Effects of dietary fatty acids on mitochondrial phospholipid compositions, oxidative status and mitochondrial gene expression of zebrafish at different ages

Betancor, M.B. *†, Almaida-Pagán, P. F. †, Hernández, A., Tocher, D. R.

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

Abbreviations: B2M, β-2-microglobulin; BACT, β-actin; BHT: butylated hydroxytoluene; cDNA, complementary DNA; CL, cardiolipin; COX, cytochrome c oxidase complex; DHA, docosahexaenoic acid; ETC, electron transport chain; FA, fatty acid; FAME, fatty acid methyl esters; HP-TLC, high performance thin layer chromatography; LA, linoleic acid; LC-PUFA, long chain polyunsaturated fatty acid;MIM, mitochondrial inner membrane; mtDNA, mitochondrial DNA; MUFA, monounsaturated fatty acids; NAC, no-amplification control; ND, NADH-coenzyme Q oxidoreductase complex; NTC, no-template control; OA, oleic acid; PC, phosphatidylcholine, PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIn, peroxidation index; PL, phospholipid; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid;qPCR, quantitative PCR; RO, rapeseed oil; ROS, reactive oxygen species; SFA, saturated fatty acids; SM, sphingomyelin; RT-PCR, real-time PCR; SEM, standar error of the mean; SGR, specific growth rate; SOD, superoxide dismutase;TBARS, thiobarbituric acid reactive substances; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TLC, thin layer chromatography; Tm, melting temperature.

* Corresponding author;

Dr. Mónica B. Betancor, Tel: +44-1786-467993, m.b.betancor@stir.ac.uk

† Both authors contributed equally to this work

Abstract

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) and accumulation of mitochondrial DNA (mtDNA) mutations are among the main mechanisms involved in this process. In the present study, mitochondrial membrane PL compositions, oxidative status (TBARS content and SOD activity) and mtDNA gene expression of muscle and liver were analyzed in zebrafish fed two diets with lipid supplied either by rapeseed oil (RO) or a blend 60:40 of RO and DHA500 TG oil (DHA). Two feeding trials were performed using zebrafish from the same population of two ages (8- and 21-months). Dietary FA composition affected fish growth in 8-month-old animals, which could be related with an increase in stress promoted by diet composition. Lipid peroxidation was considerably higher in mitochondria of 8-month-old zebrafish fed the DHA diet than in animals fed the RO diet. This could indicate higher oxidative damage to mitochondrial lipids, very likely due to increased incorporation of DHA in PL of mitochondrial membranes. Lipids would be among the first molecules affected by mitochondrial reactive oxygen species, and lipid peroxidation could propagate oxidative reactions that would damage other molecules, including mtDNA. Mitochondrial lipid peroxidation and gene expression of 21-month-old fish showed lower responsiveness to diet composition than those of younger fish. Differences found in the effect of diet composition on mitochondrial lipids between the two age groups could be indicating age-related changes in the ability to maintain structural homeostasis of mitochondrial membranes.

Keywords: Diet, fatty acid, mitochondria, zebrafish, oxidative stress, phospholipid.

Introduction

Fatty acid (FA) composition of phospholipids (PL) greatly influences both plasma and mitochondrial membranes function (Hulbert et al. 2005). Both the chain length and the number of double bonds in acyl chains have a major influence on the physical properties of the lipids that contain them. Membranes with high levels of polyunsaturated fatty acids (PUFA) are more permeable to ions such as Na^+, K^+ and H^+. This is related to a high metabolic rate as ion pumping constitutes one of the main determinants of this factor (Hulbert et al. 2005). At the same time, membranes with high levels of PUFA are more prone to attack by oxidizing agents and will then participate in free radical reactions that will propagate the oxidative damage throughout the organelle and cell (Pamplona 2011; Zimmiak 2011; Naudi et al. 2013). These processes link membrane lipid composition to animal life span as observed in a wide range of species (Hulbert 2008). In comparative studies performed in various mammals, birds and reptiles, it has been found that species with shorter life span have more unsaturated membranes than species with longer life span (Pamplona et al. 2002; Hulbert et al. 2007).

The mitochondrial free radical theory of aging can still be considered the best explanation for aging and longevity in mammals, birds and multicellular animals in general (Barja 2013). Cell culture studies of invertebrate and mammal models support the fact that reactive oxygen species (ROS), particularly those from mitochondria, play a pivotal role in aging and senescence (Barja 2004; Balaban et al. 2005; Sanz et al. 2006). With age, oxidative stress inside mitochondria increases and oxidation products accumulate resulting in damage to the organelle lipids, protein and DNA. Eventually, this damage has been suggested to lead to mitochondrial dysfunction and, thus, to cell and tissue decay with aging (Shigenaga et al. 1994; Sohal et al. 2002; Paradies et al. 2011). It is known that mitochondrial membranes in mammals have a distinctive composition of lipid classes including PL, glycolipids and cholesterol (Wiseman 1996) related to the role of mitochondria in energy metabolism and oxygen consumption (Hoch 1992). The mitochondrial inner membrane (MIM) is constituted of 80 % proteins (mainly electron transport chain, ETC, components) and 20 % lipids containing a high percentage of the PL class cardiolipin (CL), and essentially no cholesterol (Schlame et al. 2000). CL is a key molecule in mitochondrial function as it participates in the regulation of electron transport and efficiency of oxidative phosphorylation, formation and stability of ETC super-complexes, binding of cytochrome c to MIM, functioning of MIM enzymes and control of mitochondrial phase apoptosis (Paradies et al. 2002). Moreover, CL

89 has a high content of PUFA that, along with its proximity to the site of ROS production,
90 makes it particularly prone to peroxidation. Reduced CL content and/or composition could
91 lead to mitochondrial dysfunction and cell decay. Mitochondrial membranes also contain
92 small amounts of sphingomyelin (SM), which has membrane-rigidifying properties (due to its
93 low content in PUFA) and may retard the lateral propagation of free radicals (Subbaiah et al.
94 1999; Cutler and Mattson 2011). Other PL species such as phosphatidylserine (PS) and
95 phosphatidylinositol (PI) are also important as they are precursors for signalling molecules,
96 some associated with apoptosis (Hannum and Obeid 1994; Ulmann et al. 2001). In addition,
97 fish mitochondrial activity also appears to be highly modulated by nutritional stressors (Enyu
98 and Shu-Chien 2011) or PUFA levels (Kjaer et al. 2008; Pérez-Sánchez et al. 2013; Almaida-
99 Pagán et al. 2015).

100 Although lipid peroxidation is quantitatively the main oxidative process inside
101 mitochondria, other organelle molecules are also attacked by ROS and damage to proteins and
102 nucleic acids occur (Sanz et al. 2006). Mitochondrial DNA (mtDNA) is exposed to ROS as it
103 is located close to the site of ROS production. Moreover, mtDNA is not highly condensed or
104 protected by histones, as is nuclear DNA, and its repair activity is limited (Paradies et al.
105 2011). It has been suggested that mtDNA is also a primary target of ROS and, as more
106 mutations in critical coding regions accumulate, complexes of the ETC become less efficient
107 or inactive, leading to a decline in mitochondrial function (Paradies et al. 2002). Since
108 mtDNA encodes either polypeptides of ETC or components required for their synthesis,
109 mutations in mtDNA will affect the ETC as a whole. ETC-deficient cells are also prone to
110 apoptosis and increased cell loss is therefore a likely important consequence of mitochondrial
111 dysfunction in situations of high oxidative stress (Trifunovic and Larsson 2008). It is
112 important to note that, although the mitochondrial translation machinery is responsible for the
113 synthesis of 13 catalytic proteins of the respiratory chain both in mammals and fish, more
114 than 70 proteins of oxidative phosphorylation are encoded by nuclear DNA, imported from
115 the cytosol and translocated across outer and inner mitochondrial membranes (Ljubicic et al.
116 2010; Voos 2013). Thus, biogenesis and function of mitochondria requires the encompassing
117 regulation of both mitochondrial and nuclear genomes (Smits et al. 2010).

118 In summary, mitochondria are among the first responders to various stressors that
119 challenge the homeostasis of cells and organisms (Manoli et al. 2007) and are deeply involved
120 in the aging process. Mitochondrial decay is generally associated with impairment in
121 mitochondrial bioenergetics function and increased oxidative stress (Paradies et al. 2011) and

it seems clear that deterioration of mitochondrial inner membrane phospholipids, particularly of CL, and accumulation of mtDNA mutations are mechanisms involved in this process. Diet fatty acid composition is one of the main factors modifying plasma and mitochondrial membrane lipid composition (reviewed in Hulbert et al. 2005). Changes in dietary FA composition modify mitochondrial membrane composition and can alter 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 dietary fatty acid composition on mitochondrial membrane composition and mtDNA gene expression, as possible regulators of the processes associated with mitochondrial decay under situations of high oxidative stress, including aging, in a vertebrate model. Zebrafish (*Danio rerio*) of two well-differentiated ages were used in order to study the interaction between diet lipid composition and fish age on redox signalling, antioxidant enzyme activities and lipid metabolism.

Materials and methods

Animals and diets

The experiment was performed on zebrafish of two different ages (8 and 21 months) belonging to a resident colony established in 2009 at the Institute of Aquaculture, University of Stirling (for colony conditions, see Almaida-Pagán et al. 2014). Fish belonging to two different age classes (8 or 21 months) each fed on two diets (rapeseed oil and DHA) were used in the present study and the trial carried out in duplicate (8 tanks in total). One hundred and twelve zebrafish of 0.21 ± 0.04g (8 months) and 32 fish of 0.61 ± 0.12g (21 months) were maintained at the same animal density in 30 and 13 l aquaria, respectively, containing filtered freshwater which was maintained at a constant temperature of 26 ± 1ºC and under a 12h light: 12h dark photoperiod. The tanks were cleaned daily with approximately one third of the water replaced each day.

Fish were fed four times a day with one of the experimental diets at a ration corresponding to 2 % of the fish wet weight for two months. The experimental diets were prepared in the Institute of Aquaculture. The diets contained 50 % crude protein and 11 % crude lipid and their formulation is shown in Table 1. The dry ingredients were combined and mixed before the addition of the oils (rapeseed oil for the RO diet and a 60:40 blend of

rapeseed oil and DHA500 TG oil for the DHA diet) and antioxidants and mixing continued for 5 min. Water was added to 30% of the dry weight to enable pelleting. Pellets of 1.0 mm were prepared, air dried for 48 h, sieved fines of up 0.5 mm and stored at −20 °C until use. The fatty acid compositions of the diets are shown in Table 2. All diets were formulated to satisfy the nutritional requirements of freshwater fish (NRC, 2011).

After the feeding trial, fish were euthanized by exposure to the anesthetic benzocaine hydrochloride (400 mg l^{-1}) for 10 min following the cessation of opercular movement and then submerged in chilled water (5 parts ice to 1 part water). Whole zebrafish [3 fish per replicate for 8- (n = 6) and one fish per replicate for 21-month-old fish (n = 4)], were taken for immediate preparation of mitochondria (n = 4) while muscle and liver from individual fish were stabilized in RNA*later*® (Life technologies, Paisley, UK) following the manufacturer´s instructions and stored at – 20 ºC for molecular analysis (n = 6 and 4 for 8- and 21-month-old fish, respectively). The purified mitochondrial isolates were analysed to determine mitochondrial membrane lipid composition, lipid peroxidation and superoxide dismutase activity. 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.

Mitochondria isolation

Approximately, 0.6 g of fresh (non-frozen) whole fish samples were 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 a tissue disrupter (IKA T25 digital Ultra-Turrax® Fisher Scientific, Loughborough, UK). Sucrose buffer homogenates were then twice centrifuged at 600 g for 6 min. After the first centrifugation, the pellet was discarded (cell/nuclei debris) and the supernatant recentrifuged at 600 g. Resulting supernatants were then centrifuged twice at 6,800 g for 10 min. After the first centrifugation, the pellet was resuspended in sucrose buffer and recentrifuged at 6,800 g. The final pellet was resuspended in 3 ml of 100 mM potassium phosphate buffer pH 7.8, with 0.1 mM EDTA. A 250 µl aliquot of each mitochondrial homogenate was frozen in liquid nitrogen and stored at −80 °C prior to enzyme activity measurements. The remaining mitochondrial homogenates were subjected directly to lipid extraction. To verify that pellets were highly enriched in mitochondria, portions of the isolates were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer

overnight at 4 °C and then processed prior to analysis by transmission electron microscopy (Tecnai G2 Spirit BioTWIN, FEI Europe, Eindhoven, The Netherlands) as described previously (Almaida-Pagán et al. 2012). Purity of preparations was also tested by measuring total (SOD) and mitochondria-specific (SOD2 or Mn-SOD) superoxide dismutase in the mitochondria isolates (Tocher et al. 2003). SOD2 activity in mitochondria pellets always represented more than 95 % of total superoxide dismutase activity.

Lipid extraction and phospholipid class composition

Total lipid of mitochondria from whole animal was obtained by solvent extraction basically according to Folch et al. (1957). Briefly, mitochondrial pellets were homogenized in 5 ml of ice-cold chloroform/methanol (2:1, by vol.) containing 0.01 (w/v) butylated hydroxytoluene (BHT) as antioxidant, followed by addition of 1 ml of 0.88 % (w/v) KCl, mixing and layers allowed to separate on ice for 1 h. The upper aqueous layer was aspirated and the lower organic layer evaporated under a stream of oxygen-free nitrogen. All lipid extracts were stored at −20 °C under a N_2 atmosphere prior to analysis.

Phospholipid 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 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

Individual phospholipid classes from mitochondria 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

visualization under UV light (UVGL-58 Minerallight® Lamp, Ultraviolet Prod. Inc., Calif., USA). 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) 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 × 0.32 mm i.d. capillary column (CP Wax 52CB, Chrompak, London, UK) and on-column injection at 50 °C. Hydrogen was used as a carrier gas and temperature programing was from 50 °C 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 2000 g, and absorbance in the supernatant determined 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}.

Superoxide dismutase (SOD) activity

Total superoxide dismutase (SOD) activity in mitochondrial preparations was assayed by measuring inhibition of oxygen-dependent oxidation of adrenaline (epinephrine) to adenochrome by xanthine oxidase plus xanthine (Panchenko et al. 1975). Plastic semi-microcuvettes containing 0.5 ml of 100 mM potassium phosphate buffer pH 7.8/0.1 mM EDTA, 200 µl adrenaline, 200 µl xanthine, and 50 µl distilled water (uninhibited control) or 50 µl test sample were prepared and the reaction initiated by the addition of 10 µl xanthine oxidase (Sigma X4875). The reaction was followed at 480 nm in a spectrophotometer (Uvikon 860, Kontron Instruments, St. Albans, UK) and 1 unit of SOD activity was described as the amount of the enzyme that inhibited the rate of adenochrome production by 50 %. The

percent inhibition of the test sample was correlated with SOD activity using a SOD standard curve (SOD concentration vs. % Inhibition of the rate of increase of absorbance at 480 nm). For mitochondria-specific SOD2, the major isoform in mitochondria, an assay mini-cuvette, was set up as before but, before addition of xanthine oxidase, 50 µl of 30 mM KCN was added to inhibit the cytosolic Cu/Zn-SOD while the mitochondrial Mn-SOD remains unaffected (Marklund 1980).

RNA preparation, cDNA synthesis and quantification

Total RNA from tissues (muscle and liver) of randomly sampled 8- (n = 6) and 21-month-old zebrafish (n = 4) was extracted using TRI Reagent (Sigma) according to the manufacturer's protocol. Concentration and purity was determined by electrophoresis and spectrophotometry (Nanodrop ND-1000, Thermo Scientific, Wilmington, USA), followed by purification of the total RNA (RNeasy, Qiagen). Two micrograms of total RNA were reverse transcribed into cDNA using Multiscribe Reverse transcriptase first strand cDNA kit (Applied Biosystems, Foster City, California, USA), following the manufacturer's instructions, using a mixture of random hexamers and anchored oligo-dT. Negative controls (containing no enzyme) were performed to check for genomic DNA contamination.

Information about target genes was retrieved from the mitochondrion genome sequence (Genbank accession number AC024175) and used to design primers for Real-Time PCR (RT-PCR) with Primer3web version 4.0.0 (Untergrasser *et al*., 2012). Primers were designed to target five mitochondrial genes: COX3, ND3, ND4, ND4L and ND5 (Supplementary Table 1). Their encoding sequence is located between the genes encoding ATP8 and Cytb in the mitochondrial genome, an area shown to be most damaged in human mtDNA with ageing (Schon et al. 2002). Two housekeeping genes were evaluated as internal reference, β-actin (BACT) and β-2-microglobulin (B2M), which were chosen as the most stable according to geNorm (Vandesompele et al. 2002).

qPCR was performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicates in 20 µl reaction volumes containing 10 µl of SYBR Green RT-PCR Master Mix (Applied Biosystems, Paisley, UK); 1µl of the primer corresponding to the analyzed gene (10 pmol); 3 µl of molecular biology grade water and 5 µl of cDNA, with the exception of the reference genes, which were determined using 2

µl of cDNA. Amplifications were carried out with a systematic negative control (NTC, no-template control, containing no cDNA). Standard amplification parameters contained an initial activation step at 95 ºC for 15 min, followed by 35 cycles: 15 s at 95 ºC, 30 s at the annealing Tm and 30 s at 72 ºC.

Indices and statistical analysis

The long-chain polyunsaturated fatty acid (LC-PUFA) index corresponds to the sum of fatty acids with 20 or more carbons and 2 or more double bounds. 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}): $[(\ln W_t - \ln W_i)/T)] \times 100$ where W_t= mean final weight, W_i= mean initial weight and T= total experimental days (Jaya-Ram et al. 2008). Results from mitochondrial lipid analyses, lipid peroxidation (TBARS) and superoxide dismutase (SOD) are presented as mean ± SEM (n = 4). Data were checked for homogeneity of variances by the Levene's test and, where necessary, arc-sin transformed before further statistical analysis. Student t-test analysis was used to assess the differences between groups based on diet and age for each PL class, fatty acid, TBARS content and SOD activity. Interaction between diet and age was analysed by a general linear model. All statistical analyses were performed using SPSS Statistical Software System version 15.0 (SPSS Inc., Chicago, USA). Data from gene expression analysis were presented as mean ± SEM (n = 6 and 4 for 8- and 21-month-old fish, respectively). Gene expression results were analyzed using the relative expression software tool (REST 2009), which employs a pairwise fixed reallocation randomization test (10,000 randomizations) with efficiency correction (Pfaffl et al. 2002) to determine the statistical significance of expression ratios (gene expression fold changes) between two treatments. Differences were regarded as significant when $p<0.05$.

Results

Dietary fatty acid composition and fish performance

307 The RO diet contained around 8 % saturated fatty acids (SFA), 62 % monounsaturated
308 fatty acids (MUFA), 56 % of which was oleic acid (18:1n-9, OA) and almost 30 %
309 polyunsaturated fatty acids (PUFA), with 19.5 % linoleic acid (18:2n-6, LA) and almost no
310 docosahexaenoic acid (22:6n-3, DHA) (Table 2). The DHA diet contained a lower proportion
311 of monounsaturated fatty acids (46 %) and higher content in polyunsaturated fatty acids, with
312 19 % as DHA. This was reflected in the diets' peroxidation index (PIn) (60 vs. 218) (Table 2).

313 No significant mortalities were found for any of the experimental groups and no
314 differences in food intake between groups during the experiment were observed. The 8-
315 month-old zebrafish increased in weight during the feeding trial from 0.21 ± 0.04 to 0.40 ±
316 0.07 g for the RO group and to 0.30 ± 0.11 g for the DHA group, while no significant growth
317 was observed in older fish (from 0.61 ± 0.12 to 0.69 ± 0.21 g for RO and to 0.54 ± 0.13 g for
318 the DHA group). Younger fish fed the RO diet showed a higher specific growth rate (SGR)
319 than those fed the DHA diet (t= -3.187; p = 0.001) but there was not difference in SGR
320 between diets in older animals (t= -1.550; p= 0.152) (Figure 1). No significant interaction
321 between fish age and diet was observed for SGR (F= 0.630; p= 0.432).

322

323 *Effects of age and diet on mitochondrial phospholipid content of whole zebrafish*

324 The phospholipid contents and class compositions of mitochondria from 8- and 21-
325 month-old zebrafish fed the experimental diets are shown in Figure 2. Phosphatidylcholine
326 (PC), phosphatidylethanolamine (PE) and cardiolipin (CL) were the most abundant PL
327 species in all the experimental groups, constituting more than 86 % of total PL in 8-month-old
328 zebrafish and more than 82 % in 21-month-old fish.

329 Dietary fatty acid composition affected phospholipid content and composition in
330 whole zebrafish. Total phospholipid content was higher in fish fed the DHA diet than in fish
331 fed the RO diet in both 8- (72.2 vs. 60.6 %) (t= 3.076; p= 0.053) and 21-month-old animals
332 (61.5 vs. 48.4 %) (t= 2.680; p= 0.037) although it was only significant for the older fish.
333 Mitochondria from 8-month-old fish fed the RO diet had less proportions of sphingomyelin
334 (SM) (t= 2.530; p= 0.045) and phosphatidylserine (PS) (t= 3.770; p= 0.009) than those fed the
335 DHA diet. In older fish, mitochondria from fish fed the DHA diet had a lower proportion of
336 CL compared to that of fish fed the RO diet (10.5 vs. 12.8 %) (t= -4.288; p= 0.005).

Mitochondria from 8-month-old zebrafish fed the RO diet showed some significant differences in phospholipid composition compared to that of 21-month-old fish, with higher total phospholipid content (t= 5.229; p= 0.002) and lower proportions of SM (t= -4.079; p= 0.007) and PI (t= -2.657; p= 0.0038). No significant differences were found between age groups for animals fed the DHA diet. Fish age and dietary fatty acid composition did not show a significant interaction (Age*Diet) on mitochondrial phospholipid content and composition (F values for ∑PL, SM, PC, PS, PI, CL and PE were 0.037, 0.535, 0.027, 0.045, 0.701, 0.543 and 0.052, respectively; p values are shown in Figure 2).

Effects of age and diet on phospholipid fatty acid compositions of mitochondria of whole zebrafish

There were some significant differences in the fatty acid profiles of mitochondrial PC, PE and CL from whole zebrafish when the two ages were compared (Tables 3-5). These differences were particularly significant in fish fed the DHA diet. Mitochondrial PC from older fish fed the DHA diet had a lower saturated fatty acid (SFA) content (t= 6.055; p= 0.002) and higher monounsaturated fatty acids (MUFA) (t= -2.934; p= 0.032) (Table 3), PE had higher MUFA (t= -12.130; p= 0.000) and lower DHA (t= 6.909; p= 0.001) and peroxidation index (PIn) (t= 6.501; p= 0.001) (Table 4), and CL had higher SFA (t= -3.259; p= 0.047) and lower n-6 polyunsaturated fatty acids (PUFA) (t= 4.343; p= 0.035) (Table 5). Regarding the minor phospholipid classes, PS showed no differences in the main fatty acid groups between age groups (S. Table 2) while PI and SM showed many significant differences, particularly in fish fed the DHA diet, with higher DHA (t= -4.883 and -4.299; p= 0.008 and 0.013, respectively) and PIn (t= -8.773 and -4.486;p= 0.001 and 0.011, respectively) in mitochondria of older fish when compared with younger animals (S. Tables 3 and 4). Almost no significant differences were found between age groups in fish fed the RO diet.

Diet composition significantly affected the fatty acid profiles of individual phospholipid species from 8-month-old fish mitochondria and these effects were different for each phospholipid class. The fatty acid composition of PS and SM from mitochondria of 8-month-old zebrafish were largely unaffected by diet (S. Tables 2 and 4). The three main phospholipid classes in zebrafish mitochondria responded to dietary fatty acid composition in a similar way. Mitochondria from fish fed the DHA diet had lower levels of n-6 PUFA in PC,

PE and CL (t= -6.337, -6.065 and -7.485; *p*= 0.001, 0.001 and 0.002, respectively), higher DHA (t= 2.494, 8.217 and 2.619; *p*= 0.055, 0.000 and 0.059, respectively) and total n-3 PUFA content (t= 3.069, 7.895 and 4.235; *p*= 0.028, 0.000 and 0.013, respectively) and higher PIn value (only significant for PE) (t= 1.839, 5.890 and 1.314; *p*= 0.125, 0.001 and 0.010, respectively) (Tables 3-5). Mitochondrial PI from 8-month-old zebrafish responded differently to dietary fatty acids. Mitochondrial PI from fish fed the DHA diet had lower DHA (t= -4.235; *p*= 0.013), n-3 PUFA (t= -3.379; *p*= 0.028) and PIn (t= -4.578; *p*= 0.010) than PI from fish fed the RO diet (S. Table 3). Mitochondrial CL and PI from fish fed the RO diet had around 16 % more n-6 PUFA than these phospholipid classes in fish fed the DHA diet (Table 5 and S. Table 3) while the RO diet itself had just 4 % more n-6 PUFA than the DHA diet. The DHA diet contained 18 % more DHA than the RO diet but this different DHA content between the two diets was not reflected in the fatty acid composition of any single phospholipid class when the two feeding groups were compared.

When the interaction between fish age and diet (Age*Diet) on fatty acid composition was analyzed, some significant differences were found in specific phospholipid classes, particularly in PE, PI and SM, as indicated by the *P* values (Tables 4, S. Tables 3 and 4). Almost no significant differences were found for Age*Diet interaction in PC, CL and PS (Tables 3, 5, S. Table 2). PE and PI peroxidation index from 8-month-old fish showed higher susceptibility to diet fatty acid composition than those from 21-month-old fish (F= 24.359 and 6.282; *p*= 0.001 and 0.031, respectively). In contrast, CL and SM peroxidation index from older fish showed significant differences between diet groups while no differences were found in younger animals (F= 3.283 and 11.587; *p*= 0.108 and 0.009, respectively).

Lipid peroxidation

Lipid peroxidation in total lipid of mitochondria from whole zebrafish was estimated by measuring the TBARS contents (Figure 3). In 8-month-old-fish fed the DHA diet, the levels of TBARS were significantly higher than in fish fed the RO diet (t= 4.123; *p*= 0.042). No significant differences between dietary groups were found for older animals (t= -0.315; *p*= 0.782). TBARS content was lower in 21-month-old fish compared to younger fish but it was only significant for fish fed diet DHA (t= 5.433;*p*=0.048 for DHA and t= 2.484;*p*= 0.221 for RO group). The effect of diet fatty acid composition on mitochondrial lipid peroxidation was influenced by age as determined by a general linear model (F= 9.700; *p*= 0.036).

401

402 *Mitochondrial superoxide dismutase*

403 Mn-SOD or SOD2 represented the main SOD activity in zebrafish mitochondria
404 accounting more than 95 % of total SOD in all the experimental groups. SOD2 activity was
405 higher in fish fed the DHA diet but these differences were not statistically significant in either
406 of the age groups (t= 1.615 and 1.697; p= 0.304 and 0.332 for 8- and 21-month-old fish,
407 respectively) (Figure 4). SOD2 activity remained stable between 8- and 21-month-old animals
408 (t= 0.343 and -0.537; *p*= 0.771 and 0.656 for RO and DHA group, respectively) and no
409 interaction between age and diet was found (F= 0.363; *p*= 0.579).

410

411 *Mitochondrial gene expression*

412 The 8-month-old zebrafish fed the two diets showed significant differences in
413 mitochondrial gene expression in muscle and liver (Figure 5). Muscle from younger fish fed
414 the DHA diet had lower expression of all the analyzed genes, COX3, ND3, ND4, ND4L and
415 ND5, than zebrafish fed the RO diet (t= 2.387, 2.644, 4.407, 2.625 and 3.317; *p*= 0.038,
416 0.025, 0.004, 0.03 and 0.008 for COX3, ND3, ND4L, ND4 and ND5, respectively). The same
417 trend was found in 8-month-old fish liver although the differences were only significant for
418 COX3 (t= 3.099; *p*= 0.013) and ND3 (t= 2.570; *p*= 0.030) (Figure 5). Diet had no significant
419 effect on mitochondrial gene expression in 21-month-old zebrafish. Muscle from 21-month-
420 old zebrafish showed relatively stable mitochondrial gene expression, particularly of ND
421 genes, with no differences between dietary groups. Data from liver of older zebrafish showed
422 a high variation and, although there was a consistent trend of lower mitochondrial gene
423 expression in the DHA group compared to the RO group, the differences were not statistically
424 significant.

425 When mitochondrial gene expression of muscle from the two age groups was
426 compared, older fish fed the DHA diet showed a higher expression of all the ND genes,
427 although it was only significant for ND3 (t= 3.180; *p*= 0.013) and ND5 (t= 4.965; *p*= 0.001).
428 Mitochondrial gene expression was found to be higher in liver of 21-month-old zebrafish than
429 in younger fish, particularly for the RO group, these differences being significant for ND3 (t=
430 -2.405; *p*= 0.043), ND4L (t= -2.159; *p*= 0.049) and ND5 (t= -2.377; *p*= 0.045). No interaction
431 between age and diet was found for any of the studied genes in either muscle or liver

(F=1.617, 1.175, 4.217, 1.123 and 0.806 for muscle COX3, ND3, ND4L, ND4 and ND5, respectively; F= 0.604, 2.165, 3.098, 0.012 and 3.636 for liver genes) (see *p* values for Age*Diet tables in Figure 5).

Discussion

Dietary fatty acid composition affected fish growth and mitochondrial membrane composition of zebrafish and significantly influenced muscle and liver mitochondrial gene expression and these effects differed with fish age. Zebrafish fed the DHA diet showed lower growth than those fed the RO diet (significant for younger fish) despite being fed the same feed ration (2 % BW per day). This could be related to increased oxidative stress mediated by diet fatty acid composition. Lipid peroxidation in total lipid from mitochondria was double in fish fed the DHA diet than in fish fed the RO diet in 8-month-old fish. This indicated higher oxidative damage to mitochondrial lipids in young zebrafish fed the DHA diet, very likely due to the higher incorporation of long-chain polyunsaturated fatty acids (LC-PUFA), particularly DHA, in phospholipids of mitochondrial membranes in fish fed diet DHA compared to fish fed the RO diet, as shown previously in liver and muscle mitochondria of Atlantic salmon, *Salmo salar* (Kjaer et al. 2008; Ostbye et al. 2011) and in liver mitochondria of rainbow trout (Almaida-Pagán et al. 2015). Several studies indicated that increase in dietary PUFA elevated the metabolic rate which would be very likely mediated by changes in membrane composition (Hulbert et al. 2005). This conclusion is heavily influenced by recent studies of the metabolic rate of different species and particularly the development of what has been termed the `membrane pacemaker´ theory of metabolism (Hulbert 2008). Polyunsaturation of cell membrane lipids 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-term, free radical reactions that will propagate oxidative damage throughout the organelle (Hulbert 2005). This theory 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 life-cycle could compromise metabolic activity, oxidative stress and tissue function.

464 The present data showed that mitochondria from 8-month-old zebrafish fed the DHA
465 diet had higher levels of n-3 LC PUFA and higher peroxidation index (PIn) value in the three
466 main phospholipid classes, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and
467 cardiolipin (CL) (changes in PIn only significant for PE). These data are consistent with those
468 showing considerable dietary modifications of fatty acid composition of mitochondrial
469 phospholipids in rats (Lemieux 2008; Abbott et al. 2010) and fish (Ushio et al. 1997; Robin et
470 al. 2003; Guderley et al. 2008; Ostbye et al. 2011; Martin et al. 2013; Almaida-Pagán et al.
471 2015). However, they also indicate the existence of regulatory mechanisms of specific fatty
472 acids within mitochondrial membranes as the observed changes did not clearly reflect the
473 dietary compositions. Different mitochondrial phospholipid classes were affected by diet in
474 different ways suggesting the importance of fatty acid composition at the level of specific
475 phospholipids, as suggested for mammals (Carrie et al. 2000; Cha and Jones 2000, Hulbert et
476 al. 2005). Besides, phospholipid fatty acid compositions did not entirely reflect the diet fatty
477 composition. Interestingly, DHA incorporation in the main mitochondrial phospholipids was
478 significantly lower than expected considering the differences in this fatty acid between the
479 experimental diets. This can be in part explained by preferential retention of DHA in fish
480 tissues when this fatty acid is very low in the diet, as found in Atlantic salmon and other fish
481 species (Bell et al. 2004; NRC 2011) and/or increased endogenous synthesis of DHA from
482 linolenic acid (18:3n-3) in zebrafish when dietary levels of n-3 LC-PUFA are reduced
483 (Tocher et al. 2002). This could also be due to the increase in lipid peroxidation observed in
484 8-month-old zebrafish fed the DHA diet, which could reduce membrane PUFA as found in
485 senescent zebrafish (Almaida-Pagán et al. 2014). The observed increase in SM content in 8-
486 month-old zebrafish mitochondria could also suggest higher lipid peroxidation (Lucas-
487 Sánchez et al. 2013; Almaida-Pagán et al. 2014) and may represent an adaptive response in
488 the organelle to mitigate propagation of oxidative reactions through the membrane. On the
489 other hand, high levels of dietary DHA up-regulate uncoupling proteins (UCP), mitochondrial
490 inner membrane transporters that uncouple oxidative phosphorylation, in mammals (Lee et al.
491 2013). In fish, major changes in UCP mRNA expression have been associated with switches
492 in energy demand and oxidative capacities (Bermejo-Nogales et al. 2010), which may explain
493 the increased oxidation observed in 8-month-old zebrafish.

494 Assay of mtDNA gene expression showed several differences in muscle and liver
495 between feeding groups for 8-month-old zebrafish. Muscle of young fish fed the DHA diet
496 had lower expression of all the analyzed genes than zebrafish fed the RO diet. The same trend

was found in liver but differences were only significant for COX3 and ND3. In agreement, sea bream (*Sparus aurata*) fed on vegetable diets, with low n-3 LC-PUFA contents, exhibited up-regulation of several mitochondrial genes in liver after an environmental stress (Pérez-Sánchez et al. 2013). This could also indicate high oxidative stress inside the mitochondria as 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 (Schon et al. 2002). Genes in this area encode predominantly for subunits of complexes I [NADH-coenzyme Q (CoQ) oxidoreductase] and IV (cytochrome c oxidase) of the electron transport chain (ETC), 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 defects in 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 the mitochondrial genome, may increase electron leak from the ETC generating more oxidative stress, mitochondrial damage and, ultimately, mitochondrial dysfunction and bioenergetic decay associated with disease and aging. However, it is acknowledged that a limited number of mitochondrial-encoded genes were evaluated in the present study and that the trend observed may vary among tissues. In this respect, it was recently shown that the regulation of nuclear- and mitochondrial-encoded genes of the respiratory chain is different depending on the tissue metabolic capabilities in teleosts (Bermejo-Nogales et al. 2015).

The effect of dietary fatty acid composition on growth, lipid peroxidation, mitochondrial lipids and gene expression differed between the two age groups. The 21-month-old fish showed no significant growth in either feeding group and TBARS content was generally lower than in younger animals, with no differences between dietary groups. In a previous study analyzing changes in mitochondrial lipids during the life-time of zebrafish, we found that zebrafish reached their highest weight around the 18th month of their life-cycle a time during which mitochondrial lipid peroxidation was also at its maximum (Almaida-Pagán et al. 2014). However, fish had a considerably lower weight and TBARS content at the end of

their life-cycle (24-month-old) indicating that this could be a natural process in zebrafish, regardless of diet. Older zebrafish mitochondrial gene expression was also less affected by the diet fatty acid composition with no differences between feeding groups. This lower responsiveness of lipid peroxidation and mitochondrial gene expression in older fish fed the DHA diet could be due to the fact that younger fish were growing during the feeding trial while older fish were not, resulting in greater incorporation of dietary fatty acids into tissues of young fish. However, this would not fully explain the differences observed in phospholipid fatty acid compositions between age groups. Mitochondrial lipids of 21-month-old zebrafish were affected differently by dietary fatty acid composition than 8-month-old fish. Interestingly, mitochondrial PC and PE from 21-month-old zebrafish showed less difference between the two feeding groups than younger fish, while CL and SM reflected more strongly the DHA diet when compared with the younger fish. Moreover, older fish fed the DHA diet had mitochondria with a significantly lower CL content than those fed the RO diet while no difference was found in younger fish. It is well-known that the aging process involves a reduction in the rate of lipid metabolism and turnover of fatty acids (Hansford and Castro 1982). Therefore, these differences could also indicate age-related changes in the animal's capacity for maintaining the structural homoeostasis of mitochondrial membranes.

In conclusion, the present results suggested there was high oxidative stress in 8-month-old zebrafish fed a diet with high DHA. Despite of the existence of mechanisms regulating mitochondrial phospholipid content and phospholipid fatty acid composition, these processes appear to be overridden by the change in dietary fatty acid composition and the main constituents of mitochondrial membranes suffered marked changes in their composition. This situation affected mitochondrial lipid peroxidation, mitochondrial membrane composition and gene expression, and influenced animal growth. Following the membrane pacemaker theory of animal metabolism (Sanz et al. 2006; Hulbert 2007), lipids would be among the first molecules affected by mitochondrial free radicals, and lipid peroxidation could be the propagator of oxidative damage reactions which would attack other organelle molecules, including mtDNA. These changes could affect the organelle function and cell viability by affecting ETC efficiency, ROS production and signalling systems. The effects of dietary fatty acid composition differed between the two age groups, which could be related to observed differences in specific growth rate. Nevertheless, many of the observed changes in phospholipid fatty acid composition with diet in one age group were in the opposite direction in the other age group, or simply remained unchanged which could indicate age-related

563 changes in the animal's capacity for maintaining the structural homoeostasis of mitochondrial
564 membranes.

565

566 **Acknowledgments**

567 This research and P.F.A.-P. were funded by a Marie Curie Intra-European Fellowship within
568 the 7th Community Framework Programme (PIEF-GA-2011-297964, OLDMITO). The
569 authors report no conflicts of interest.

570 **Compliance with ethical standards**

571 The authors confirm that there are not potential conflicts of interests. Fish were treated in
572 accordance with British national ethical requirements and the experiments conducted under
573 the UK Government Home Office project Licence number PPL 60/03969 in accordance with
574 the amended Animals Scientific Procedures Act 1986 implementing EU directive 2010/63.

575

576

577 **References**

578 Abbott SK, Else PL, Hulbert AJ (2010) Membrane fatty acid composition of rat skeletal
579 muscle is most responsive to the balance of dietary n-3 and n-6 PUFA. Br J Nutr 103: 522-
580 529.

581 Almaida-Pagán PF, de Costa J, Mendiola P, Tocher DR (2012) Age-related changes in
582 mitochondrial membrane composition of rainbow trout (*Oncorhynchus mykiss*) heart and
583 brain. Comp Biochem Physiol B 163: 129-137.

584 Almaida-Pagán PF, Lucas-Sánchez A, Tocher DR (2014) Changes in mitochondrial
585 membrane composition and oxidative status during rapid growth, maturation and aging in
586 zebrafish, *Danio rerio*. BBA Mol Cell Biol L 1841: 1003-1011.

587 Almaida-Pagán PF, De Santis C, Rubio-Mejía OL, Tocher DR (2015) Dietary fatty acids
588 affect mitochondrial phospholipid compositions and mitochondrial gene expression of
589 rainbow trout liver at different ages. J Comp Physiol B 185: 73-86.

590 Balaban RS, Nemoto S, Finkel T (2005) Mitochondria, Oxidants, and Aging. Cell 120: 483-
591 495.

592 Barja G (2004) Free radicals and aging. TRENDS Neurosci. 27: 595-600.

593 Barja G (2013) Updating the Mitochondrial Free Radical Theory of Aging: An Integrated
594 View, Key Aspects, and Confounding Concepts. Antioxid Redox Signal 19: 1420-1445.

595 Barzanti V, Battino M, Baracca A, Cavazzoni M, Cocchi M, Noble R, Maranesi M, Turchetto
596 E, Lenaz G (1994) The Effect of Dietary-Lipid Changes on the Fatty-Acid Composition and
597 Function of Liver, Heart and Brain Mitochondria in the Rat at Different Ages. Brit J Nutr: 71,
598 193-202.

599 Bell JG, Henderson RJ, Tocher DR, Sargent JR (2004) Replacement of dietary fish oil with
600 increasing levels of linseed oil: modification of flesh fatty acid compositions in Atlantic
601 salmon (*Salmo salar*) using a fish oil finishing diet. Lipids 39: 223-232.

602 Bermejo-Nogales A, Calduch-Giner JA, Pérez-Sánchez J (2010) Gene expression survey of
603 mitochondrial uncoupling proteins (UCP1/UCP3) in gilthead sea bream (*Sparus aurata* L.). J
604 Comp Physiol B 180: 685-694.

605 Bermejo-Nogales A, Calduch-Giner JA, Pérez-Sánchez J (2015) Unraveling the molecular
606 signatures of oxidative phosphorylation to cope with the nutritionally changing metabolic
607 capabilities of liver and muscle tissues in farmed fish. PLOS One 10: e0122889.

608 Burk RF, Trumble MJ, Lawrence RA (1980) Rat hepatic cytosolic GSH-dependent enzyme
609 protection against lipid peroxidation in the NADPH microsomal lipid peroxidation system.
610 BBA 618: 35-41.

611 Carrie I, Clement M, De Javel D, Frances H, Bourre JM (2000) Specific phospholipid fatty
612 acid composition of brain regions in mice: effects of n-3 polyunsaturated fatty acid deficiency
613 and phospholipid supplementation. J. Lipid Res 41: 465-472.

614 Cha MC, Jones PJH (2000) Energy restriction dilutes the changes related to dietary fat type in
615 membrane phospholipid fatty acid composition in rats. Metabolism 49: 977-983.

616 Christie WW (2003) Lipid analysis: isolation, separation, identification and structural analysis
617 of lipids. Oily Press, Somerset, UK, 2003.

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

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

622 Fang L, Miller YI (2012) Emerging applications for zebrafish as a model organism to study
623 oxidative mechanisms and their roles in inflammation and vascular accumulation of oxidized
624 lipids. Free Radical Biol Med 53: 1411-1420.

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

627 Guderley H, Kraffe E, Bureau W (2008) Dietary fatty acid composition changes
628 mitochondrial phospholipids and oxidative capacities in rainbow trout red muscle, J Comp
629 Physiol B 178: 385-399.

630 Hannum YA, Obeid LM (1994) Ceramide and the eukaryotic stress response. Biochem Soc
631 Trans 25: 1171-1175.

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

635 Henderson RJ, Tocher DR (1992) Thin-layer chromatography, in: Hamilton, R. J., Hamilton,
636 S. (Eds.) Lipid Analysis. A Practical Approach, IRL Press, Oxford, UK, pp. 65-111.

637 Hoch FL (1992) Cardiolipins and biomembrane function. BBA 1113: 71-133.

638 Hulbert AJ (2005) On the importance of fatty acid composition of membranes for aging. J
639 Theor Biol 234: 277-288.

640 Hulbert AJ (2007) Membrane fatty acids as pacemaker of animal metabolism. Lipids 42: 811-
641 819.

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

644 Hulbert AJ, Turner N, Storlien LH, Else PL (2005) Dietary fats and membrane function:
645 implications for metabolism and disease. Biol Rev 80: 155-169.

646 Hulbert AJ, Pamplona R, Buffenstein R, Buttemer WA (2007) Life and Death: Metabolic
647 Rate, Membrane Composition, and Life Span of Animals. Physiol Rev 87: 1175-1213.

648 Jaya-Ram A, Kuah M, Lim P, Kolkovski S, Shu-Chien AC (2008) Influence of dietary HUFA
649 levels on reproductive performance, tissue fatty acid profile and desaturase and elongase
650 mRNAs expression in female zebrafish *Danio rerio*. Aquaculture 277: 275-281.

651 Kjaer MA, Todorcevic M, Torstensen B, Vegusdal A, Ruyter B (2008) Dietary n-3 HUFA
652 Affects Mitochondrial Fatty Acid β-Oxidation Capacity and Susceptibility to Oxidative Stress
653 in Atlantic Salmon. Lipids 43: 813-827.

654 Lee M-K, Kim I-H, Kim Y (2013) Effects of eicosapentaenoic acid and docosahexaenoic acid
655 on uncoupling protein 3 gene expression in C_2C_{12} muscle cells. Nutrients 5: 1660-1671.

656 Lemieux H, Blier PU, Tardif JC (2008) Does membrane fatty acid composition modulate
657 mitochondrial functions and their thermal sensitivity? Comp Biochem Physiol Part A 149: 20-
658 29.

659 Ljubibic V, Joseph A-M, Saleem A, Uguccioni G, Collu-Marchese M, Lai RYJ, Nguyen LM,
660 Hood DA (2010) Transcriptional and post-transcriptional regulation of mitocondrial
661 biogenesis in skeletal muscle: Effects of exercise and aging. BBA Gen Subjects 1800: 223-
662 234.

663 Lucas-Sánchez A, Almaida-Pagán PF, Tocher DR, Mendiola P, de Costa J (2013) Age-related
664 changes in mitochondrial membrane composition of *Nothobranchius rachovii*. J Gerontol
665 Biol Sci 69: 142-151.

666 Manoli I, Alesci S, Blackman MR, Sun YA, Rennert OM, Chrousos GP (2007) Mitochondria
667 as key components of the stress response. Trends Endocrin Met 18: 190-198.

668 Marklund S (1980) Distribution of CuZn superoxide dismutase and Mn superoxide dismutase
669 in human tissues and extracellular fluids. Acta Physiol Scand Suppl 492: 19-23.

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

National Research Council (NRC) (2011) Nutrient requirements of fish and shrimp. The National Academies Press, Washington DC.

Naudi A, Jové M, Ayala V, Portero-Ortín M, Barja G, Pamplona R (2013) Membrane lipid unsaturation as physiological adaptation to animal longevity. Front Physiol 4: 1-13.

Olsen RE, Henderson RJ (1989) The rapid analysis of neutral and polar marine lipids using double-development HPTLC and scanning densitometry. J Exp Mar Biol Ecol 129: 189-197.

Ostbye TK, Kjaer MA, Rorá AMB, Torstensen B, Ruyter B (2011) High n-3 HUFA levels in the diet of Atlantic salmon affect muscle and mitochondrial membrane lipids and their susceptibility to oxidative stress. Aquacult Nutr 17: 177-190.

Pamplona R (2011) Advanced lipoxidation end-products. Chem Biol Interact 192: 14-20.

Pamplona R., Barja G, Portero-Otin M (2002) Membrane fatty acid unsaturation, protection against oxidative stress, and maximum life span — a homeoviscous-longevity adaptation? Ann NY Acad Sci 959: 475-490.

Panchenko LF, Brusov OS, Gerasimov M, Loktaeva TD (1975) Intramitochondrial localization and release of rat liver superoxide dismutase. Febs Letters 55: 84-87.

Paradies G, Petrosillo G, Pistolese M, Ruggiero FM (2002) Reactive oxygen species affect mitochondrial electron transport complex I activity through oxidative cardiolipin damage. Gene 286: 135-141.

Paradies G, Petrosillo G, Paradies V, Ruggiero FM (2011) Mitochondrial dysfunction in brain aging: Role of oxidative stress and cardiolipin. Neurochem. Int. 58: 447-457.

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST©) for group wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30: e36.

696 Richter C (1995) Oxidative Damage to Mitochondrial DNA and its relationship to Ageing. Int
697 J Biochem Cell Biol 27: 647-653.

698 Robin JH, Regost C, Kaushik SJ (2003) Fatty acid profile of fish following a change in
699 dietary fatty acid source: model of fatty acid composition with a dilution hypothesis.
700 Aquaculture 225: 283-293.

701 Sanz A, Pamplona R, Barja G (2006) Is the mitochondrial free radical theory of aging intact?
702 Antioxid Redox Sign 8: 582-599.

703 Schlame M, Rua D, Greenberg ML (2000) The biosynthesis and functional role of cardiolipin.
704 Prog Lipid Res 39: 257-288.

705 Shigenaga MK, Hagen TM, Ames BN (1994) Oxidative damage and mitochondrial decay in
706 aging. Proc Nat Acad Sci US 91: 10771-10778.

707 Schon EA, Naini A, Shanske S (2002) Identification of mutations in mitochondrial DNA from
708 patients suVering mitochondrial disease. Methods Mol Biol 197: 55–74.

709 Smits P, Smeitink J, van den Heuvel L (2010) Mitochondrial translation and beyond:
710 processes implicated in combined oxidative phosphorylation deficiencies. J Biomed
711 Biotechnol 737385.

712 Sohal RS, Mockett RJ, Orr WC (2002) Mechanisms of aging: an appraisal of the oxidative
713 stress hypothesis. Free Radical Bio Med 33: 575-586.

714 Subbaiah PV, Subramanian VS, Wang K (1999) Novel physiological function of
715 sphingomyelin in plasma. J Biol Chem 274: 36409-36414.

716 Tocher DR, Agaba M, Hastings N, Bell JG, Dick JR, Teale AJ (2002) Nutritional regulation
717 of hepatocyte fatty acid desaturation and polyunsaturated fatty acid composition in zebrafish
718 (*Danio rerio*) and tilapia (*Oreochromis niloticus*). Fish Physiol Biochem 24: 309-320.

719 Tocher DR, Mourente G, Van der Eecken A, Evjemo JO, Díaz E, Wille M, Bell JG, Olsen Y
720 (2003) Comparative study of antioxidant defence mechanisms in marine fish fed variable
721 levels of oxidised oil and vitamin E. Aquacult Int 11: 195-216.

722 Trifunovic A, Larsson NG (2008) Mitochondrial dysfunction as a cause of ageing. J Int Med
723 263: 167-178.

724 Ulmann L, Mimouni V, Roux S, Porsolt R, Poisson JP (2001) Brain and hippocampus fatty
725 acid composition in phospholipid classes of aged-relative cognitive deficit rats. Prostag.
726 Leukotr. EES 64: 189-195.

727 Untergrasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG (2012)
728 Primer3 p new capabilities and interfaces. Nucleic Acids Res 40: e115.

729 Ushio H, Muramatsu M, Ohshima T, Koizumi C (1997) Fatty acid compositions of biological
730 membranes from fast skeletal muscle of carp. Fisheries Sci 62: 427-434.

731 Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F
732 (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging
733 of multiple internal control genes. Genome Biol doi: 10.1186/gb-2002-3-7-research0034.

734 Voos W (2013) Chaperone-protease networks in mitochondrial protein homeostasis. BBA
735 Mol Cel Res 1883: 388-399.

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

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

740 Zimmiak P (2011) Relationship of electrophilic stress to aging, Free Radic Biol Med 51:
741 1087-1105.

742

743 **Figure legends**

744 **Figure 1. Specific growth rate (SGR % day^{-1}) of 8- and 21-month-old zebrafish fed the**
745 **two experimental diets for two months**. Results are means ± SEM (n = 56 for 8- and n = 16
746 for 21-month-old fish). `+´ symbols represent differences between feeding groups and
747 asterisks denote significant differences between age groups as determined by Student t-test (p
748 < 0.05).

Figure 2. Phospholipid content (percentage of total lipid weight) and phospholipid class composition (percentage of total phospholipids) of mitochondria isolated from 8- and 21-month-old zebrafish fed two different diets. Results are means ± SEM. (n = 4). `+´ symbols represent differences between feeding groups for each phospholipid class as determined by a Student t-test ($p < 0.05$). Asterisks indicate significant differences between age groups for each phospholipid class as determined by a Student t-test ($p<0.05$). Table represents *P* values for interaction Diet and Age for each phospholipid class as calculated by a general linear model ($p<0.05$). CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipid; ∑PL, total phospholipids; PS, phosphatidylserine; SM, sphingomyelin.

Figure 3. TBARS contents (ng g lipid^{-1}) of 8- and 21-month-old zebrafish fed the two experimental diets. Data expressed as mean ± SEM (n = 4). `+´ symbols represent differences between feeding groups and asterisks denote significant differences between age groups as determined by a Student t-test ($p<0.05$).

Figure 4. Mitochondrial superoxide dismutase (Mn-SOD) activity (units min^{-1} mg prot^{-1}) of 8- and 21-month-old zebrafish fed the two experimental diets. Data are expressed as mean ± SEM (n = 4).

Figure 5. Relative expression of COX3, ND3, ND4L, ND4 and ND5 genes of 8- and 21-month-old zebrafish fed the two experimental diets. Data are expressed as mean ± SEM (n = 6 for younger and n = 4 for older fish). `+´ symbols represent differences between feeding groups and asterisks denote significant differences between age groups as determined by a Student t-test ($p< 0.05$). The insert Table presents significant values for the interaction between Age and Diet for each gene as calculated by a general linear model ($p < 0.05$).

Table 1. Components (g/kg of dry diet) of experimental diets

	RO	DHA
Ingredients of capsules' content (g per 100 g dry weight)		
Vit-free casein[1]	480	480
Potato Starch[2]	150	150
Fishmeal[3]	50	50
Mineral mix[4]	47	47
Vitamin mix[5]	10	10
Arginine	4	4
Leucine	4	4
Methionine	3	3
Cystine	2	2
Orange G	1	1
a-cellulose	139.6	139.6
INCROMEGA DHA 500 TG[6]	0	44
Rapeseed oil[7]	110	66
Antioxidant mix[8]	0.4	0.4
Proximate analysis of the capsules (% dry matter)		
Dry matter	88.8	87.8
Crude protein	49.4	49.7
Crude lipid	11.0	10.9
NFE	33.4	33.1
Ash	6.2	6.3

[1]Vitamin-free micropulverised (ICN Biomedical Ltd., High Wycombe, UK).

[2]Passeli WA4 (Avebe Ltd., Ulceby, South Humberside, UK).

[3]Norse-LT94 (Norsidmel AS, Fyllingsdalen, Norway).

[4]Supplied (per kg diet): KH_2PO_4, 22 g; $FeSO_4 \cdot 7H_2O$, 1.0 g; $ZnSO_4 \cdot 7H_2O$, 0.13 g; $MnSO_4 \cdot 4H_2O$, 52.8mg; $Cu\text{-}SO_4 \cdot 5H_2O$, 12 mg; $CoSO_4 \cdot 7H_2O$, 2 mg.

[5]Supplied (mg kg $diet^{-1}$): ascorbic acid, 1000; myo-inositol, 400; nicotinic acid, 150; calcium pantothenate, 44; allrac-α-tocopheryl acetate, 40; riboflavin, 20; pyridoxine hydrochloride, 12; menadione, 10; thiamine hydrochloride, 10; retinyl acetate, 7;3; folic acid, 5; biotin, 1; cholecalciferol, 0.06; cyanocobalamin, 0.02.

[6] CRODA International Plc (East Yorkshire, UK).

[7] The Cooperative Food (UK).

[8]Dissolved in propylene glycol and contained (g l^{-1}): butylated hydroxy anisole, 60; propyl gallate, 60; citric acid, 40.

All the other ingredients were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, UK).

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

	RO	DHA
16:0	5.1	4.2
18:0	1.7	2.2
∑Saturated	8.2	7.9
18:1n-9	55.9	39.7
18:1n-7	3.3	2.8
20:1n-9	1.5	1.9
∑Monounsaturated	62.1	46
18:2n-6	19.5	13.1
∑n-6 PUFA	19.5	15.4
18:3n-3	9.3	6.3
20:5n-3	0.1	3.7
22:5n-3	0	1.1
22:6n-3	0.7	19
∑n-3 PUFA	10.1	30.6
∑PUFA	29.7	46
PIn	45.9	207.7

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

PIn. Peroxidation index; PUFA, polyunsaturated fatty acids.

792

793

794 Table 3. Forward and reverse primers used in gene expression studies.

Gene	Sequence (5'→3')	Amplicon size (bp)	Efficiency	T°
COX3	F: AAAGGGTTGCGGTACGGTAT	241	0.92	59
	R: TTCGTTCTCCTTCCATGAGG			
ND3	F: CCCGCCTACCATTTTCATT	185	0.90	59
	R: TTGGGCTCATTCGTAGGCTAGT			
ND4	F: GATTCAAACCCCCTGAGGAT	201	0.91	59
	R: AGTGCTAGGTTGGCCAGATT			
ND4L	F: TTCACCGTGTTCACCTCCTA	159	0.90	59
	R: CACTTGCTTCACAGGCAGAA			
ND5	F: CACATCTGCACTCACGCTTT	177	0.93	59
	R: AAGGAAGGGGGTACCCATAA			
BACT	F: CTCTTCCAGCCTTCCTTCCT	246	0.99	60
	R: CACCGATCCAGACGGAGTAT			
B2M	F: CCACTCCGAAAGTTCATGTGT	221	0.97	60
	R: ATCTCCTTTCTCTGGGGTGAA			

795

796

797

798 Table 4. Fatty acid composition (percentage of total fatty acids) of phosphatidylcholine of mitochondria

799 isolated from whole zebrafish fed with one of two experimental diets.

	8-month-old		21-month-old		Age*Diet
	RO	DHA	RO	DHA	*P*
16:0	25.6±1.6	27.4±0.5*	25.5±1.7+	23.2±0.8	0.012
18:0	6.0±0.3+	5.5±0.1	5.9±0.8	5.4±0.5	0.887
∑Saturated	32.7±1.5	34.1±0.6*	32.5±0.9+	30.5±0.8	0.012
18:1n-9	27.9±1.3	27.0±1.4	29.3±1.7	29.7±1.5	0.439
18:1n-7	2.1±0.1*	2.0±0.2	2.8±0.3+	2.3±0.1	0.059
20:1n-9	1.0±0.3	0.9±0.2	1.0±0.2	1.4±0.3	0.17
24:1n-9	1.2±0.6	0.9±0.1*	0.9±0.3+	2.2±0.4	0.002
∑Monounsaturated	34.0±1.3	33.3±2.2*	36.2±2.3	38.3±2.3	0.216
18:2n-6	4.0±0.3+	2.7±0.1*	4.5±0.2+	3.1±0.1	0.809
20:2n-6	0.0±0.0	0.2±0.1*	0.5±0.1+	1.3±0.3	0.039
20:3n-6	1.4±0.1+	0.3±0.1	1.4±0.3+	0.2±0.0	0.618
20:4n-6	3.5±0.4+	1.9±0.2	3.4±1.1+	1.7±0.1	0.849
22:5n-6	0.7±0.1	0.8±0.1*	0.7±0.1+	1.2±0.2	0.006
∑n-6 PUFA	11.3±1.4+	6.0±0.3*	11.0±1.6+	7.7±0.2	0.111
20:5n-3	2.0±1.0	2.7±0.3	2.0±0.3+	3.3±0.8	0.465
22:6n-3	18.2±1.2*	21.7±2.6	15.7±1.5	17.8±1.7	0.429
∑n-3 PUFA	21.3±1.4+	25.6±2.4	19.2±1.6	22.2±2.4	0.553
∑PUFA	33.3±2.1	32.6±2.6	31.3±1.7	31.2±2.4	0.817
∑n-3 LC-PUFA	21.0±1.4+	25.4±2.3	18.8±0.3+	21.7±2.3	0.49
n3/n6	1.9±0.2+	4.3±0.2*	1.8±0.3+	2.9±0.3	0.001
PIn	193.4±10.6	214.8±20.4	175.7±12.4	190.5±17.4	0.684

800 Data expressed as mean ± SD (n=4). `+´ symbols within a row and for each age group represent significant
801 differences between diet treatments as determined by a t-student (p<0.05).
802 Asterisks denote statistical differences between 8- and 21-month-old zebrafish for each diet group
803 (RO and DHA) when compared using a t-test (p<0.05). Right column represent significance values
804 for the interaction between Diet and Age as indicated by a general linear model (p<0.05).
805 RO, rapeseed oil group; DHA, DHA500 TG oil group; LC-PUFA, long-chain polyunsaturated fatty acids;
806 PIn, peroxidation index; PUFA, polyunsaturated fatty acids.
807 Fatty acids representing less than 1% of total fatty acids are not shown.
808

809 Table 5. Fatty acid composition (percentage of total fatty acids) of phosphatidylethanolamine of

810 mitochondria isolated from whole zebrafish fed with one of two experimental diets.

	8-month-old		21-month-old		Age*Diet
	RO	DHA	RO	DHA	*P*
16:0 DMA	1.5±0.3*	1.5±0.2	3.1±0.2+	1.2±0.5	<0.001
16:0	7.7±0.7	7.8±1.0	7.6±1.2	7.1±1.6	0.676
18:0 DMA	4.1±1.8	4.5±1.0	6.6±0.5+	3.2±0.2	0.012
18:0	16.3±1.0	16.1±0.6	16.0±0.7	15.7±1.0	0.863
∑Saturated	30.4±0.9*	31.9±0.9	34.0±1.1	31.0±2.5	0.014
16:1n-9	1.1±0.0*	0.5±0.6*	0.0±0.0+	3.0±0.9	<0.001
18:1n-9 DMA	0.7±0.3*	0.8±0.1	2.0±0.8	0.7±0.3	0.014
18:1n-9	12.8±0.5+	8.6±1.3*	13.2±3.1	17.7±0.9	0.001
18:1n-7	1.7±0.1	1.5±0.1*	2.0±0.2	2.4±0.3	0.005
∑Monounsaturated	18.3±1.4+	12.6±1.2*	20.3±3.1	25.6±1.7	<0.001
18:2n-6	1.7±0.2+	1.0±0.1*	1.7±0.1	1.7±0.4	0.008
20:4n-6	7.2±0.8+	4.8±0.6*	8.3±1.8+	3.5±0.6	0.058
22:5n-6	0.9±0.2+	1.3±0.1	0.8±0.0+	1.4±0.1	0.111
∑n-6 PUFA	12.2±0.9+	7.8±1.2	13.5±2.1+	7.8±0.9	0.377
20:5n-3	0.9±0.1+	1.9±0.4	1.3±0.3	1.5±0.1	0.017
22:5n-3	0.8±0.1+	1.3±0.3	1.4±0.4	0.8±0.1	0.034
22:6n-3	33.8±1.2+	41.6±1.5*	27.7±5.2	29.1±3.3	0.072
∑n-3 PUFA	36.0±1.1+	45.0±1.9*	31.3±6.0	31.9±3.8	0.049
∑PUFA	51.2±2.0	55.4±1.7*	45.7±3.9	43.4±3.3	0.05
∑n-3 LC-PUFA	35.6±1.2+	44.8±2.0*	30.7±6.0	31.4±3.1	0.048
n3/n6	3.0±0.3+	5.8±0.7*	2.4±0.9	4.1±0.7	0.149
PIn	330.6±11.8+	386.8±15.0*	289.8±38.2	269.9±33.5	0.031

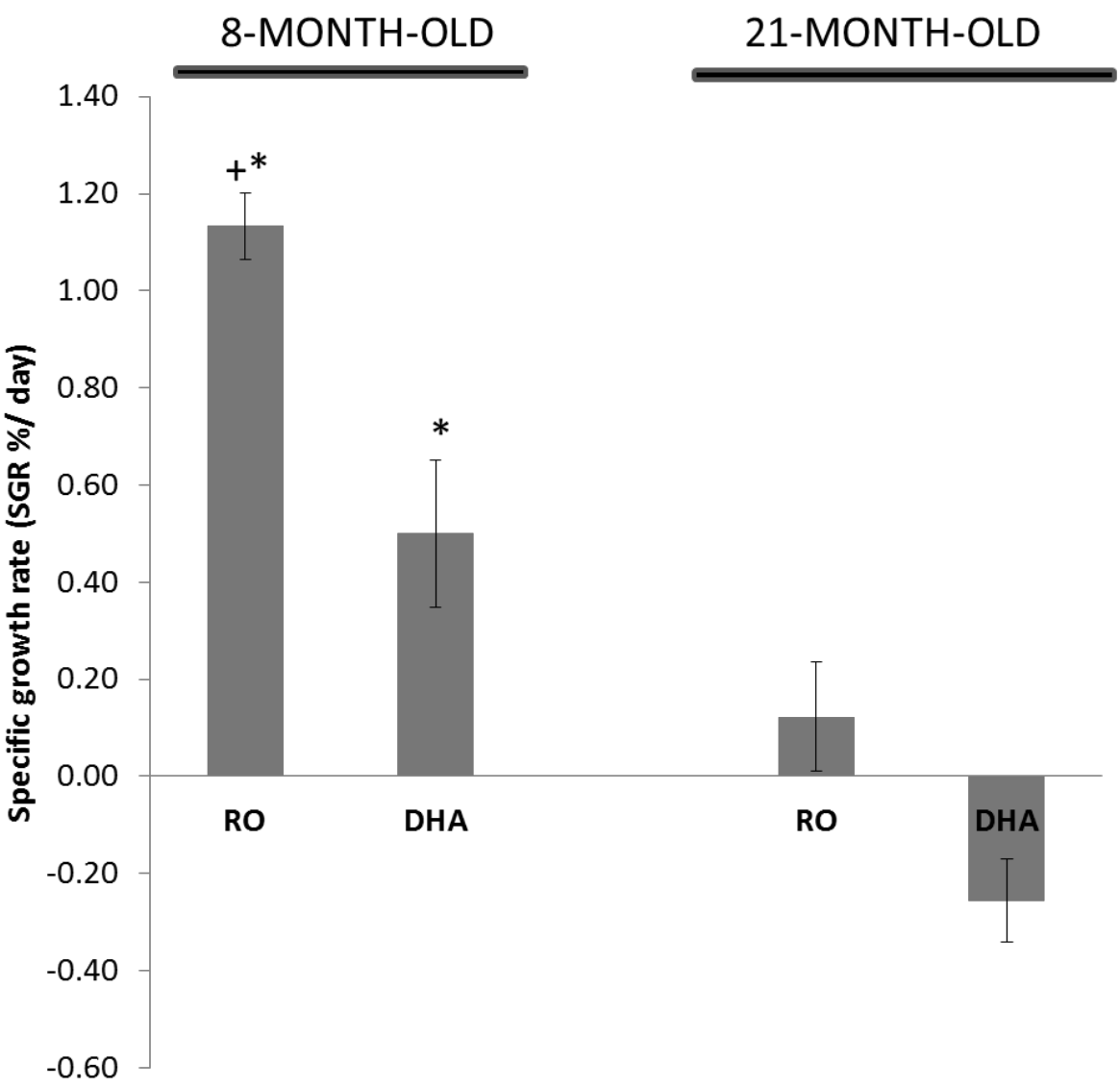
811 Data expressed as mean ± SD (n=4). `+´ symbols within a row and for each age group represent
812 significant differences between diet treatments as determined by a t-student (p<0.05).
813 Asterisks denote statistical differences between 8- and 21-month-old zebrafish for each diet
814 group (RO and DHA) when compared using a t-test (p<0.05). Right column represent significance
815 values for the interaction between Diet and Age as indicated by a general linear model (p<0.05).
816 DMA, dimethyl acetal; RO, rapeseed oil group; DHA, DHA500 TG oil group; LC-PUFA, l
817 ong-chain polyunsaturated fatty acids; PIn, peroxidation index; PUFA, polyunsaturated fatty acids.
818 Fatty acids representing less than 1% of total fatty acids are not shown.
819

Table 6. Fatty acid composition (percentage of total fatty acids) of cardiolipin of mitochondria isolated from whole zebrafish fed with one of two experimental diets.

	8-month-old		21-month-old		Age*Diet
	RO	DHA	RO	DHA	*P*
16:0	5.7±0.5	7.8±1.7	7.4±2.3	8.3±0.5	0.542
18:0	2.3±0.1+*	4.6±1.3*	5.1±1.3+	10.4±0.5	0.043
∑Saturated	9.0±0.1+*	14.0±2.9*	14.0±3.4+	21.6±1.6	0.421
16:1n-7	1.6±0.8	2.7±0.8	3.4±1.2	3.6±1.5	0.472
18:1n-9	16.1±0.9	17.5±0.6	19.6±4.5	14.9±1.9	0.12
18:1n-7	9.7±0.7	11.8±1.8	8.0±1.9	11.6±3.4	0.521
20:1n-9	1.8±0.4	2.7±1.0	1.3±0.5	1.7±0.1	0.647
∑Monounsaturated	30.1±2.2+	37.5±3.3	34.4±5.0	32.7±0.3	0.072
18:2n-6	26.9±2.4+	18.0±2.8*	22.8±4.7+	11.0±0.4	0.494
20:2n-6	2.1±0.3	1.9±0.2	1.8±0.7	1.9±0.7	0.618
20:3n-6	7.6±1.2+	1.6±0.4	5.9±2.9	0.6±0.3	0.77
20:4n-6	2.8±0.8	1.9±0.5	2.2±0.6	2.7±0.4	0.09
∑n-6 PUFA	40.1±2.4+	24.3±2.7*	33.8±7.0+	18.3±2.0	0.239
18:3n-3	2.9±0.5	3.1±0.6	2.6±0.5	1.6±0.2	0.153
20:3n-3	1.0±0.1	1.4±0.3	0.9±0.2	0.9±0.1	0.289
20:5n-3	0.9±0.1+*	1.7±0.3	1.3±0.2	1.7±0.1	0.209
22:6n-3	11.1±2.2+	15.6±2.0	10.2±2.8+	20.3±3.6	0.103
∑n-3 PUFA	16.7±1.8+	23.1±1.9	16.3±3.5+	25.7±3.3	0.409
∑PUFA	61.0±2.1+	48.5±4.5	51.6±8.0	45.8±1.9	0.35
∑n-3 LC-PUFA	13.8±2.3+	19.9±1.8	13.4±3.0+	23.8±3.3	0.212
n3/n6	0.4±0.1+	1.0±0.1	0.5±0.1+	1.4±0.3	0.031
PIn	170.2±17.8	189.8±18.7	156.3±26.0+	223.5±23.0	0.108

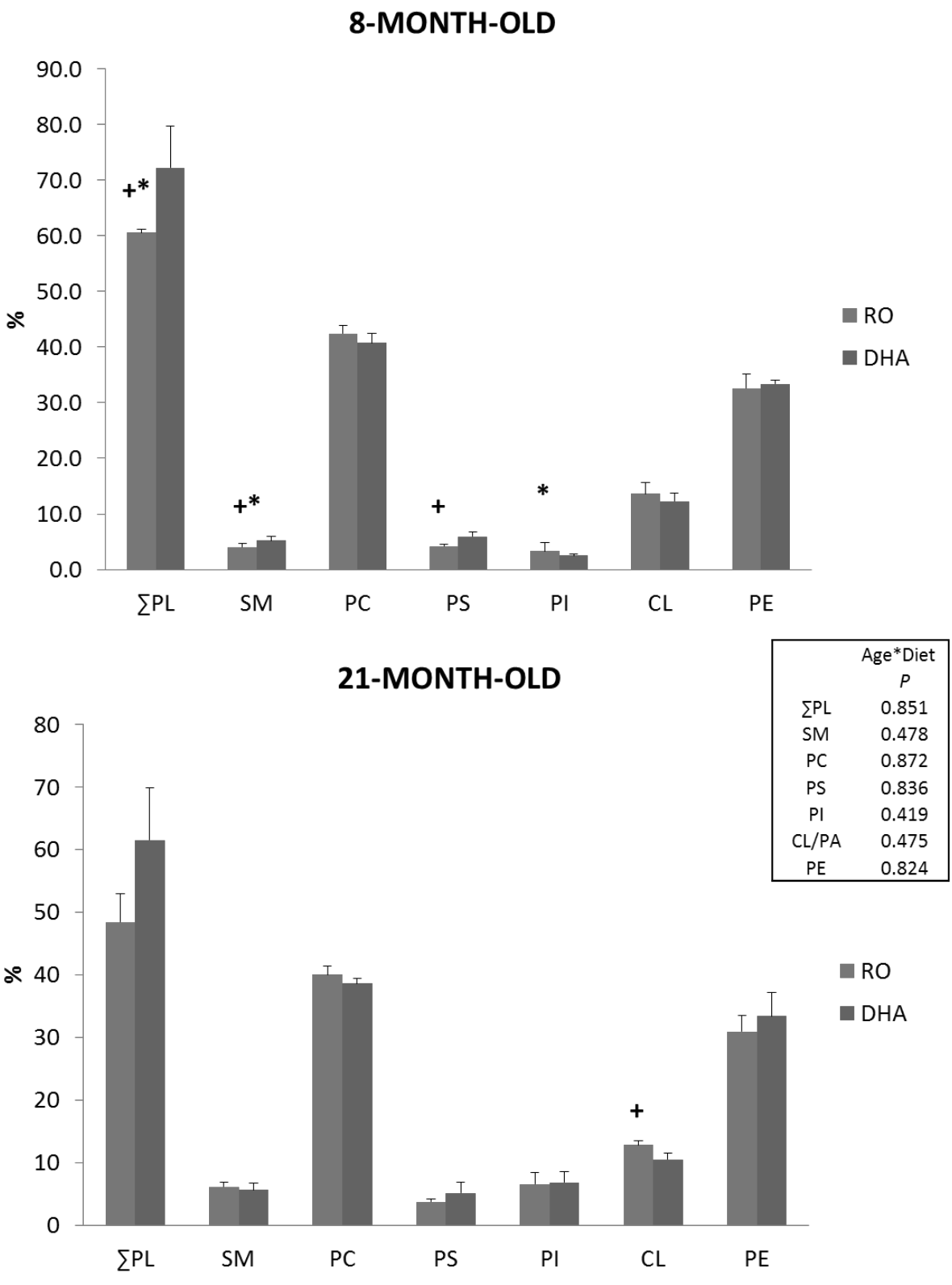
Data expressed as mean ± SD (n=4). `+´ symbols within a row and for each age group represent significant differences between diet treatments as determined by a t-student (p<0.05). Asterisks denote statistical differences between 8- and 21-month-old zebrafish for each diet group (RO and DHA) when compared using a t-test (p<0.05). Right column represent significance values for the interaction between Diet and Age as indicated by a general linear model (p<0.05). RO, rapeseed oil group; DHA, DHA500 TG 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.

830 Fig.1



831
832

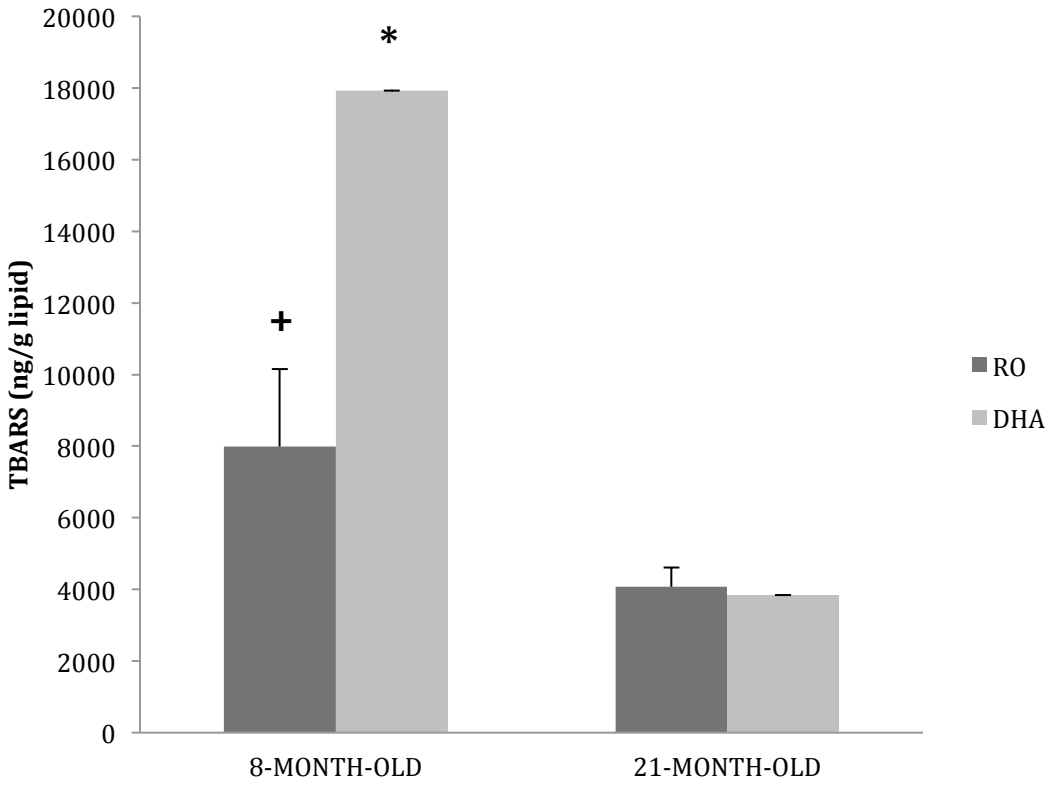
833 Fig.2



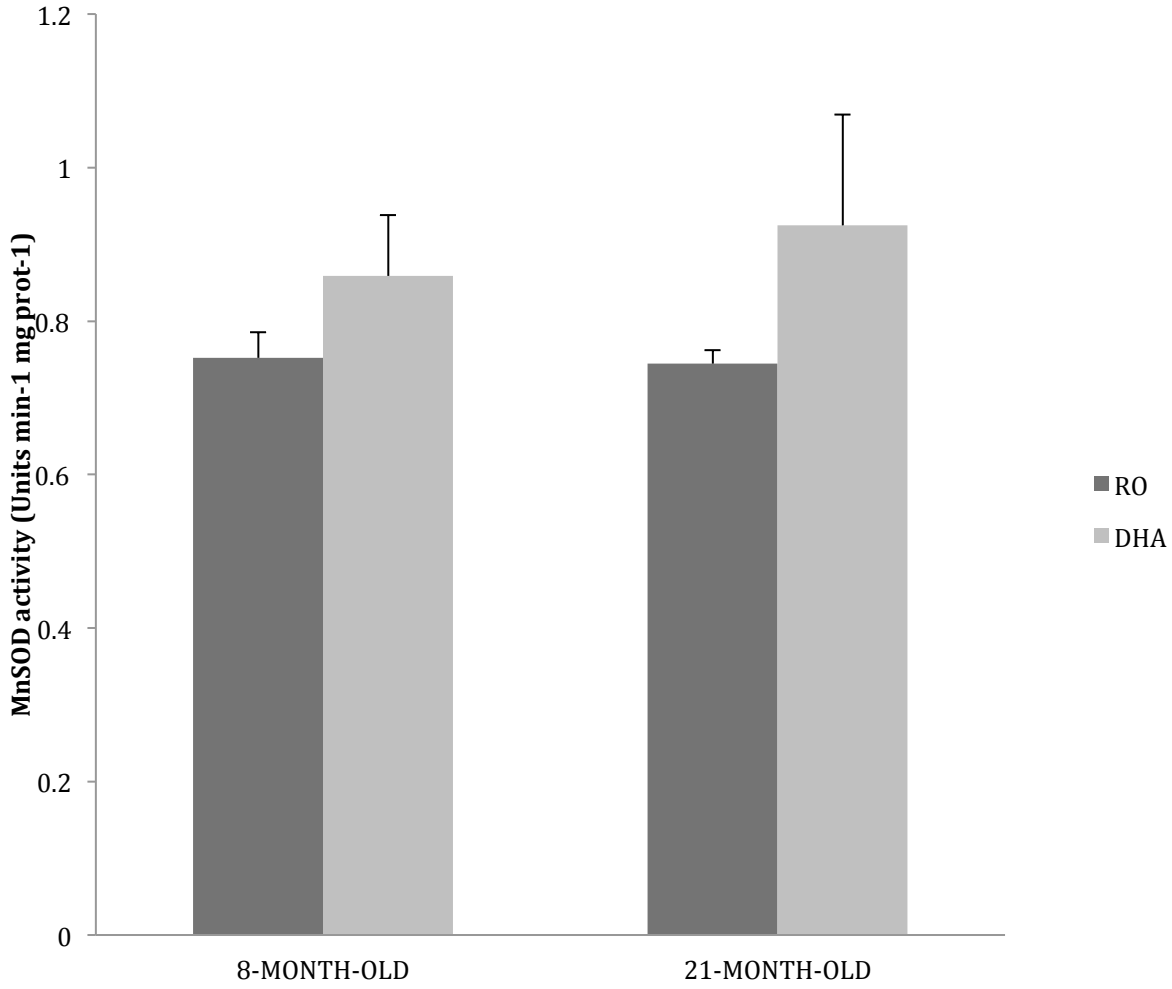
	Age*Diet
	P
∑PL	0.851
SM	0.478
PC	0.872
PS	0.836
PI	0.419
CL/PA	0.475
PE	0.824

834
835

836 Fig.3
837

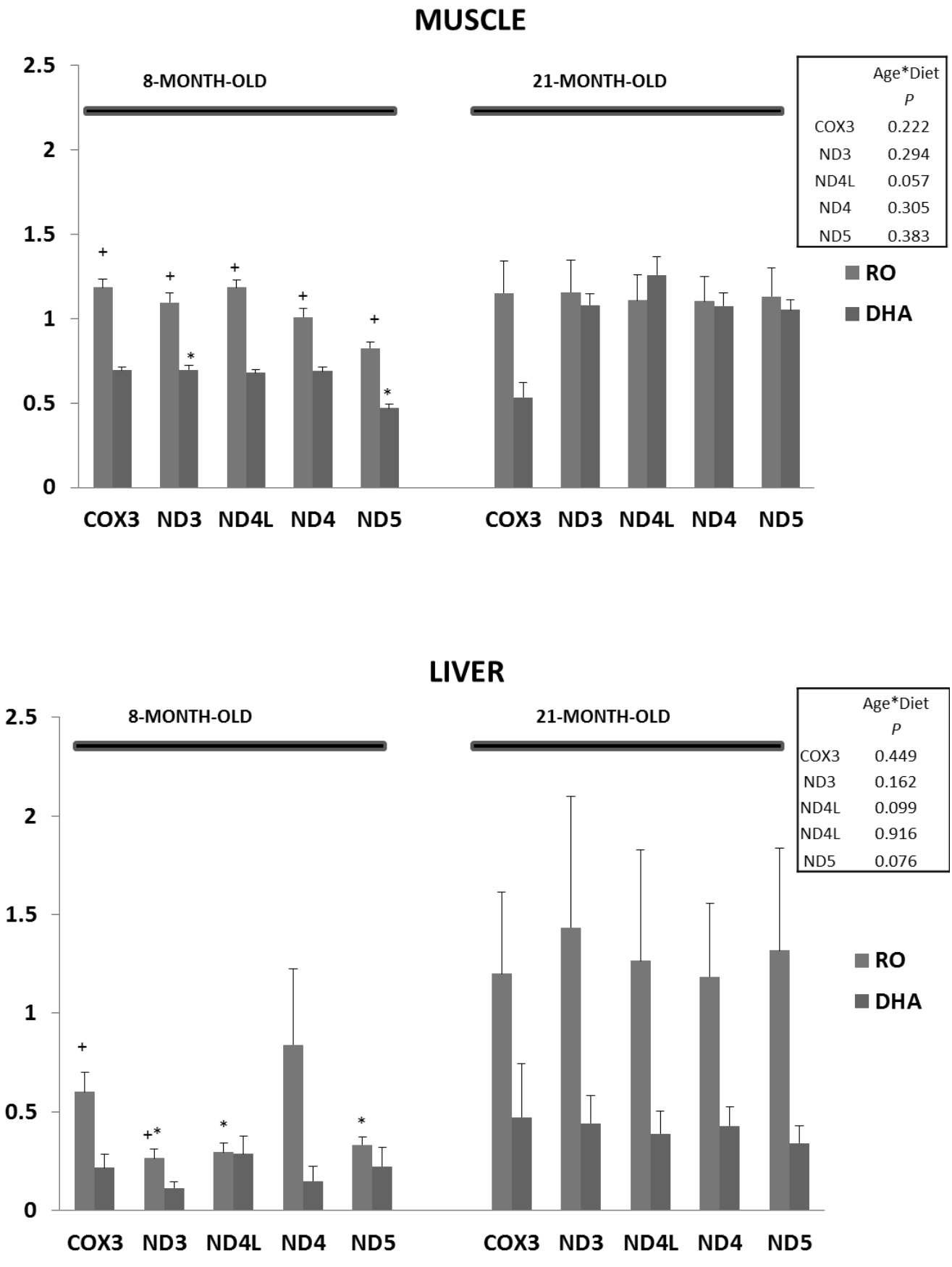

20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
TBARS (ng/g lipid)
*
+
8-MONTH-OLD
21-MONTH-OLD
RO
DHA

838 Fig.4


1.2
1
0.8
0.6
0.4
0.2
0
MnSOD activity (Units min-1 mg prot-1)
8-MONTH-OLD
21-MONTH-OLD
RO
DHA

839

840 Fig.5


MUSCLE
8-MONTH-OLD
21-MONTH-OLD
2.5
2
1.5
1
0.5
0
COX3 ND3 ND4L ND4 ND5
COX3 ND3 ND4L ND4 ND5
Age*Diet
P
COX3 0.222
ND3 0.294
ND4L 0.057
ND4 0.305
ND5 0.383
RO
DHA
LIVER
8-MONTH-OLD
21-MONTH-OLD
2.5
2
1.5
1
0.5
0
COX3 ND3 ND4L ND4 ND5
COX3 ND3 ND4L ND4 ND5
Age*Diet
P
COX3 0.449
ND3 0.162
ND4L 0.099
ND4L 0.916
ND5 0.076
RO
DHA

841

842