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

Almaida-Pagán, P. F.*, De Santis, C., Rubio-Mejía, O. L., Tocher, D. R.

Institute of Aquaculture, School of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland, United Kingdom

* Correspondence to:

Pedro F Almaida-Pagán
School of Natural Sciences
University of Stirling
Stirling, FK9 4LA
United Kingdom

Phone: + 44-1786 467993
Fax: + 44-1786 472133
E-mail: pedro.almaida@stir.ac.uk

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; HP-TLC, 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, 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.

Running title: Effects of diet lipid composition on rainbow trout liver mitochondria

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 a high DHA oil (DHA) during six 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.

1. 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 remodelling 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

84 polyunsaturation of cell membrane lipids (high number of double bonds) results in more fluid
85 membranes that can promote higher molecular activity of membrane proteins and, in turn,
86 increase the metabolic activity of cells, tissues and, consequently, whole animals (Pamplona *et*
87 *al.* 2002; Sanz *et al.* 2006). At the same time, membranes with increased levels of PUFA are
88 more prone to attack by oxidizing agents and will then participate in long, free radical reactions
89 that will propagate oxidative damage throughout the organelle (Hulbert *et al*. 2005). This
90 hypothesis would explain the huge differences in life span existing among animal species with
91 different levels of unsaturation in their membranes, but could also explain how situations such
92 as thermal or dietary changes in an animal life-cycle, could compromise metabolic activity,
93 oxidative stress and tissue function.

94 The key role of FA composition in membrane processes suggests that dietary effects
95 upon membrane lipid structure should be, at least in part, regulated. Both factors, the overall
96 status of the membrane and the specific characteristics of lipid components, must be balanced
97 as they, very likely, participate in maintaining membrane function (Paradies *et al*., 1992;
98 Zabelinskii *et al*. 1999; Guderley *et al.* 2008; Modi *et al*. 2008). It is known that mitochondrial
99 membranes have different compositions of lipids such as phospholipids (PL), glycolipids and
100 cholesterol compared with plasma membranes (Wiseman 1996), this being related with the role
101 of the organelle in oxygen consumption (Hoch 1992). Mitochondrial inner membrane (MIM)
102 uniquely contains cardiolipin (CL), a key molecule associated with complexes I, III and IV of the
103 electron transport chain (ETC), F_1F_0ATPase and nucleotide translocase (ANT) (Yamaoka *et al*.
104 1988; Paradies *et al*. 2002). CL has a high content of PUFA which makes it, along with its
105 proximity to the site of ROS production, particularly prone to peroxidation (Paradies *et al*. 2011).
106 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 life-span (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 signalling molecules, some associated with apoptosis (Hannum and Obeid, 1994). 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 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.

2. **Methods**

2.1. Experimental fish and sampling

A population of rainbow trout, *Oncorhynchus mykiss*, was maintained from eggs (Howietoun Fishery, Stirling, UK) to 3 years under controlled feeding and rearing conditions

152 throughout in the freshwater aquarium facilities at the Institute of Aquaculture, University of
153 Stirling. Fish were fed twice a day *ad libitum* with a commercial feed (50% protein, 20% fat,
154 Skretting, Northwich, UK) and kept under ambient water temperature conditions before being
155 transferred to experimental thermo-regulated tanks. When fish reached one year of age, 240
156 fish (72.4 ± 12.6 g average body weight) were distributed into six tanks equipped with a
157 thermostat to maintain the temperature at 12 ± 1 ºC, kept under a 12L:12D photoperiod and fed,
158 in duplicate, one of three experimental diets consisting of the same base formulation, but with
159 lipid supplied either by fish oil (FO diet), rapeseed oil (RO) or an oil containing high
160 docosahexaenoic acid, 22:6n-3 (DHA) (Table 1). Fish were fed twice a day *ad libitum* for 6-
161 weeks and then euthanized by exposure to the anesthetic benzocaine hydrochloride (400 mg
162 l−1) for 10 min following the cessation of opercular movement. Livers were collected for
163 analyses. The experiment was repeated identically when the remaining fish (60 fish of 625.1 ±
164 33.8 g of average body weight; 10/tank) reached 3 years of age. For 1-year-old fish, livers of 5
165 animals were pooled to provide sufficient material for the different analyses and also to reduce
166 inter-individual variability. For 3-year-old animals, two livers per pool were taken after
167 considering fish size and numbers. A portion of each pool was immediately taken into
168 RNA*later*® (Life technologies, Paisley, UK) following the manufacturer instructions and stored at
169 – 20 ºC for molecular analysis, while the remainder of the tissue sample was processed to
170 obtain enriched mitochondrial preparations. The purified mitochondrial isolates were analyzed to
171 determine mitochondrial membrane lipid composition and peroxidation. Fish were treated in
172 accordance with British national ethical requirements established by the UK Government Home
173 Office and guidelines determined by the Animals (Scientific Procedures) Act 1986.

174

175 *2.2. Experimental Diets*

176 Practical pelleted diets (2- and 4-mm diameter for 1- and 3-year-old trout, respectively)
177 containing 47% crude protein and 24% crude lipid were formulated using the same basal
178 ingredients and varying only in the lipid source used: Southern hemisphere fish oil (FO),
179 rapeseed oil (RO) and Incromega TG0525 (DHA) (Table 1). The fatty acid compositions of the
180 diets are shown in Table 2. The control diet (FO) contained 31.7% saturated fatty acids (SFA),
181 mainly 16:0, 27.8% monounsaturated fatty acids (MUFA), 11.7% of which was 18:1n-9, and
182 40.6% PUFA, with 6.8% as n-6 PUFA and 30.3% as n-3 PUFA. The FO diet contained a high
183 proportion of the n-3 long-chain polyunsaturated fatty acid (LC-PUFA), eicosapentaenoic acid
184 (20:5n-3, EPA) and DHA, in approximately equal amounts. Diet RO had lower levels of SFA
185 (12.3%), higher percentages of MUFA (56.0% with 48.2% as 18:1n-9) and lower PUFA (31.7%)
186 with just 2.1% EPA and 3.6% DHA. Finally, the DHA diet contained similar proportions of SFA,
187 MUFA and PUFA to the FO diet but had a lower percentage of EPA (6.6%) and higher
188 percentage of DHA (22.3%). The diets were formulated to meet all the known nutritional
189 requirements of salmonid fish (National Research Council, 2011).

190

191 *2.3. Mitochondria isolation*

192 Approximately 2 g of liver pate was homogenized in 8 mL ice-cold sucrose buffer (0.4 M
193 phosphate buffer pH 7.4, 0.25 M sucrose, 0.15 M KCl, 40 mM KF and 1 mM N-acetyl-cysteine)
194 using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK). Homogenates
195 were centrifuged at 600 g for 6 min, the pellet discarded (cell/nuclei debris) and the supernatant
196 re-centrifuged at 600 g. Resulting supernatants were then centrifuged at 6800 g for 10 min and
197 the resulting pellet (mitochondrial fraction) used for lipid extraction. To verify that pellets were

198 highly enriched with mitochondria, a portion was fixed in 2.5% glutaraldehyde in 0.1 M
199 cacodylate buffer overnight at 4 °C, and then processed as specified by Rajapakse *et al*. (2001)
200 prior to analysis by transmission electron microscopy (Tecnai™ G2 Spirit BioTWIN, FEI Europe,
201 Eindhoven, The Netherlands).

202

203 *2.4. Lipid extraction and phospholipid class composition*

204 Total lipid contents of livers and diet samples were determined gravimetrically after
205 extraction by homogenization in chloroform/methanol (2:1, v/v) containing 0.01% butylated
206 hydroxytoluene (BHT) as antioxidant, basically according to Folch *et al*. (1957). Phospholipid
207 (PL) classes were separated by high-performance thin layer chromatography (HPTLC) using
208 10×10 cm silica gel plates (VWR, Lutterworth, England) and methyl acetate/isopropanol/
209 chloroform/methanol/0.25% (w/v) KCl (25:25:25:10:9, by vol.) as solvent system (Olsen and
210 Henderson 1989). The lipid classes were visualized by charring at 160 °C for 15 min after
211 spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid and
212 quantified by densitometry using a CAMAG-3 TLC scanner (version Firmware 1.14.16)
213 (Henderson and Tocher 1992). Scanned images were recorded automatically and analyzed by
214 computer using winCATS (Planar Chromatography Manager, version 1.2.0).

215

216 *2.5. Phospholipid fatty acid composition*

217 Phospholipid classes were separated by preparative-TLC, using silica gel plates (20 x
218 20 cm) (VWR) and the solvent system as above. Individual phospholipid classes were identified
219 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 visualization 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 ºC overnight following addition of 2 mL of 1% (v/v) sulphuric 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 x 0.32 mm i.d. capillary column (CP Wax 52CB, Chrompak, London, U.K.) and on-column injection at 50 ºC. Hydrogen was used as carrier gas and temperature programming was from 50 ºC to 150 ºC at 40 ºC min^{-1} and then to 230 ºC at 2.0 ºC min^{-1}. Individual methyl esters were identified by comparison with known standards. Data were collected and processed using Chromcard for Windows (version 1.19).

2.6. 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 2000 *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}.

243 *2.7. RNA preparation, cDNA synthesis and quantification*

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

252 Information about target genes was retrieved from the mitochondrion genome sequence
253 (Genbank accession number DQ288271) and used to design primers for Real-Time PCR (RT-
254 PCR) with PerlPrimer v1.1.17 (Marshall 2004). Primers were designed to target seven
255 mitochondrial genes: COX3, ND1, ND3, ND4, ND4L, ND5 and ND6 (Table 3). Three
256 housekeeping genes were evaluated as internal reference (elongation factor 1α, glyceraldehyde
257 3-phosphate dehydrogenase and β-actin) but they were not stable but variable between diets or
258 age groups and were not used for normalization (data not shown). RT-qPCR reactions were
259 carried out on a Biometra thermal cycler (Gottingen, Germany) using Luminaris Color HiGreen
260 qPCR Master mix (Thermo Scientific, Leicestershire, UK) according to the instructions provided
261 by the manufacturer. To avoid technical variation between runs all treatment and control
262 samples from both age groups were analyzed on the same plate together with a $\log_{10}$ serial
263 dilution of pooled samples used to calculate PCR efficiency (E). Each reaction was conducted in
264 a total volume of 20 µl in duplicates. All primer pairs were designed to have an optimal
265 annealing temperature of 60ºC. 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 [$E = 10^{\wedge(-1/slope)}$] (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 [$Ratio_{(test/calibrator)} = E \wedge Ct(calibrator) - Ct(test)$] and log transformed before statistical analyses. The average of the 1-year-old FO treatment was used as the calibrator.

2.8. *Indexes and statistical analysis*

The LC-PUFA index corresponds to the sum of fatty acids with 20 or more carbons and 2 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 Wt−ln Wi)/T)] × 100 where Wt = mean final weight, Wi = 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

288 performed using SPSS Statistical Software System version 15.0 (SPSS Inc., Chicago, USA).
289 Differences were regarded as significant when $P<0.05$.

290

291 **3. Results**

292 3.1. *Fish performance* There were no differences in feed intake between the different
293 dietary groups for either 1- (~1.5% BW /day) or 3-year-old trout (~0.8% BW/day). The 1-year-old
294 fish showed a considerably higher SGR (around 1.5) than 3-year-old trout (~ 0.45) during the
295 feeding trial (Fig. 1). No statistically significant differences among the feeding groups were
296 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
297 significant interaction between fish age and diet was observed for SGR ($p= 0.549$).

298

299 3.2. *Effects of diet on mitochondrial phospholipid content of trout liver*

300 The phospholipid (PL) content and PL class composition of liver mitochondria from 1-
301 and 3-year-old rainbow trout fed the experimental diets are shown in Fig. 2. Phosphatidylcholine
302 (PC) and phosphatidylethanolamine (PE) were the most abundant PL species in all the
303 experimental groups, constituting more than 71% of total PL in 1-year-old trout and more than
304 68% in 3-year-old animals. Among the remaining PL classes and focusing only in the control
305 group (FO) in both age groups, the rank order was cardiolipin (CL) (9.5 and 11.4% for 1- and 3-
306 year-old trout, respectively), followed by phosphatidylinositol (PI) (4.4 and 6.6%) with lower
307 amounts of phosphatidylserine (PS) and sphingomyelin (SM). Mitochondria from liver of 1-year-
308 old fish fed the FO diet had a different PL composition to that of 3-year-old fish, with significantly

309 more PE (p= 0.000), SM (p = 0.000) and CL (p = 0.020) while the content of PS (p = 0.027) and
310 PI (p = 0.000) was lower.

311 Dietary FA composition affected PL content and composition in rainbow trout. In 1-year-
312 old trout several significant changes were found among experimental groups. The RO group
313 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
314 [F(2,9 = 6.173, p = 0.010] and lower proportions of total PL [F(2,9 = 30.389, p = 0.000], CL
315 [F(2,9 = 55.566, p = 0.000] and SM [F(2,9 = 12.062, p = 0.001] compared with the FO and DHA
316 groups. The FO and DHA groups had a similar PL composition with only CL showing a
317 significant difference (p = 0.009), being higher in fish fed FO compared to fish fed DHA (Fig. 2).
318 However, the DHA group had the lowest content of total PL. A different situation was found in 3-
319 year-old fish, with fewer differences among the feeding groups. The effect of diet fatty acid
320 composition on mitochondrial PL composition was age-dependent for all classes except PS,
321 (Age*Diet, Fig. 2).

322

323 3.3. *Effects of diet on phospholipid fatty acid compositions of liver mitochondria*

324 Fatty acid compositions of the three main phospholipid classes (PC, PE and CL) from
325 liver of 1- and 3-year-old rainbow trout are presented in Tables 4-6. The fatty acid compositions
326 of the minor PL classes, PI, PS and SM, are included as Supplementary Tables 1-3. The FA
327 profiles of mitochondrial PC, PE and CL from trout liver were basically similar in both age
328 groups (Tables 4-6). Further differences were found in PI (S. Table 1) and, particularly, in PS
329 which showed higher DHA (p = 0.000) and PIn (p = 0.000) in 1-year-old trout fed the FO diet
330 when compared to 3-year-old fish (S. Table 2). Diet lipid composition significantly affected the
331 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 4-6, S. Tables 1 and 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 and 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

356 DHA diet (p = 0.294, 1.000 and 0.560 for PC, PE and CL, respectively). In PC and PE, this was
357 mainly due to the fact that EPA content decreased (4%, p = 0.000, and 3%, p = 0.048,
358 respectively) while DHA increased (7%, p = 0.000, and 3%, p = 0.035, respectively). PIn
359 significantly increased in mitochondrial PC (p = 0.003) and PE (p = 0.032) but not in CL (p =
360 1.000) from 1-year-old trout fed the DHA diet compared with those from fish fed the FO diet.
361 Regarding the minor PL classes, SM showed virtually no differences between DHA and FO
362 groups (S. Table 3) while PI and PS from fish fed the DHA diet showed lower n-3 LC-PUFA,
363 particularly PS (around 20% less, p = 0.001) and PIn (p = 0.000) than those from trout fed the
364 FO diet (S. Tables 1 and 2).

365 The effects of diet on mitochondrial PL FA compositions varied between the two age
366 groups as indicated by the significant Age*Diet interactions (Tables 4-6, S. Tables 1-3). In
367 mitochondrial PC and PE from 3-year-old trout fed the DHA diet, lower DHA levels compared to
368 that of 1-year-old fish were observed (Tables 4 and 5). This was reflected in the different impact
369 of diet composition on PIn of mitochondrial PC and PE between the two age groups (p = 0.015
370 and 0.003, respectively). Diet DHA had a similar impact on mitochondrial CL FA composition in
371 both age groups. A similar trend was found in the three minor PL classes. Mitochondrial PI, PS
372 and SM from 3-year-old fish fed the DHA diet showed fewer changes than those from 1-year-old
373 trout (S. Tables 1-3). Regarding the RO diet, CL from 3-year-old trout showed less n-6 PUFA
374 than 1-year-old fish (28.2 vs. 33.2%, p = 0.001). While mitochondrial CL from 1-year-old fish fed
375 the RO diet had around 20% more n-6 PUFA than that from fish fed the FO diet, mitochondrial
376 CL from 3-year-old animals fed the RO diet had just around 14% more n-6 PUFA than that from
377 3-year-old fish fed the FO diet. There were no significant differences in n-6 PUFA levels in the
378 remaining PL classes between the two age groups when fed the RO diet.

379

380 3.4. Lipid peroxidation

381 Lipid peroxidation in rainbow trout mitochondria, estimated by measuring the TBARS
382 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
385 group (1164.4 vs. 590.1 ng/g lipid, respectively). Although 3-year-old fish fed the DHA diet
386 showed lower lipid peroxidation (652.3 ng/g lipid) than that of the FO group, it was not
387 statistically significant ($p = 0.060$). Regarding age differences, lipid peroxidation was statistically
388 higher in 1-year-old trout fed the DHA diet than in 3-year-old fish fed the same diet ($p = 0.020$)
389 (Fig. 3). No significant differences were found between age groups in fish fed the FO or RO
390 diets. The effect of diet lipid composition on mitochondria lipid peroxidation was not influenced
391 by age as determined by two-way ANOVA ($p = 0.430$).

392

393 3.5. Mitochondrial gene expression

394 One-year old rainbow trout fed the different diets showed some consistent trends in
395 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
397 studied genes compared with the FO group while trout fed the DHA diet had in general lower
398 expression than the FO group. Values for 3-year-old trout were in general more stable between
399 diet groups. When the two age groups were compared, 3-year-old fish showed generally lower
400 gene expression than 1-year-old animals (except for fish fed the DHA diet) (Fig. 4),but statistical

401 significant differences were only found in fish fed the RO diet for COX3 (p= 0.021), ND1 (p=
402 0.013), ND4 (p= 0.018) and ND5 (p= 0.022). The interaction between age and diet was only
403 significant for ND4 (p= 0.037).

404

405 **4. Discussion**

406 *4.1. Diet fatty acid composition and mitochondrial lipids*

407 Dietary lipid composition markedly influenced PL contents and FA compositions of
408 individual PL classes of mitochondrial membranes from rainbow trout liver, but had generally
409 low impact upon mitochondrial DNA (mtDNA) gene expression. The effects differed in relation to
410 fish age. One-year-old trout fed RO and, mainly, DHA diet showed a significantly lower total PL
411 content (percentage of total lipid) in their liver mitochondrial membranes compared with the FO
412 group. Moreover, the RO group showed significantly higher proportions of PC, PE and PI and
413 lower percentages of CL and SM, while trout fed the DHA diet only showed a significantly lower
414 CL content compared with the FO group. These changes in the proportions of individual PL
415 classes may alter mitochondrial membrane function by affecting charge distribution across the
416 membrane, membrane permeability properties, catalytic activities or specific enzymes and ETC
417 function (Daum 1985). Especially interesting was the observed loss of CL considering the
418 critical role that CL plays in the MIM as a regulator of processes related to oxidative
419 phosphorylation and mitochondrial integrity (Paradies *et al*. 2002). Loss of CL has been shown
420 in a variety of tissues in mammals and fish (Chicco and Sparagna 2007; Almaida-Pagán *et al*.
421 2012) and suggested as one of the first signs of damage caused by high oxidative stress in
422 mitochondrial membranes. Decreased SM has also been related with oxidative stress since SM
423 can retard the lateral propagation of free radicals through the membrane and it is an important
424 mediator of mitochondrial pathways including apoptosis (Hannum and Obeid 1997; Subbaiah *et*
425 *al*. 1999; Cutler and Mattson 2001).

426 The FA compositions of individual PL classes from liver mitochondria of 1-year-old trout
427 were strongly affected by the diet lipid composition but the observed changes did not entirely

428 reflect differences among the diets. These data agree with those showing marked modifications
429 of FA of total mitochondrial PL by diet in rats (Lemieux et al. 2008; Abbott et al. 2010) and fish
430 (Ushio *et al*. 1997; Robin *et al*. 2003; Guderley *et al*. 2008; Østbye *et al*. 2011; Martin *et al*.
431 2013), and indicates the existence of mechanisms regulating FA within mitochondrial PL
432 classes. Moreover, these data point to the importance of FA composition of specific PL classes
433 in mitochondrial membranes. Individual mitochondrial PL classes were affected by the diet in
434 different ways. CL in liver mitochondria from one-year-old fish fed the DHA diet had almost the
435 same FA composition than that from the FO group, while CL from the RO group had around 20
436 % more n-6 PUFA than that from the FO group, considerably larger than the difference in n-6
437 PUFA content between the two diets (12%). The FA compositions of PC and PE reflected more
438 clearly the diet composition although changes in these two PL classes were attenuated
439 compared with the diets. The influence of diet on liver mitochondrial DHA level was generally
440 smaller than expected, considering the large differences between diets, especially between RO
441 and DHA (3.6 vs. 22.3%). This can be in part explained by preferential retention of DHA in fish
442 tissues when this FA is very low in the diet, as found in Atlantic salmon and other fish species
443 (Bell *et al*. 2004, NRC 2011) and/or increased endogenous synthesis of DHA from 18:3n-3 in
444 rainbow trout when dietary levels of n-3 LC-PUFA are reduced (Tocher *et al*. 2002). Selective
445 incorporation of FA from the diet and biosynthesis of FA are regulatory mechanisms that have
446 been shown in trout (Guderley *et al*. 2008). Another explanation may be an increase in the
447 degree of oxidative stress and, particularly, lipid peroxidation that would decrease membrane
448 levels of PUFAs (Hulbert *et al*. 2007). The observed decrease in mitochondrial total PL content
449 in RO and especially, DHA groups could indicate the existence of high oxidative stress as was
450 found in liver and muscle mitochondria of Atlantic salmon fed diets with a high level of n-3 LC-
451 PUFA (Kjaer *et al*. 2008; Østbye *et al*. 2011). These studies also showed a decrease in CL
452 content in mitochondrial membranes that agrees with data in the present study. PL classes
453 incorporating LC-PUFA would become more susceptible to oxidative attack and would
454 contribute to the observed reduction in total PL. Among the minor PL classes, a noteable
455 decrease in DHA content in mitochondrial PS from fish fed the RO and DHA diets was found
456 which could be also indicating high oxidative stress since PS is the PL with the highest PIn.

457 However, these data were not consistent with lipid peroxidation results from the present study
458 that showed no significant differences among feeding groups.

459 *4.2. Diet lipid composition and mitochondrial genes*

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

484 *4.3. Fish age*

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

500 *4.4. Membrane pacemaker hypothesis (MPH)*

501 In conclusion, the present study showed marked changes in mitochondrial PL content
502 and composition from trout liver when diet lipid composition was modified. These changes were
503 PL specific and showed some regulatory mechanisms operated on mitochondrial lipids from
504 trout liver as they did not clearly reflect differences among the diets. The main PL classes from
505 trout mitochondria showed the PIn was higher in fish fed the DHA diet than in the RO group.
506 Following the MPH, this would render the former more prone to oxidative attack and would
507 explain the decrease in total PL and CL content observed in 1-year-old animals. Membranes
508 with higher n-3 LC-PUFA content are also related with higher metabolism and ROS production
509 (Pamplona *et al*. 2002; Sanz *et al*. 2006) which, eventually, could affect mtDNA. TBARS values,
510 however, did not show any differences in lipid peroxidation among feeding groups. Changes
511 observed in liver mitochondrial lipids from trout fed the RO diet could not be explained following
512 the MPH. The three main PL classes in liver mitochondria had lower PIn and, therefore, were
513 less susceptible to oxidative attack. In this case, fish could be suffering stress due to the

514 influence of dietary lipid composition, which has been shown to induce changes in metabolism
515 (Tocher *et al*. 2001). Therefore, changes observed in liver mitochondrial PL content and
516 composition from trout fed the RO diet could be reflecting the existence of compensatory
517 mechanisms in mitochondrial membranes as a response to dietary FA composition. In both
518 cases, mitochondrial function could be compromised by diet lipid composition which would
519 affect animal well-being and longevity. Mitochondrial lipid composition and mtDNA expression
520 from trout liver were affected by diet in a different way when the two age groups were
521 compared. The observed changes could in part be explained by the different SGR of the two
522 age groups, this being related to different fatty acid incorporation into fish tissues, but may also
523 indicate age-related changes in the animal's capacity for maintaining the structural
524 homoeostasis of mitochondrial membranes.

525

526 **Acknowledgments**

527 The authors gratefully acknowledge our colleagues Professor Gordon Bell for formulation and
528 manufacture of the experimental feeds and Niall Auchinachie for fish husbandry. This research
529 and P.F.A.-P. were funded by a Marie Curie Intra-European Fellowship within the 7th
530 Community Framework Programme (PIEF-GA-2011-297964, OLDMITO). The authors report no
531 conflicts of interest.

532

533

534 **References**

535 Abbott SK, Else PL, Hulbert AJ (2010) Membrane fatty acid composition of rat skeletal muscle
536 is most responsive to the balance of dietary n-3 and n-6 PUFA. Br J Nutr 103:522-9.

537 Almaida-Pagán PF, de Costa J, Mendiola P, Tocher DR (2012) Age-related changes in
538 mitochondrial membrane composition of rainbow trout (*Oncorhynchus mykiss*) heart and brain.
539 Comp Biochem Physiol B 163:129-37.

540 Barzanti V, Battino M, Baracca A, Cavazzoni M, Cocchi M, Noble R, Maranesi M, Turchetto E,
541 Lenaz G (1994) The effect of dietary-lipid changes on the fatty-acid composition and function of
542 liver, heart and brain mitochondria in the rat at different ages. Br J Nutr 71(2):193-202.

543 Bell JG, Henderson RJ, Tocher DR, Sargent JR (2004) Replacement of dietary fish oil with
544 increasing levels of linseed oil: modification of flesh fatty acid compositions in Atlantic salmon
545 (Salmo salar) using a fish oil finishing diet. Lipids 39(3):223-32.

546 Burk RF, Trumble MJ, Lawrence RA (1980) Rat hepatic cytosolic GSH-dependent enzyme
547 protection against lipid peroxidation in the NADPH microsomal lipid peroxidation system.
548 Biochim Biophys Acta 618:35-41.

549 Chicco AJ, Sparagna GC (2007) Role of cardiolipin alterations in mitochondrial dysfunction and
550 disease. Am J Physiol Cell Physiol 292:C33-C44.

551 Chomczynski P, Mackey K (1995) Modification of the TRI reagent procedure for isolation of
552 RNA from polysaccharide- and proteoglycan-rich sources. BioTechniques 19:942-5.

553 Christie WW (2003) Lipid analysis: isolation, separation, identification and structural analysis of
554 lipids. 3rd ed. Oily Press, Somerset, UK.

555 Clandinin MT, Field CJ, Hargraves K, Morson L, Zsigmond E (1985) Role of diet fat in
556 subcellular structure and function. Can J Physiol Pharm 63(546):556.

557 Cortopassi GA, Wong A (1999) Mitochondria in organismal aging and degeneration. Biochim
558 Biophys Acta 1410:183-93.

559 Crimi M, Esposti MD (2011) Apoptosis-induced changes in mitochondrial lipids. Biochim
560 Biophys Acta Mol Cell Res 1813:551-7.

561 Cutler RG, Mattson MP (2001) Sphingomyelin and ceramide as regulators of development and
562 lifespan. Mech Ageing Dev 122:895-908.

563 Daum G (1985) Lipids of mitochondria. Biochim Biophys Acta 822:1-42.

564 Folch J, Lees M, Sloane-Stanley GH (1957) A simple method for the isolation and purification of
565 total lipids from animal tissues. J Biol Chem 226:497-509.

566 Guderley H, Kraffe E, Bureau W (2008) Dietary fatty acid composition changes mitochondrial
567 phospholipids and oxidative capacities in rainbow trout red muscle. J Comp Physiol B 178:385-
568 99.

569 Hannum YA, Obeid LM (1994) Ceramide and the eukaryotic stress response. Biochem Soc
570 Trans 25:1171-5.

571 Hansford RG, Castro F (1982) Age-linked changes in the activity of enzymes of the
572 tricarboxylate cycle and lipid oxidation, and of carnitine content in muscles of the rat. Mech
573 Ageing Dev 19:191-201.

574 Hazel JR, Williams EE (1990) The role of alterations in membrane lipid composition in enabling
575 physiological adaptation of organisms to their physical environment. Prog Lipid Res 29:167-227.

576 Henderson RJ, Tocher DR (1992) Thin-layer chromatography. In: Hamilton RJ, Hamilton S,
577 editors. Lipid Analysis. A Practical Approach. Oxford, UK.: IRL Press; p 65-111.

578 Hoch FL (1992) Cardiolipins and biomembrane function. Biochim Biophys Acta 1113:71-133.

579 Hulbert AJ (2007) Membrane fatty acids as pacemaker of animal metabolism. Lipids 42:811-9.

580 Hulbert AJ (2008) The links between membrane composition, metabolic rate and lifespan.
581 Comp Biochem Physiol Part A 150:196-203.

582 Hulbert AJ, Turner N, Storlien LH, Else PL (2005) Dietary fats and membrane function:
583 implications for metabolism and disease. Biol Rev 80:155-69.

584 Hulbert AJ, Faulks SC, Buffenstein R (2006) Oxidation-resistant membrane phospholipids can
585 explain longevity differences among the longest living rodents and similarly-sized mice. J
586 Gerontol A Biol Sci Med Sci 61:1009-18.Hulbert AJ, Pamplona R, Buffenstein R, Buttemer A
587 (2007) Life and death: Metabolic rate, membrane composition and life span of animals. Physiol
588 Rev 87: 1175–1213.

589 Jaya-Ram A, Kuah M, Lim P, Kolkovski S, Shu-Chien AC (2008) Influence of dietary HUFA
590 levels on reproductive performance, tissue fatty acid profile and desaturase and elongase
591 mRNAs expression in female zebrafish *Danio rerio*. Aquaculture 277:275-81.

592 Kjaer MA, Todorčević M, Torstensen B, Vegusdal A, Ruyter B (2008) Dietary n-3 HUFA Affects
593 mitochondrial fatty acid b-Oxidation Capacity and Susceptibility to Oxidative Stress in Atlantic
594 Salmon. Lipids 43:813-27.

595 Kraffe E, Marty Y, Guderley H (2007) Changes in mitochondrial oxidative capacities during
596 thermal acclimation of rainbow trout *Oncorhynchus mykiss*: roles of membrane proteins,
597 phospholipids and their fatty acid compositions. J Exp Biol 210:149-65.

598 Lemieux H, Blier PU, Tardif JC (2008) Does membrane fatty acid composition modulate
599 mitochondrial functions and their thermal sensitivity? Comp Biochem Physiol A Mol Integr
600 Physiol 149:20-9.

601 Manoli I, Alesci S, Blackman MR, Sun YA, Rennert OM, Chrousos GP (2007) Mitochondria as
602 key components of the stress response. TRENDS Endocrin Met 18(5):190-8.

603 Marshall O (2004) PerlPrimer: cross-platform, graphical primer design for standard, bisulphite
604 and real-time PCR. Bioinformatics 20(15):2471-2.

605 Martin N, Bureau DP, Marty Y, Kraffe E, Guderley H (2013) Dietary lipid quality and
606 mitochondrial membrane composition in trout: responses of membrane enzymes and oxidative
607 capacities. J Comp Physiol B 183:393-408.

608 Modi HR, Katyare SS (2008) Ageing-Induced alterations in lipid/phospholipid profiles of rat brain
609 and liver mitochondria: Implications for mitochondrial energy-linked functions. J Membrane Biol
610 221:51-60.

611 Monteiro JP, Oliveira PJ, Jurado AS (2013) Mitochondrial membrane lipid remodelling in
612 pathophysiology: A new target for diet and therapeutic interventions. Prog Lipid Res 52(4):513-
613 28.

614 Moyes CD, Ballantyne JS (2011) Membranes and temperature: homeoviscous adaptation. In:
615 Farrell A (ed.) Encyclopedia of Fish Physiology: From Genome to Environment. Academic
616 Press. Elsevier Inc, pp 1725-31.

617 National Research Council (NRC) (2011) Nutrient requirements of fish and shrimp. The National
618 Academies Press, Washington D.C.

619 Olsen RE, Henderson RJ (1989) The rapid analysis of neutral and polar marine lipids using
620 double-development HPTLC and scanning densitometry. J Exp Mar Biol Ecol 129:189-97.

621 Østbye TK, Kjaer MA, Rorá AMB, Torstensen B, Ruyter B (2011) High n-3 HUFA levels in the
622 diet of Atlantic salmon affect muscle and mitochondrial membrane lipids and their susceptibility
623 to oxidative stress. Aquacult Nutr 17(2):177-90.

624 Otto D, Moon T (1996) Endogenous antioxidant systems of two teleost fish, the rainbow trout
625 and the black bullhead. Fish Physiol Biochem 15:349-58.

626 Pamplona R, Barja G, Portero-Otin M (2002) Membrane fatty acid unsaturation, protection
627 against oxidative stress, and maximum life span — a homeoviscous-longevity adaptation? Ann
628 N Y Acad Sci 959:475-90.

629 Paradies G, Ruggiero FM, Quagliariello E (1992) Age-dependent changes in the activity of
630 anion carriers and in the lipid composition in rat heart mitochondria. Ann NY Acad Sci 673:160-
631 4.

632 Paradies G, Petrosillo G, Pistolese M, Ruggiero FM (2002) Reactive oxygen species affect
633 mitochondrial electron transport complex I activity through oxidative cardiolipin damage. Gene
634 286:135-41.

635 Paradies G, Petrosillo G, Paradies V, Ruggiero FM (2011) Mitochondrial dysfunction in brain
636 aging: Role of oxidative stress and cardiolipin. Neurochem Int 58:447-57.

637 Rajapakse N, Shimizu K, Payne M, Busija D (2001) Isolation and characterization of intact
638 mitochondria from neonatal rat brain. Brain Res Protoc 8:176-83.

639 Richter C (1995) Oxidative damage to mitochondrial DNA and its relationship to ageing. Int J
640 Biochem Cell Biol 27(7):647-53.

641 Robin JH, Regost C, Kaushik SJ (2003) Fatty acid profile of fish following a change in dietary
642 fatty acid source: model of fatty acid composition with a dilution hypothesis. Aquaculture
643 225:283-93.

644 Rohrbach S (2009) Effects of dietary polyunsaturated fatty acids on mitochondria. Curr Pharm
645 Des 15:4103-16.

646 Sanz A, Pamplona R, Barja G (2006) Is the mitochondrial free radical theory of aging intact?
647 Antioxid Redox Sign 8:582-99.

648 Shigenaga MK, Hagen TM, Ames BN (1994) Oxidative damage and mitochondrial decay in
649 aging. Proc Nat Acad Sci U S 91:10771-8.

650 Subbaiah PV, Subramanian VS, Wang K (1999) Novel physiological function of sphingomyelin
651 in plasma. J Biol Chem 274(51):36409-14.

652 Tocher DR, Bell JG, MacGlaughlin P, McGhee F, Dick JR (2001) Hepatocyte fatty acid
653 desaturation and polyunsaturated fatty acid composition of liver in salmonids: effects of dietary
654 vegetable oil. Comp Biochem Physiol B 130:257-70.

655 Tocher DR, Agaba M, Hastings N, Bell JG, Dick JR, Teale AJ (2002) Nutritional regulation of
656 hepatocyte fatty acid desaturation and polyunsaturated fatty acid composition in zebrafish
657 (Danio rerio) and tilapia (Oreochromis niloticus). Fish Physiol Biochem 24:309-20.

658 Trifunovic A, Larsson NG (2008) Mitochondrial dysfunction as a cause of ageing. J Intern Med
659 263:167-78.

660 Ushio H, Muramatsu M, Ohshima T, Koizumi C (1997) Fatty acid compositions of biological
661 membranes from fast skeletal muscle of carp. Fisheries Sci 62(3):427-34.

662 Vu TH, Tanji K, Pallotti F, Golzi V, Hirano M, DiMauro S, Bonilla E (2000) Analysis of mtDNA
663 deletions in muscle by in situ hybridization. Muscle Nerve 23:80-5.

664 Wiseman H (1996) Dietary influences on membrane function: importance in protection against
665 oxidative damage and disease. J Nutr Biochem 7:2-15.

666 Witting LA, Horwitt MK (1964) Effect of Degree of Fatty Acid Unsaturation in Tocopherol
667 Deficiency-induced Creatinuria. J Nutr 82:19-33.

668 Yamaoka S, Urade R, Makoto K (1988) Mitochondrial function in rats is affected by modification
669 of membrane phospholipids with dietary sardine oil. J Nutr 118:290-6.

670 Zabelinskii SA, Chebotareva MA, Kostkin VB, Krivchenko AI (1999) Phospholipids and their
671 fatty acids in mitochondria, synaptosomes and myelin from the liver and brain of trout and rat: a
672 new view on the role of fatty acids in membranes. Comp Biochem Physiol Part B 124:187-93.

673

674 **Figure legends**

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

678 Figure 2. Phospholipid content (percentage of total lipid weight) and phospholipid class
679 composition (percentage of total phospholipids) of mitochondria isolated from liver of 1- and 3-
680 year-old rainbow trout fed three different diets. Results are means ± SEM. (n=4). Different

681 superscript letters represent differences between feeding groups for each phospholipid class as
682 determined by two-way ANOVA (P<0.05). Table represents P values for interaction Diet and
683 Age for each phospholipid class (P<0.05). Asterisks denote significant differences between age
684 groups for each phospholipid class when compared using a Bonferroni test (P<0.05). CL,
685 cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol;
686 PL, phospholipid; ∑PL, total phospholipids; PS, phosphatidylserine; SM, sphingomyelin. SM
687 content found in mitochondria of 3-year-old trout fed the RO and DHA diet was lower than 0.4%.

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

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

699

Table 1. Ingredients and feed composition by proximate analysis (n= 4)

	SHFO	RO	DHA
Ingredients (g per 100 g dry weight)			
Fish meal[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
Carboxy-methyl-celluolose (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 fat	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

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

	SHFO	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	218.0	77.7	248.5

Fatty acids representing less than 1.0% of total fatty acids are not shown.
PIn, peroxidation index; PUFA, polyunsaturated fatty acids.

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%

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.

	1 year			3 years			Age*Diet
Fatty acid	SHFO	RO	DHA	SHFO	RO	DHA	*P*
14:0	2.5±0.1^{b*}	1.0±0.1^{a}	1.2±0.1^{a*}	2.1±0.4^{c}	1.1±0.1^{a}	1.6±0.1^{b}	0.003
16:0	23.0±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±0.2^{a*}	4.9±0.5	3.5±1.1	5.1±0.8	0.166
∑Saturated	30.6±0.4^{c*}	22.9±0.8^{a}	26.4±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±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±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±0.1^{a*}	2.3±0.3^{b}	1.0±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±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±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±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±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±1.2^{a*}	47.0±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±1.3^{a*}	54.4±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±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±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±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±0.1^{a*}	442.4±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 (SHFO, RO and DHA) when

compared using a Bonferroni test (p<0.05). Right column represent signification values for the interaction between Diet and Age (p<0.05). SHFO, 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.
Fatty acids representing less than 1% of total fatty acids are not shown.

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.

	1 year			3 years			Age*Diet
Fatty acid	SHFO	RO	DHA	SHFO	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

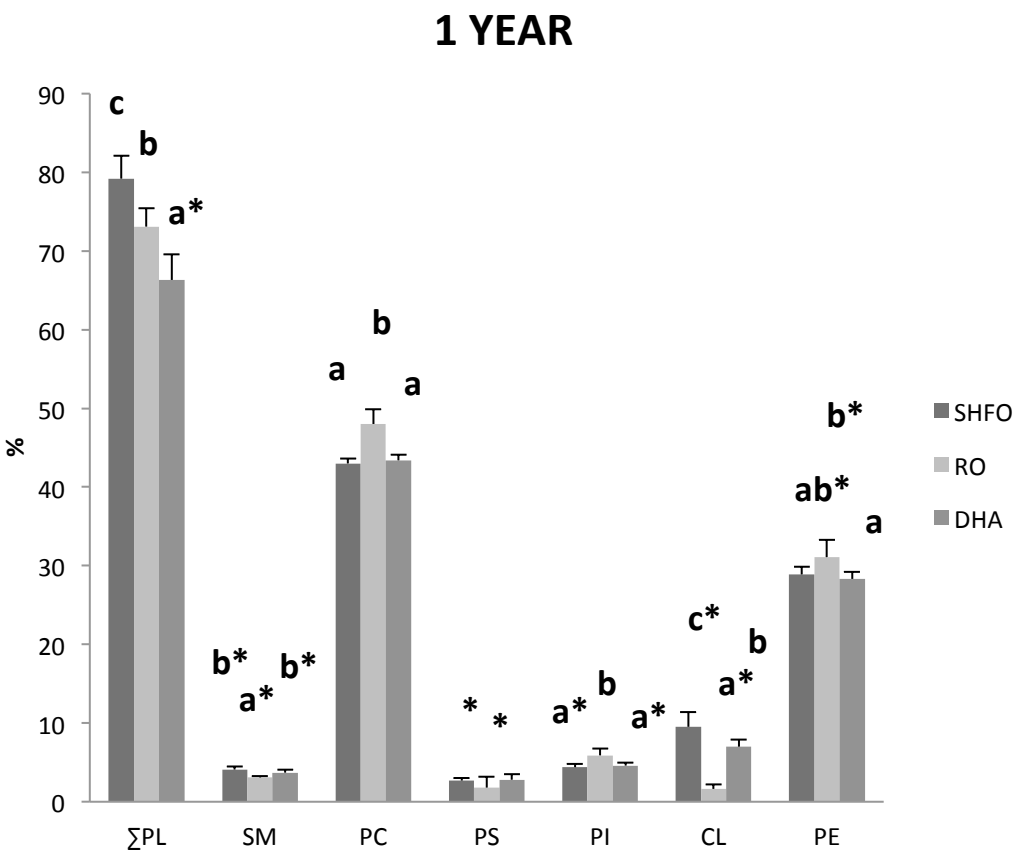
Data expressed as mean ± SD (n=4). Different superscript letters within a row and for each age group represent significant differences between diet treatments as determined by a two-way ANOVA (p<0.05). Asterisks denote statistical differences between 1- and 3-year-old trout for each diet group (SHFO,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

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.

	1 year			3 years			Age*Diet
Fatty acid	SHFO	RO	DHA	SHFO	RO	DHA	*P*
14:0	2.3±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±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±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±0.5^{c*}	1.4±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±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±0.1*	1.4±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±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±0.4^{a*}	21.1±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±0.3^{a*}	6.3±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±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±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±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±0.2^{b*}	0.6±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±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±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±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 (SHFO, RO and DHA) when

compared using a Bonferroni test (p<0.05). Right column represent signification values for the interaction between Diet and Age (p<0.05). SHFO, 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.
Fatty acids representing less than 1% of total fatty acids are not shown.



	Age*Diet
	P
∑PL	<0.001
SM	<0.001
PC	<0.001
PS	0.131
PI	0.033
CL	<0.001
PE	0.002

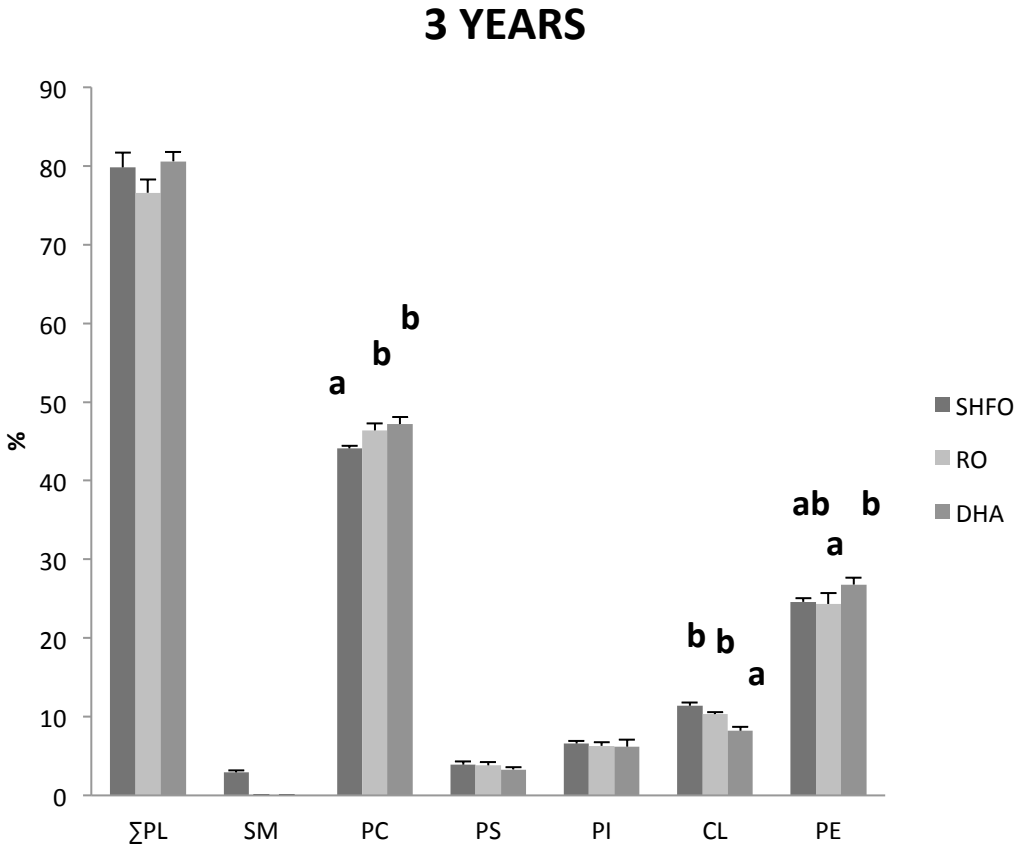


Figure 1. Almaida-Pagán *et al*.

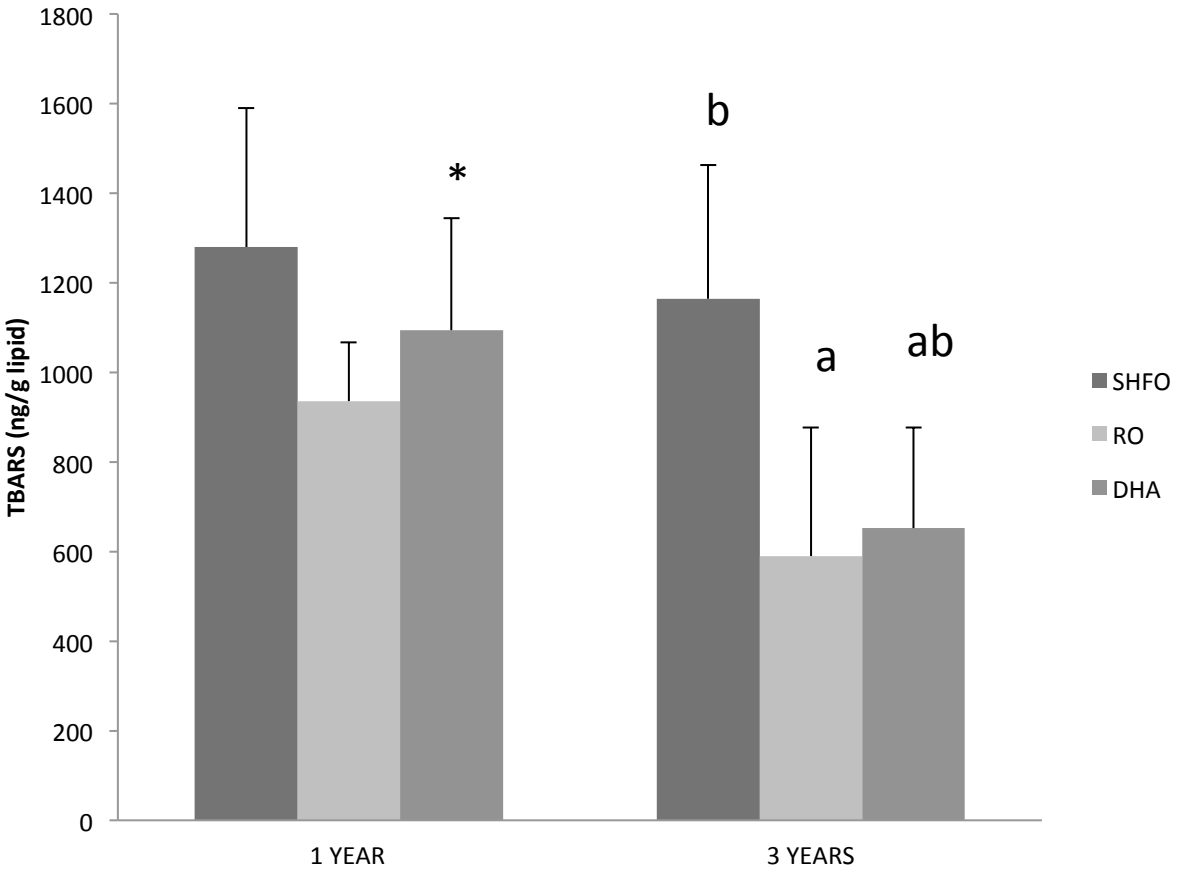
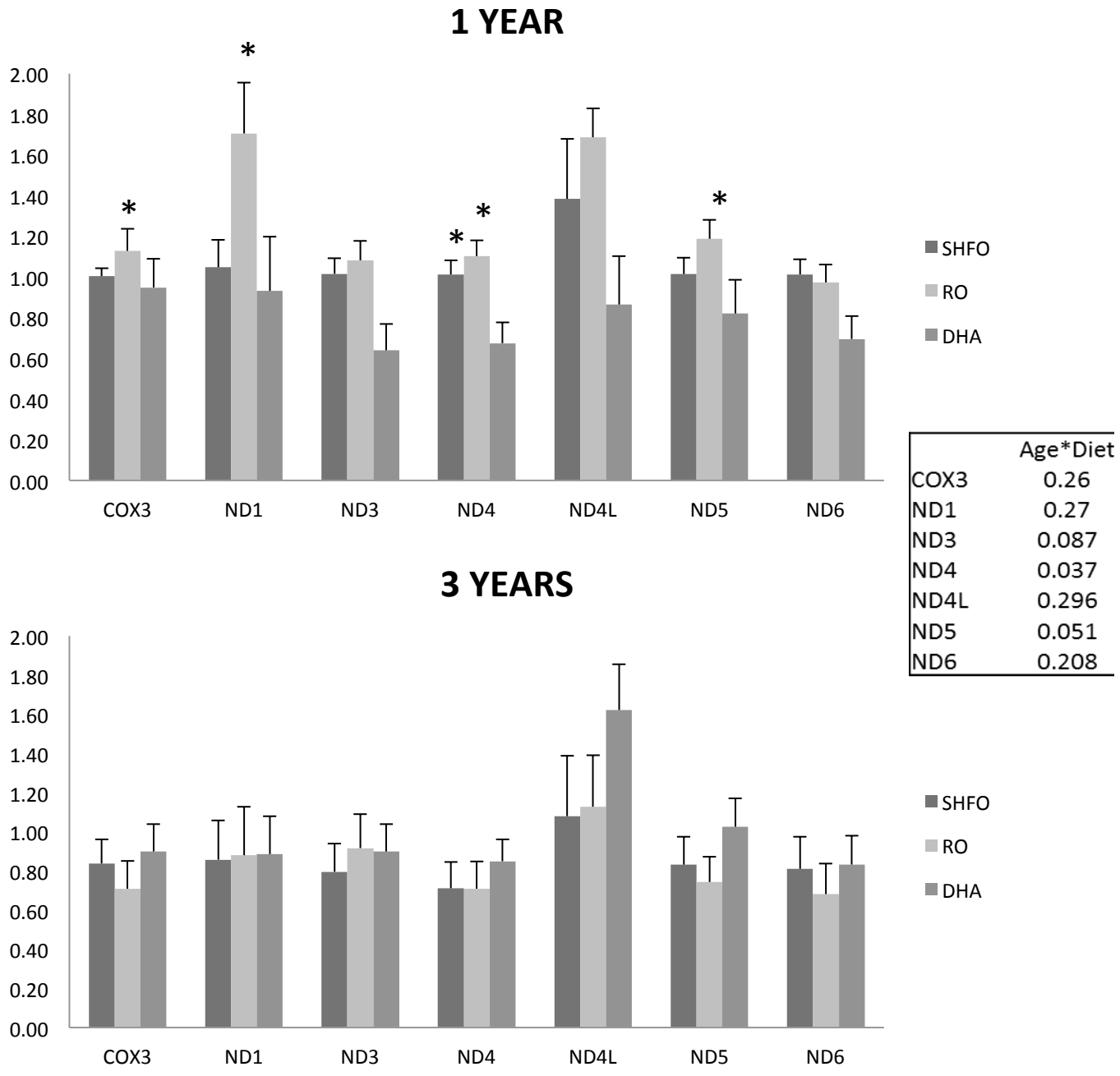


Figure 2. Almaida-Pagán *et al*.



	Age*Diet
COX3	0.26
ND1	0.27
ND3	0.087
ND4	0.037
ND4L	0.296
ND5	0.051
ND6	0.208

Figure 3. Almaida-Pagán *et al*.

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

	Liver			3 years			Age*Diet
Fatty acid	SHFO	RO	DHA	SHFO	RO	DHA	*P*
16:0	4.3±0.3*	5.1±1.1	3.8±0.2*	9.2±1.2^{b}	4.7±0.7^{a}	5.6±1.2^{a}	<0.001
18:0	35.0±0.9*	30.4±1.5*	33.1±1.5*	23.2±4.4	26.8±1.9	28.4±3.2	0.008
∑Saturated	40.3±0.8*	36.5±2.7	37.7±1.5	33.7±3.2	32.2±1.5	34.7±2.1	0.239
18:1n-9	4.2±0.1^{a*}	8.6±1.2^{b}	5.4±0.8^{a}	9.4±1.9^{b}	10.5±0.^{b}	6.7±1.6^{a}	0.009
18:1n-7	1.0±0.1*	0.7±0.1	0.9±0.1*	2.0±0.5^{b}	1.0±0.2^{a}	1.4±0.3^{ab}	0.025
20:1n-9	1.0±0.1*	1.5±0.2*	1.2±0.2*	2.4±0.5	2.2±0.3	1.9±0.2	0.029
∑Monounsaturated	7.1±0.2^{a*}	12.6±2.2^{b}	8.6±1.5^{a}	16.4±3.0^{b}	14.2±1.0^{b}	10.8±2.1^{a}	0.001
18:2n-6	0.4±0.0^{a*}	1.4±0.3^{b}	0.6±0.2^{a}	1.3±0.4^{ab}	1.5±0.2^{b}	0.8±0.2^{a}	0.011
20:4n-6	34.5±2.3^{a}	32.9±3.8^{a}	41.1±1.2^{b}	31.4±4.2^{a}	34.9±2.2^{a}	40.0±1.2^{b}	0.197
∑n-6 PUFA	36.0±2.3^{a}	39.0±4.1^{ab}	43.2±0.9^{b}	35.0±4.2^{a}	41.3±0.6^{b}	42.7±1.2^{b}	0.438
20:5n-3	1.1±0.1^{b*}	0.8±0.1^{b*}	0.4±0.1^{a*}	2.2±0.4^{b}	1.5±0.5^{ab}	0.8±0.3^{a}	0.036
22:5n-3	0.8±0.0^{b*}	0.3±0.1^{a*}	0.3±0.0^{a*}	1.4±0.3^{b}	0.5±0.2^{a}	0.5±0.1^{a}	0.374
22:6n-3	13.8±3.0^{b}	9.4±3.7^{ab}	9.1±0.5^{a}	10.3±1.2	9.6±0.2	10.1±0.4	0.081
∑n-3 PUFA	15.8±3.0^{b}	10.8±3.7^{ab}	10.0±0.4^{a}	14.4±1.4^{b}	12.0±0.5^{a}	11.7±0.3^{a}	0.302
∑PUFA	52.6±0.8	50.9±0.6*	53.8±0.2	50.0±3.8	53.5±0.5	54.6±0.9	0.015
∑n-3 LC-PUFA	16.3±3.4	9.0±2.5*	9.7±0.5*	14.1±1.6^{b}	12.0±0.1^{a}	11.5±0.3^{a}	0.003
n-3/n-6	0.5±0.1^{b}	0.2±0.1^{a}	0.2±0.0^{a}	0.4±0.1	0.3±0.0	0.3±0.0	0.744
PIn	274.0±19.2^{c*}	213.8±8.1^{a*}	248.8±3.1^{b*}	243.6±10.8^{a}	240.3±7.4^{a}	258.5±0.8^{b}	0.016

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 (SHFO, RO and DHA) when compared using a Bonferroni test (p<0.05). Right column represent signification values for the interaction between Diet and Age (p<0.05). SHFO, 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.
Fatty acids representing less than 1% of total fatty acids are not shown.

Supplementary Table 2. Fatty acid composition (percentage of total fatty acids) of phosphatidyserine of mitochondria isolated from liver of 1- and 3-year old rainbow trout fed with one of three experimental diets.

	Liver			3 years			Age*Diet
Fatty acid	SHFO	RO	DHA	SHFO	RO	DHA	*P*
16:0	8.5±1.4^{a*}	13.5±1.7^{b*}	10.6±0.9^{a}	12.3±2.4	10.4±1.5	11.8±0.9	0.002
18:0	9.0±1.5^{a*}	21.5±1.1^{b}	25.2±0.9^{b*}	15.9±1.4^{a}	18.9±4.2^{ab}	21.0±1.0^{b}	<0.001
∑Saturated	18.6±2.4^{a*}	36.8±2.4^{b}	37.2±0.9^{b}	30.3±3.2	30.9±5.9	34.2±0.8	<0.001
18:1n-9	2.9±0.5^{a*}	5.2±0.0^{b*}	2.8±0.2^{a}	4.2±0.6^{b}	4.5±0.5^{b}	3.2±0.3^{a}	0.001
18:1n-7	1.3±0.3*	1.2±0.1	1.3±0.1	2.7±0.9^{b}	1.4±0.1^{a}	1.9±0.6^{ab}	0.068
20:1n-9	0.7±0.2^{a*}	2.0±0.5^{b}	1.2±0.2^{a}	1.9±0.6	1.9±0.2	1.6±0.4	0.017
∑Monounsaturated	6.7±1.2^{a*}	10.9±1.1^{b*}	6.9±0.6^{a}	10.7±1.8	8.9±0.6	7.9±1.3	0.001
18:2n-6	0.6±0.2^{a}	1.5±0.1^{b}	0.4±0.1^{a}	0.6±0.3^{a}	1.3±0.2^{b}	0.5±0.1^{a}	0.515
20:4n-6	1.3±0.2	0.7±0.2	0.9±0.1*	1.2±0.4	0.9±0.4	1.6±0.4	0.128
22:5n-6	0.8±0.0^{a}	0.7±0.0^{a}	2.0±0.1^{b}	0.5±0.1^{a}	0.8±0.2^{a}	2.4±0.3^{b}	0.009
∑n-6 PUFA	3.4±0.4^{a}	6.1±2.0^{b}	4.4±0.2^{b*}	3.4±0.5^{a}	5.1±0.6^{b}	6.2±0.4^{b}	0.015
20:5n-3	8.2±0.7^{b*}	1.0±0.2^{a}	0.7±0.1^{a}	5.0±0.4^{b}	2.4±1.1^{a}	1.9±0.6^{a}	0.001
22:5n-3	3.1±0.3^{b}	0.6±0.0^{a}	0.4±0.0^{a}	3.0±0.6^{b}	1.3±0.9^{a}	0.9±0.3^{a}	0.306
22:6n-3	58.9±3.4^{c*}	42.1±1.3^{a*}	48.5±0.4^{b}	46.3±4.9	50.0±3.8	48.2±2.7	<0.001
∑n-3 PUFA	70.8±3.7^{c*}	44.2±0.9^{a*}	50.0±0.4^{b}	54.6±5.0	54.5±6.6	51.4±2.0	<0.001
∑PUFA	74.7±3.4^{b*}	52.2±1.3^{a*}	55.8±0.5^{a}	59.0±3.8	60.2±4.5	57.9±1.7	<0.001
∑n-3 LC-PUFA	70.6±4.6^{c*}	43.9±1.1^{a*}	49.9±0.4^{b}	54.4±4.8	55.8±4.2	52.0±0.9	<0.001
n-3/n-6	21.2±4.0^{c}	7.7±2.1^{b}	11.4±0.7^{a*}	16.4±4.0^{b}	11.4±2.2^{ab}	8.7±0.5^{a}	0.018
PIn	547.7±46.5^{c*}	361.3±4.6^{a*}	417.7±1.4^{b}	427.9±49.0	448.6±73.8	434.6±13.4	<0.001

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 (SHFO, RO and DHA) when compared using a Bonferroni test (p<0.05). Right column represent signification values for the interaction between Diet and Age (p<0.05). SHFO, 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.
Fatty acids representing less than 1% of total fatty acids are not shown.

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

	Liver			3 years			Age*Diet
Fatty acid	SHFO	RO	DHA	SHFO	RO	DHA	*P*
14:0	4.3±0.4^{b*}	1.8±0.2^{a*}	2.1±0.2^{a*}	6.0±0.9^{b}	3.8±1.2^{a}	3.7±0.5^{a}	0.901
16:0	19.3±2.5^{a}	24.1±1.7^{b*}	25.2±1.5^{b*}	16.7±3.9^{b}	8.5±1.1^{a}	12.8±0.1^{b}	<0.001
18:0	7.8±0.6*	7.6±0.4*	7.3±0.4	5.9±0.3	5.7±0.4	6.5±0.9	0.252
∑Saturated	33.2±2.3	35.1±1.2*	35.9±1.5*	30.3±4.4^{b}	19.8±1.0^{a}	24.1±1.3^{a}	0.002
16:1n-9	1.2±0.5	0.8±0.4*	0.6±0.2	1.7±0.5^{ab}	2.2±0.2^{b}	1.3±0.3^{a}	0.002
16:1n-7	1.6±0.5^{ab}	0.9±0.1^{a*}	2.0±0.1^{b}	2.9±1.7^{b}	0.2±0.1^{a}	0.0±0.0^{a}	<0.001
18:1n-9	12.1±4.8^{a}	25.3±2.1^{b*}	23.3±2.7^{b*}	12.3±2.9^{b}	6.2±1.3^{a}	9.8±1.1^{b}	<0.001
18:1n-7	1.4±0.2	1.4±0.1*	1.4±0.1	2.1±0.6^{c}	0.7±0.1^{a}	1.0±0.1^{b}	<0.001
24:1n-9	35.8±5.2^{b}	14.3±4.9^{a*}	16.7±2.8^{a*}	30.6±6.3^{a}	56.9±1.2^{b}	50.3±4.7^{b}	<0.001
∑Monounsaturated	53.6±2.2	45.3±3.2*	45.4±2.4*	53.2±2.5	67.1±1.0	63.8±2.9	0.062
18:2n-6	0.8±0.0^{a}	8.0±1.1^{b*}	1.3±0.1^{a}	1.1±0.3	1.7±0.2	0.8±0.3	<0.001
∑n-6 PUFA	3.1±0.3^{a*}	13.7±2.0^{b*}	4.7±0.7^{a*}	5.3±1.9	3.6±0.2	2.9±0.7	<0.001
20:5n-3	0.7±0.0	0.3±0.1	1.1±0.2	1.7±1.3	1.0±0.8	1.0±0.4	0.319
22:6n-3	7.4±1.5^{b}	4.3±0.9^{a}	11.3±2.9^{b*}	7.3±1.5	6.7±1.1	7.0±0.2	0.01
∑n-3 PUFA	8.7±1.6^{b}	5.4±1.1^{a*}	13.1±3.1^{b*}	10.1±3.0	8.6±1.7	8.5±0.7	0.017
Total PUFA	13.2±1.8^{a}	19.6±2.1^{b*}	18.7±3.0^{b*}	16.5±4.2	13.1±1.9	12.0±1.6	0.009
∑n-3 LC-PUFA	8.2±1.4^{ab}	5.0±1.1^{a}	12.8±3.1^{b*}	9.6±2.9	8.0±1.9	8.3±0.6	0.018
n-3/n-6	2.9±0.8^{b}	0.4±0.1^{a*}	2.8±0.8^{b}	2.0±0.7	2.4±0.4	3.0±0.4	0.005
PIn	74.8±4.2^{a}	61.6±11.8^{a}	110.3±29.1^{b*}	97.1±1.4^{b}	81.5±0.8^{a}	78.2±8.1^{a}	0.021

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 (SHFO, RO and DHA) when compared using a Bonferroni test (p<0.05). Right column represent signification values for the interaction between Diet and Age (p<0.05). SHFO, 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.
Fatty acids representing less than 1% of total fatty acids are not shown.