson 2008). This is not surprising taking into consideration that 7 out of the 13 mtDNA encoded polypeptides in the ETC are found in complex I while 3 are found in complex IV. Moreover, there is a strong connection between complexes I and IV, and CL. It has been reported that CL is specifically required for electron transfer in complex I of the mitochondrial ETC (Paradies et al. 2002), complex I being considered the main site of ROS production in mitochondria. It is reasonable to suggest that the defect of mitochondrial complex I activity in addition to that of complex IV (very tightly bonded to CL), due to oxidation/depletion of CL molecules and/or mutations affecting the corresponding area in mitochondrial genome, may increase electron leak from the ETC, generating more oxidative stress, mitochondrial damage and, ultimately, mitochondrial dysfunction and bioenergetics decay associated with disease and aging.

Fish age

Some differences were observed in the effects of dietary FA composition on mitochondrial membranes and mtDNA expression between age groups. Compared with 1-year-old fish, more mature animals showed less susceptibility to diet composition, especially to DHA diet. This can be due mainly to the fact that younger fish had a higher growth rate resulting in greater incorporation of dietary lipids into their tissues, but it could also indicate age-related changes in the animal's capacity for maintaining the structural homoeostasis of mitochondrial membranes. Mitochondrial membrane PL composition was significantly different in 1- and 3-year-old fish and many of the observed changes in one age group were in the opposite direction in the other, or simply remained unchanged. Moreover, mtDNA gene expression was generally lower in 3-year-old trout fed the FO and RO diets and, although only significant for ND4, there were different trends in most of the studied genes. These observations could be associated with the well-known feature of the aging process involving a reduction in the rate of lipid metabolism and turnover of FA (Hansford and Castro 1982). Martin et al. (2013) also found that several functional properties of mitochondria were affected by trout body mass which could also influence the effect of diet composition on mitochondrial processes.

Membrane pacemaker hypothesis (MPH)

In conclusion, the present study showed marked changes in mitochondrial PL content and composition from trout liver when diet lipid composition was modified. These changes were PL specific and showed some regulatory mechanisms operated on mitochondrial lipids from trout liver as they did not clearly reflect differences among the diets. The main PL classes from trout mitochondria showed the PIn was higher in fish fed the DHA diet than in the RO group. Following the MPH, this would render the former more prone to oxidative attack and would explain the decrease in total PL and CL content observed in 1-year-old animals. Membranes with higher n-3 LC-PUFA content are also related with higher metabolism and ROS production (Pamplona et al. 2002; Sanz et al. 2006) which, eventually, could affect mtDNA. TBARS values, however, did not show any differences in lipid peroxidation among feeding groups. Changes observed in liver mitochondrial lipids from trout fed the RO diet could not be explained following the MPH. The three main PL classes in liver mitochondria had lower PIn and, therefore, were less susceptible to oxidative attack. In this case, fish could be suffering stress due to the influence of dietary lipid composition, which has been shown to induce changes in metabolism (Tocher et al. 2001). Therefore, changes observed in liver mitochondrial PL content and composition from trout fed the RO diet could be reflecting the existence of compensatory mechanisms in mitochondrial membranes as a response to dietary FA composition. In both cases, mitochondrial function could

be compromised by diet lipid composition which would affect animal well-being and longevity. Mitochondrial lipid composition and mtDNA expression from trout liver were affected by diet in a different way when the two age groups were compared. The observed changes could in part be explained by the different SGR of the two age groups, this being related to different fatty acid incorporation into fish tissues, but may also indicate age-related changes in the animal's capacity for maintaining the structural homoeostasis of mitochondrial membranes.

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