

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

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

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29 **Abstract**

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

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

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56 **Introduction**

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

71 The mitochondrial free radical theory of aging can still be considered the best
72 explanation for aging and longevity in mammals, birds and multicellular animals in general
73 (Barja 2013). Cell culture studies of invertebrate and mammal models support the fact that
74 reactive oxygen species (ROS), particularly those from mitochondria, play a pivotal role in
75 aging and senescence (Barja 2004; Balaban et al. 2005; Sanz et al. 2006). With age, oxidative
76 stress inside mitochondria increases and oxidation products accumulate resulting in damage to
77 the organelle lipids, protein and DNA. Eventually, this damage has been suggested to lead to
78 mitochondrial dysfunction and, thus, to cell and tissue decay with aging (Shigenaga et al.
79 1994; Sohal et al. 2002; Paradies et al. 2011). It is known that mitochondrial membranes in
80 mammals have a distinctive composition of lipid classes including PL, glycolipids and
81 cholesterol (Wiseman 1996) related to the role of mitochondria in energy metabolism and
82 oxygen consumption (Hoch 1992). The mitochondrial inner membrane (MIM) is constituted
83 of 80 % proteins (mainly electron transport chain, ETC, components) and 20 % lipids
84 containing a high percentage of the PL class cardiolipin (CL), and essentially no cholesterol
85 (Schlame et al. 2000). CL is a key molecule in mitochondrial function as it participates in the
86 regulation of electron transport and efficiency of oxidative phosphorylation, formation and
87 stability of ETC super-complexes, binding of cytochrome c to MIM, functioning of MIM
88 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 (Ljubcic 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

122 it seems clear that deterioration of mitochondrial inner membrane phospholipids, particularly
123 of CL, and accumulation of mtDNA mutations are mechanisms involved in this process. Diet
124 fatty acid composition is one of the main factors modifying plasma and mitochondrial
125 membrane lipid composition (reviewed in Hulbert et al. 2005). Changes in dietary FA
126 composition modify mitochondrial membrane composition and can alter organelle function
127 (Clandinin et al. 1985; Barzanti et al. 1994; Guderley et al. 2008; Martin et al. 2013), which
128 can lead to an imbalance in organelle oxidative status. The aim of the present study was to
129 determine the impact of dietary fatty acid composition on mitochondrial membrane
130 composition and mtDNA gene expression, as possible regulators of the processes associated
131 with mitochondrial decay under situations of high oxidative stress, including aging, in a
132 vertebrate model. Zebrafish (*Danio rerio*) of two well-differentiated ages were used in order
133 to study the interaction between diet lipid composition and fish age on redox signalling,
134 antioxidant enzyme activities and lipid metabolism.

135

136 **Materials and methods**

137 *Animals and diets*

138 The experiment was performed on zebrafish of two different ages (8 and 21 months)
139 belonging to a resident colony established in 2009 at the Institute of Aquaculture, University
140 of Stirling (for colony conditions, see Almailda-Pagán et al. 2014). Fish belonging to two
141 different age classes (8 or 21 months) each fed on two diets (rapeseed oil and DHA) were
142 used in the present study and the trial carried out in duplicate (8 tanks in total). One hundred
143 and twelve zebrafish of 0.21 ± 0.04 g (8 months) and 32 fish of 0.61 ± 0.12 g (21 months) were
144 maintained at the same animal density in 30 and 13 l aquaria, respectively, containing filtered
145 freshwater which was maintained at a constant temperature of $26 \pm 1^\circ\text{C}$ and under a 12h light:
146 12h dark photoperiod. The tanks were cleaned daily with approximately one third of the water
147 replaced each day.

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

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

158 After the feeding trial, fish were euthanized by exposure to the anesthetic benzocaine
159 hydrochloride (400 mg l^{-1}) for 10 min following the cessation of opercular movement and
160 then submerged in chilled water (5 parts ice to 1 part water). Whole zebrafish [3 fish per
161 replicate for 8- ($n = 6$) and one fish per replicate for 21-month-old fish ($n = 4$)], were taken for
162 immediate preparation of mitochondria ($n = 4$) while muscle and liver from individual fish
163 were stabilized in RNAlater[®] (Life technologies, Paisley, UK) following the manufacturer's
164 instructions and stored at -20°C for molecular analysis ($n = 6$ and 4 for 8- and 21-month-old
165 fish, respectively). The purified mitochondrial isolates were analysed to determine
166 mitochondrial membrane lipid composition, lipid peroxidation and superoxide dismutase
167 activity. Fish were treated in accordance with British national ethical requirements established
168 by the UK Government Home Office and guidelines determined by the Animals (Scientific
169 Procedures) Act 1986.

170

171 *Mitochondria isolation*

172 Approximately, 0.6 g of fresh (non-frozen) whole fish samples were homogenized in
173 8-ml ice-cold sucrose buffer (0.4 M phosphate buffer pH 7.4, 0.25 M sucrose, 0.15 M KCl, 40
174 mM KF, and 1 mM N-acetylcysteine) using a tissue disrupter (IKA T25 digital Ultra-Turrax[®]
175 Fisher Scientific, Loughborough, UK). Sucrose buffer homogenates were then twice
176 centrifuged at 600 g for 6 min. After the first centrifugation, the pellet was discarded
177 (cell/nuclei debris) and the supernatant recentrifuged at 600 g. Resulting supernatants were
178 then centrifuged twice at 6,800 g for 10 min. After the first centrifugation, the pellet was
179 resuspended in sucrose buffer and recentrifuged at 6,800 g. The final pellet was resuspended
180 in 3 ml of 100 mM potassium phosphate buffer pH 7.8, with 0.1 mM EDTA. A 250 μl aliquot
181 of each mitochondrial homogenate was frozen in liquid nitrogen and stored at -80°C prior to
182 enzyme activity measurements. The remaining mitochondrial homogenates were subjected
183 directly to lipid extraction. To verify that pellets were highly enriched in mitochondria,
184 portions of the isolates were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer

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

191

192 *Lipid extraction and phospholipid class composition*

193 Total lipid of mitochondria from whole animal was obtained by solvent extraction
194 basically according to Folch et al. (1957). Briefly, mitochondrial pellets were homogenized in
195 5 ml of ice-cold chloroform/methanol (2:1, by vol.) containing 0.01 (w/v) butylated
196 hydroxytoluene (BHT) as antioxidant, followed by addition of 1 ml of 0.88 % (w/v) KCl,
197 mixing and layers allowed to separate on ice for 1 h. The upper aqueous layer was aspirated
198 and the lower organic layer evaporated under a stream of oxygen-free nitrogen. All lipid
199 extracts were stored at -20 °C under a N₂ atmosphere prior to analysis.

200 Phospholipid classes were separated by high-performance thin-layer chromatography
201 (HPTLC) using 10 × 10 cm silica gel plates (VWR, Lutterworth, England) and methyl
202 acetate/isopropanol/chloroform/methanol/0.25 % (w/v) KCl (25:25:25:10:9, by vol.) as
203 solvent system (Olsen and Henderson 1989). The lipid classes were visualized by charring at
204 160 °C for 15 min after spraying with 3 % (w/v) aqueous cupric acetate containing 8 % (v/v)
205 phosphoric acid and quantified by densitometry using a CAMAG-3 TLC scanner (version
206 Firmware 1.14.16) (Henderson and Tocher 1992). Scanned images were recorded
207 automatically and analyzed by computer using winCATS (Planar Chromatography Manager,
208 version 1.2.0).

209

210 *Phospholipid fatty acid composition*

211 Individual phospholipid classes from mitochondria were separated by preparative-
212 TLC, using silica gel plates (20 × 20 cm) (VWR) and the solvent system as above. Individual
213 phospholipid classes were identified by comparison with known standards after spraying with
214 1 % (w/v) 2',7'-dichlorofluorescein in 97 % (v/v) methanol containing 0.05% (w/v) BHT, and

215 visualization under UV light (UVGL-58 Minerallight® Lamp, Ultraviolet Prod. Inc., Calif.,
216 USA). Each phospholipid class was scraped from the plate into a test tube and subjected
217 directly (on silica) to acid-catalyzed transmethylation at 50 °C overnight following addition of
218 2 ml of 1% (v/v) sulphuric acid in methanol in order to prepare fatty acid methyl esters
219 (FAME) (Christie 2003). FAME were separated and quantified by gas-liquid chromatography
220 (Carlo Erba Vega 8160, Milan, Italy) using a 30 m × 0.32 mm i.d. capillary column (CP Wax
221 52CB, Chrompak, London, UK) and on-column injection at 50 °C. Hydrogen was used as a
222 carrier gas and temperature programming was from 50 °C to 150 °C at 40 °C min⁻¹ and then to
223 230 °C at 2.0 °C min⁻¹. Individual methyl esters were identified by comparison with known
224 standards. Data were collected and processed using Chromcard for Windows (version 1.19).

225

226 *Measurement of thiobarbituric acid reactive substances (TBARS)*

227 Approximately 1 mg of total lipid extract from liver mitochondria was used for the
228 measurement of TBARS using an adaptation of the protocol of Burk et al. (1980). Briefly, 50
229 µl of 0.2 % (w/v) BHT in ethanol was added to the sample followed by 0.5 ml of 1% (w/v)
230 TBA and 0.5 ml 10 % (w/v) TCA, both solutions freshly prepared. The reagents were mixed
231 in a stoppered test tube and heated at 100 °C for 20 min. After cooling, particulate matter was
232 removed from the homogenate by centrifugation at 2000 g, and absorbance in the supernatant
233 determined at 532 nm against a blank sample. The concentration of TBARS, expressed as ng
234 g of lipid⁻¹, was calculated using the absorption coefficient 0.156 µM⁻¹ cm⁻¹.

235

236 *Superoxide dismutase (SOD) activity*

237 Total superoxide dismutase (SOD) activity in mitochondrial preparations was assayed by
238 measuring inhibition of oxygen-dependent oxidation of adrenaline (epinephrine) to
239 adenochrome by xanthine oxidase plus xanthine (Panchenko et al. 1975). Plastic semi-
240 microcuvettes containing 0.5 ml of 100 mM potassium phosphate buffer pH 7.8/0.1 mM
241 EDTA, 200 µl adrenaline, 200 µl xanthine, and 50 µl distilled water (uninhibited control) or
242 50 µl test sample were prepared and the reaction initiated by the addition of 10 µl xanthine
243 oxidase (Sigma X4875). The reaction was followed at 480 nm in a spectrophotometer
244 (Uvikon 860, Kontron Instruments, St. Albans, UK) and 1 unit of SOD activity was described
245 as the amount of the enzyme that inhibited the rate of adenochrome production by 50 %. The

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

252

253 *RNA preparation, cDNA synthesis and quantification*

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

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

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

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

281

282 *Indices and statistical analysis*

283 The long-chain polyunsaturated fatty acid (LC-PUFA) index corresponds to the sum
284 of fatty acids with 20 or more carbons and 2 or more double bounds. The peroxidation index
285 (PIn) was used as an estimate of PL susceptibility to oxidation and was calculated using the
286 formula: $\text{PIn} = 0.025 \times (\text{percentage of monoenoics}) + 1 \times (\text{percentage of dienoics}) + 2 \times$
287 $(\text{percentage of trienoics}) + 4 \times (\text{percentage of tetraenoics}) + 6 \times (\text{percentage of pentaenoics}) +$
288 $8 \times (\text{percentage of hexaenoics})$ (Witting and Horwitt, 1964). Specific growth rate (SGR %
289 day^{-1}): $[(\ln W_t - \ln W_i) / T] \times 100$ where W_t = mean final weight, W_i = mean initial weight and
290 T = total experimental days (Jaya-Ram et al. 2008). Results from mitochondrial lipid analyses,
291 lipid peroxidation (TBARS) and superoxide dismutase (SOD) are presented as mean \pm SEM
292 ($n = 4$). Data were checked for homogeneity of variances by the Levene's test and, where
293 necessary, arc-sin transformed before further statistical analysis. Student t-test analysis was
294 used to assess the differences between groups based on diet and age for each PL class, fatty
295 acid, TBARS content and SOD activity. Interaction between diet and age was analysed by a
296 general linear model. All statistical analyses were performed using SPSS Statistical Software
297 System version 15.0 (SPSS Inc., Chicago, USA). Data from gene expression analysis were
298 presented as mean \pm SEM ($n = 6$ and 4 for 8- and 21-month-old fish, respectively). Gene
299 expression results were analyzed using the relative expression software tool (REST 2009),
300 which employs a pairwise fixed reallocation randomization test (10,000 randomizations) with
301 efficiency correction (Pfaffl et al. 2002) to determine the statistical significance of expression
302 ratios (gene expression fold changes) between two treatments. Differences were regarded as
303 significant when $p < 0.05$.

304

305 **Results**

306 *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 (PI_n) (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 $0.40 \pm$
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 %) ($t = -4.288$; $p = 0.005$).

337 Mitochondria from 8-month-old zebrafish fed the RO diet showed some significant
338 differences in phospholipid composition compared to that of 21-month-old fish, with higher
339 total phospholipid content ($t= 5.229$; $p= 0.002$) and lower proportions of SM ($t= -4.079$; $p=$
340 0.007) and PI ($t= -2.657$; $p= 0.0038$). No significant differences were found between age
341 groups for animals fed the DHA diet. Fish age and dietary fatty acid composition did not
342 show a significant interaction (Age*Diet) on mitochondrial phospholipid content and
343 composition (F values for Σ PL, SM, PC, PS, PI, CL and PE were 0.037, 0.535, 0.027, 0.045,
344 0.701, 0.543 and 0.052, respectively; p values are shown in Figure 2).

345
346 *Effects of age and diet on phospholipid fatty acid compositions of mitochondria of whole*
347 *zebrafish*

348 There were some significant differences in the fatty acid profiles of mitochondrial PC,
349 PE and CL from whole zebrafish when the two ages were compared (Tables 3-5). These
350 differences were particularly significant in fish fed the DHA diet. Mitochondrial PC from
351 older fish fed the DHA diet had a lower saturated fatty acid (SFA) content ($t= 6.055$; $p=$
352 0.002) and higher monounsaturated fatty acids (MUFA) ($t= -2.934$; $p= 0.032$) (Table 3), PE
353 had higher MUFA ($t= -12.130$; $p= 0.000$) and lower DHA ($t= 6.909$; $p= 0.001$) and
354 peroxidation index (PIn) ($t= 6.501$; $p= 0.001$) (Table 4), and CL had higher SFA ($t= -3.259$;
355 $p= 0.047$) and lower n-6 polyunsaturated fatty acids (PUFA) ($t= 4.343$; $p= 0.035$) (Table 5).
356 Regarding the minor phospholipid classes, PS showed no differences in the main fatty acid
357 groups between age groups (S. Table 2) while PI and SM showed many significant
358 differences, particularly in fish fed the DHA diet, with higher DHA ($t= -4.883$ and -4.299 ; $p=$
359 0.008 and 0.013 , respectively) and PIn ($t= -8.773$ and -4.486 ; $p= 0.001$ and 0.011 ,
360 respectively) in mitochondria of older fish when compared with younger animals (S. Tables 3
361 and 4). Almost no significant differences were found between age groups in fish fed the RO
362 diet.

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

369 PE and CL ($t = -6.337, -6.065$ and -7.485 ; $p = 0.001, 0.001$ and 0.002 , respectively), higher
370 DHA ($t = 2.494, 8.217$ and 2.619 ; $p = 0.055, 0.000$ and 0.059 , respectively) and total n-3
371 PUFA content ($t = 3.069, 7.895$ and 4.235 ; $p = 0.028, 0.000$ and 0.013 , respectively) and
372 higher PIn value (only significant for PE) ($t = 1.839, 5.890$ and 1.314 ; $p = 0.125, 0.001$ and
373 0.010 , respectively) (Tables 3-5). Mitochondrial PI from 8-month-old zebrafish responded
374 differently to dietary fatty acids. Mitochondrial PI from fish fed the DHA diet had lower DHA
375 ($t = -4.235$; $p = 0.013$), n-3 PUFA ($t = -3.379$; $p = 0.028$) and PIn ($t = -4.578$; $p = 0.010$) than PI
376 from fish fed the RO diet (S. Table 3). Mitochondrial CL and PI from fish fed the RO diet had
377 around 16 % more n-6 PUFA than these phospholipid classes in fish fed the DHA diet (Table
378 5 and S. Table 3) while the RO diet itself had just 4 % more n-6 PUFA than the DHA diet.
379 The DHA diet contained 18 % more DHA than the RO diet but this different DHA content
380 between the two diets was not reflected in the fatty acid composition of any single
381 phospholipid class when the two feeding groups were compared.

382 When the interaction between fish age and diet (Age*Diet) on fatty acid composition
383 was analyzed, some significant differences were found in specific phospholipid classes,
384 particularly in PE, PI and SM, as indicated by the P values (Tables 4, S. Tables 3 and 4).
385 Almost no significant differences were found for Age*Diet interaction in PC, CL and PS
386 (Tables 3, 5, S. Table 2). PE and PI peroxidation index from 8-month-old fish showed higher
387 susceptibility to diet fatty acid composition than those from 21-month-old fish ($F = 24.359$ and
388 6.282 ; $p = 0.001$ and 0.031 , respectively). In contrast, CL and SM peroxidation index from
389 older fish showed significant differences between diet groups while no differences were found
390 in younger animals ($F = 3.283$ and 11.587 ; $p = 0.108$ and 0.009 , respectively).

391

392 *Lipid peroxidation*

393 Lipid peroxidation in total lipid of mitochondria from whole zebrafish was estimated
394 by measuring the TBARS contents (Figure 3). In 8-month-old-fish fed the DHA diet, the
395 levels of TBARS were significantly higher than in fish fed the RO diet ($t = 4.123$; $p = 0.042$).
396 No significant differences between dietary groups were found for older animals ($t = -0.315$; $p =$
397 0.782). TBARS content was lower in 21-month-old fish compared to younger fish but it was
398 only significant for fish fed diet DHA ($t = 5.433$; $p = 0.048$ for DHA and $t = 2.484$; $p = 0.221$ for
399 RO group). The effect of diet fatty acid composition on mitochondrial lipid peroxidation was
400 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

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

435

436 **Discussion**

437 Dietary fatty acid composition affected fish growth and mitochondrial membrane
438 composition of zebrafish and significantly influenced muscle and liver mitochondrial gene
439 expression and these effects differed with fish age. Zebrafish fed the DHA diet showed lower
440 growth than those fed the RO diet (significant for younger fish) despite being fed the same
441 feed ration (2 % BW per day). This could be related to increased oxidative stress mediated by
442 diet fatty acid composition. Lipid peroxidation in total lipid from mitochondria was double in
443 fish fed the DHA diet than in fish fed the RO diet in 8-month-old fish. This indicated higher
444 oxidative damage to mitochondrial lipids in young zebrafish fed the DHA diet, very likely
445 due to the higher incorporation of long-chain polyunsaturated fatty acids (LC-PUFA),
446 particularly DHA, in phospholipids of mitochondrial membranes in fish fed diet DHA
447 compared to fish fed the RO diet, as shown previously in liver and muscle mitochondria of
448 Atlantic salmon, *Salmo salar* (Kjaer et al. 2008; Ostbye et al. 2011) and in liver mitochondria
449 of rainbow trout (Almáida-Pagán et al. 2015). Several studies indicated that increase in
450 dietary PUFA elevated the metabolic rate which would be very likely mediated by changes in
451 membrane composition (Hulbert et al. 2005). This conclusion is heavily influenced by recent
452 studies of the metabolic rate of different species and particularly the development of what has
453 been termed the 'membrane pacemaker' theory of metabolism (Hulbert 2008).
454 Polyunsaturation of cell membrane lipids results in more fluid membranes that can promote
455 higher molecular activity of membrane proteins and, in turn, increase the metabolic activity of
456 cells, tissues and, consequently, whole animals (Pamplona et al. 2002; Sanz et al. 2006). At
457 the same time, membranes with increased levels of PUFA are more prone to attack by
458 oxidizing agents and will then participate in long-term, free radical reactions that will
459 propagate oxidative damage throughout the organelle (Hulbert 2005). This theory would
460 explain the huge differences in life span existing among animal species with different levels
461 of unsaturation in their membranes, but could also explain how situations such as thermal or
462 dietary changes in an animal life-cycle could compromise metabolic activity, oxidative stress
463 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; Almailda-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 (Almailda-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; Almailda-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

497 was found in liver but differences were only significant for COX3 and ND3. In agreement,
498 sea bream (*Sparus aurata*) fed on vegetable diets, with low n-3 LC-PUFA contents, exhibited
499 up-regulation of several mitochondrial genes in liver after an environmental stress (Pérez-
500 Sánchez et al. 2013). This could also indicate high oxidative stress inside the mitochondria as
501 the studied genes belong to a specific region of the mitochondrial genome that has been
502 reported to be especially prone to oxidative attack in several tissues of humans and mice
503 (Schon et al. 2002). Genes in this area encode predominantly for subunits of complexes I
504 [NADH-coenzyme Q (CoQ) oxidoreductase] and IV (cytochrome c oxidase) of the electron
505 transport chain (ETC), which appear to be particularly prone to age-related decline in activity
506 in several tissues (Shigenaga et al. 1994; Richter 1995; Trifunovic and Larsson 2008). This is
507 not surprising taking into consideration that 7 out of the 13 mtDNA encoded polypeptides in
508 the ETC are found in complex I while 3 are found in complex IV. Moreover, there is a strong
509 connection between complexes I and IV, and CL. It has been reported that CL is specifically
510 required for electron transfer in complex I of the mitochondrial ETC (Paradies et al. 2002),
511 complex I being considered the main site of ROS production in mitochondria. It is reasonable
512 to suggest that defects in mitochondrial complex I activity in addition to that of complex IV
513 (very tightly bonded to CL), due to oxidation/ depletion of CL molecules and/or mutations
514 affecting the corresponding area in the mitochondrial genome, may increase electron leak
515 from the ETC generating more oxidative stress, mitochondrial damage and, ultimately,
516 mitochondrial dysfunction and bioenergetic decay associated with disease and aging.
517 However, it is acknowledged that a limited number of mitochondrial-encoded genes were
518 evaluated in the present study and that the trend observed may vary among tissues. In this
519 respect, it was recently shown that the regulation of nuclear- and mitochondrial-encoded
520 genes of the respiratory chain is different depending on the tissue metabolic capabilities in
521 teleosts (Bermejo-Nogales et al. 2015).

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

530 their life-cycle (24-month-old) indicating that this could be a natural process in zebrafish,
531 regardless of diet. Older zebrafish mitochondrial gene expression was also less affected by the
532 diet fatty acid composition with no differences between feeding groups. This lower
533 responsiveness of lipid peroxidation and mitochondrial gene expression in older fish fed the
534 DHA diet could be due to the fact that younger fish were growing during the feeding trial
535 while older fish were not, resulting in greater incorporation of dietary fatty acids into tissues
536 of young fish. However, this would not fully explain the differences observed in phospholipid
537 fatty acid compositions between age groups. Mitochondrial lipids of 21-month-old zebrafish
538 were affected differently by dietary fatty acid composition than 8-month-old fish.
539 Interestingly, mitochondrial PC and PE from 21-month-old zebrafish showed less difference
540 between the two feeding groups than younger fish, while CL and SM reflected more strongly
541 the DHA diet when compared with the younger fish. Moreover, older fish fed the DHA diet
542 had mitochondria with a significantly lower CL content than those fed the RO diet while no
543 difference was found in younger fish. It is well-known that the aging process involves a
544 reduction in the rate of lipid metabolism and turnover of fatty acids (Hansford and Castro
545 1982). Therefore, these differences could also indicate age-related changes in the animal's
546 capacity for maintaining the structural homeostasis of mitochondrial membranes.

547 In conclusion, the present results suggested there was high oxidative stress in 8-
548 month-old zebrafish fed a diet with high DHA. Despite of the existence of mechanisms
549 regulating mitochondrial phospholipid content and phospholipid fatty acid composition, these
550 processes appear to be overridden by the change in dietary fatty acid composition and the
551 main constituents of mitochondrial membranes suffered marked changes in their composition.
552 This situation affected mitochondrial lipid peroxidation, mitochondrial membrane
553 composition and gene expression, and influenced animal growth. Following the membrane
554 pacemaker theory of animal metabolism (Sanz et al. 2006; Hulbert 2007), lipids would be
555 among the first molecules affected by mitochondrial free radicals, and lipid peroxidation
556 could be the propagator of oxidative damage reactions which would attack other organelle
557 molecules, including mtDNA. These changes could affect the organelle function and cell
558 viability by affecting ETC efficiency, ROS production and signalling systems. The effects of
559 dietary fatty acid composition differed between the two age groups, which could be related to
560 observed differences in specific growth rate. Nevertheless, many of the observed changes in
561 phospholipid fatty acid composition with diet in one age group were in the opposite direction
562 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

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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

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- 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).

749 **Figure 2. Phospholipid content (percentage of total lipid weight) and phospholipid class**
750 **composition (percentage of total phospholipids) of mitochondria isolated from 8- and 21-**
751 **month-old zebrafish fed two different diets.** Results are means \pm SEM. (n = 4). '+' symbols
752 represent differences between feeding groups for each phospholipid class as determined by a
753 Student t-test ($p < 0.05$). Asterisks indicate significant differences between age groups for
754 each phospholipid class as determined by a Student t-test ($p < 0.05$). Table represents *P* values
755 for interaction Diet and Age for each phospholipid class as calculated by a general linear
756 model ($p < 0.05$). CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI,
757 phosphatidylinositol; PL, phospholipid; Σ PL, total phospholipids; PS, phosphatidylserine;
758 SM, sphingomyelin.

759 **Figure 3. TBARS contents (ng g lipid⁻¹) of 8- and 21-month-old zebrafish fed the two**
760 **experimental diets.** Data expressed as mean \pm SEM (n = 4). '+' symbols represent
761 differences between feeding groups and asterisks denote significant differences between age
762 groups as determined by a Student t-test ($p < 0.05$).

763 **Figure 4. Mitochondrial superoxide dismutase (Mn-SOD) activity (units min⁻¹ mg prot⁻¹)**
764 **of 8- and 21-month-old zebrafish fed the two experimental diets.** Data are expressed as
765 mean \pm SEM (n = 4).

766 **Figure 5. Relative expression of COX3, ND3, ND4L, ND4 and ND5 genes of 8- and 21-**
767 **month-old zebrafish fed the two experimental diets.** Data are expressed as mean \pm SEM (n
768 = 6 for younger and n = 4 for older fish). '+' symbols represent differences between feeding
769 groups and asterisks denote significant differences between age groups as determined by a
770 Student t-test ($p < 0.05$). The insert Table presents significant values for the interaction
771 between Age and Diet for each gene as calculated by a general linear model ($p < 0.05$).

772

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

| | RO | DHA |
|---|-------|-------|
| <i>Ingredients of capsules¹ content (g per 100 g dry weight)</i> | | |
| 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 |
| <i>Proximate analysis of the capsules (% dry matter)</i> | | |
| 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 |

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

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

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

776 ⁴Supplied (per kg diet): KH₂PO₄, 22 g; FeSO₄ · 7H₂O, 1.0 g; ZnSO₄ · 7H₂O, 0.13 g; MnSO₄ · 4H₂O,
777 52.8mg; Cu-SO₄ · 5H₂O, 12 mg; CoSO₄ · 7H₂O, 2 mg.

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

782 ⁶CRODA International Plc (East Yorkshire, UK).

783 ⁷The Cooperative Food (UK).

784 ⁸Dissolved in propylene glycol and contained (g l⁻¹): butylated hydroxy anisole, 60; propyl gallate, 60;
785 citric acid, 40.

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

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790

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 |
| ΣSaturated | 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 |
| ΣMonounsaturated | 62.1 | 46 |
| 18:2n-6 | 19.5 | 13.1 |
| Σn-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 | 1.9 |
| Σn-3 PUFA | 10.1 | 30.6 |
| ΣPUFA | 29.7 | 46 |
| PI _n | 45.9 | 207.7 |

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

PI_n. 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: GATTCAAACCCCTGAGGAT | 201 | 0.91 | 59 |
| | R: AGTGCTAGGTTGGCCAGATT | | | |
| ND4L | F: TTCACCGTGTTACCTCCTA | 159 | 0.90 | 59 |
| | R: CACTTGCTTCACAGGCAGAA | | | |
| ND5 | F: CACATCTGCACTCACGCTTT | 177 | 0.93 | 59 |
| | R: AAGGAAGGGGTACCCATAA | | | |
| 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 | <i>P</i> |
| 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 |
| PI _n | 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 PI_n, 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.

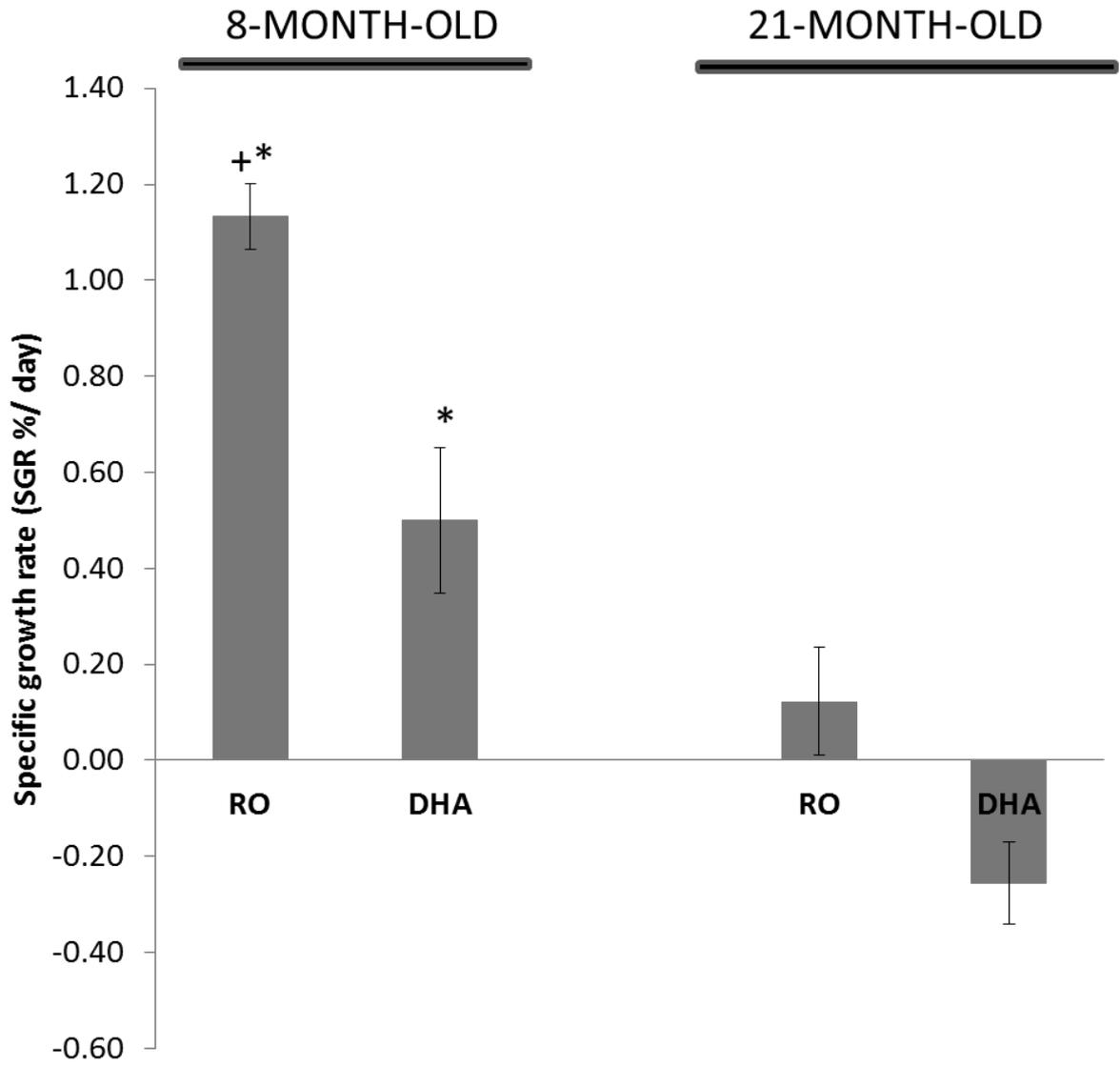
| | 8-month-old | | 21-month-old | | Age*Diet |
|------------------|-------------|-------------|--------------|------------|----------|
| | RO | DHA | RO | DHA | <i>P</i> |
| 16:0 DMA | 1.5±0.3* | 1.5±0.2 | 3.1±0.2+ | 1.2±0.5 | <0.001 |
| 16:0 | 7.7±0.7 | 7.8±1.0 | 7.6±1.2 | 7.1±1.6 | 0.676 |
| 18:0 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 |
| ΣSaturated | 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 |
| ΣMonounsaturated | 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 |
| Σn-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 |
| Σn-3 PUFA | 36.0±1.1+ | 45.0±1.9* | 31.3±6.0 | 31.9±3.8 | 0.049 |
| ΣPUFA | 51.2±2.0 | 55.4±1.7* | 45.7±3.9 | 43.4±3.3 | 0.05 |
| Σn-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 |
| PI _n | 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; PI_n, peroxidation index; PUFA, polyunsaturated fatty acids.
 818 Fatty acids representing less than 1% of total fatty acids are not shown.
 819

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

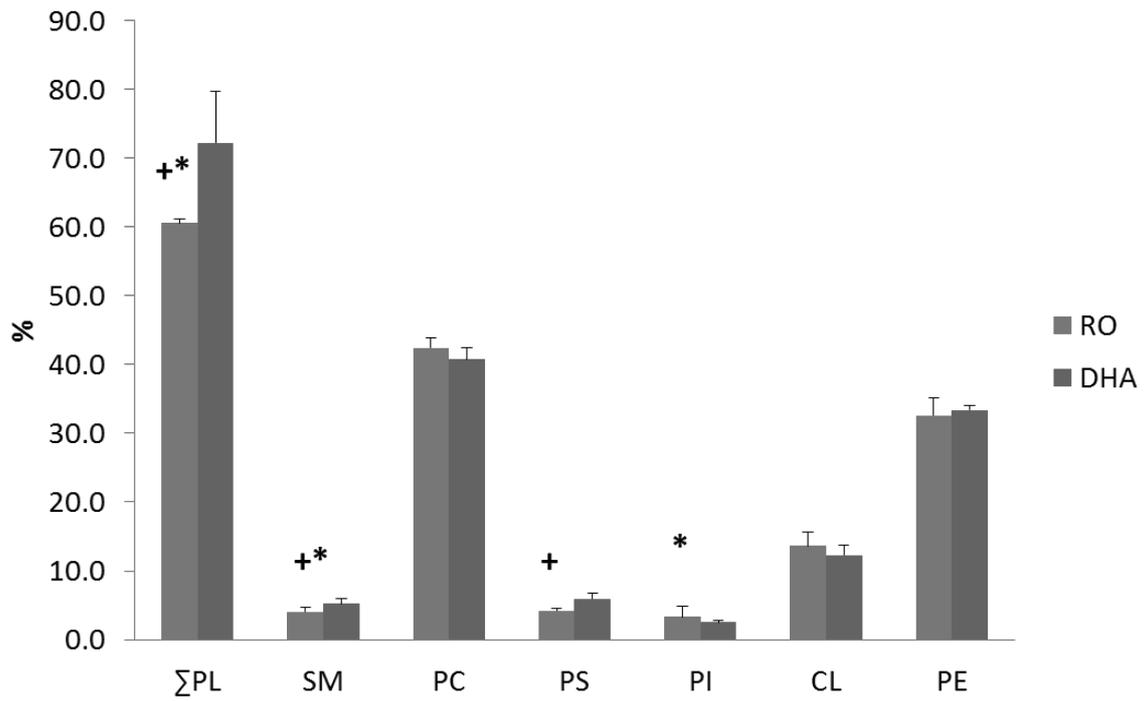
| | 8-month-old | | 21-month-old | | Age*Diet |
|------------------|-------------|------------|--------------|------------|----------|
| | RO | DHA | RO | DHA | <i>P</i> |
| 16:0 | 5.7±0.5 | 7.8±1.7 | 7.4±2.3 | 8.3±0.5 | 0.542 |
| 18:0 | 2.3±0.1+* | 4.6±1.3* | 5.1±1.3+ | 10.4±0.5 | 0.043 |
| ∑Saturated | 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 |
| PI _n | 170.2±17.8 | 189.8±18.7 | 156.3±26.0+ | 223.5±23.0 | 0.108 |

822 Data expressed as mean ± SD (n=4). '+' symbols within a row and for each age group represent significant
 823 differences between diet treatments as determined by a t-student (*p*<0.05). Asterisks denote statistical
 824 differences between 8- and 21-month-old zebrafish for each diet group (RO and DHA) when compared
 825 using a t-test (*p*<0.05). Right column represent significance values for the interaction between Diet and
 826 Age as indicated by a general linear model (*p*<0.05). RO, rapeseed oil group; DHA, DHA500 TG oil group;
 827 LC-PUFA, long-chain polyunsaturated fatty acids; PI_n, peroxidation index; PUFA, polyunsaturated fatty acids.
 828 Fatty acids representing less than 1% of total fatty acids are not shown.
 829

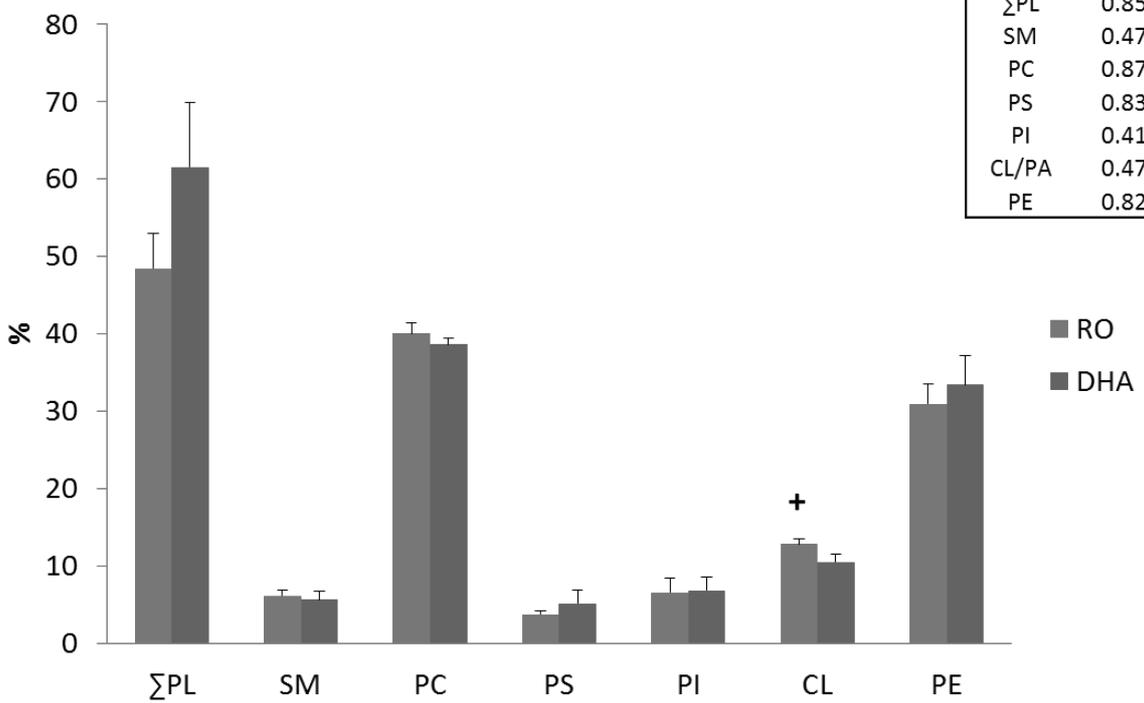


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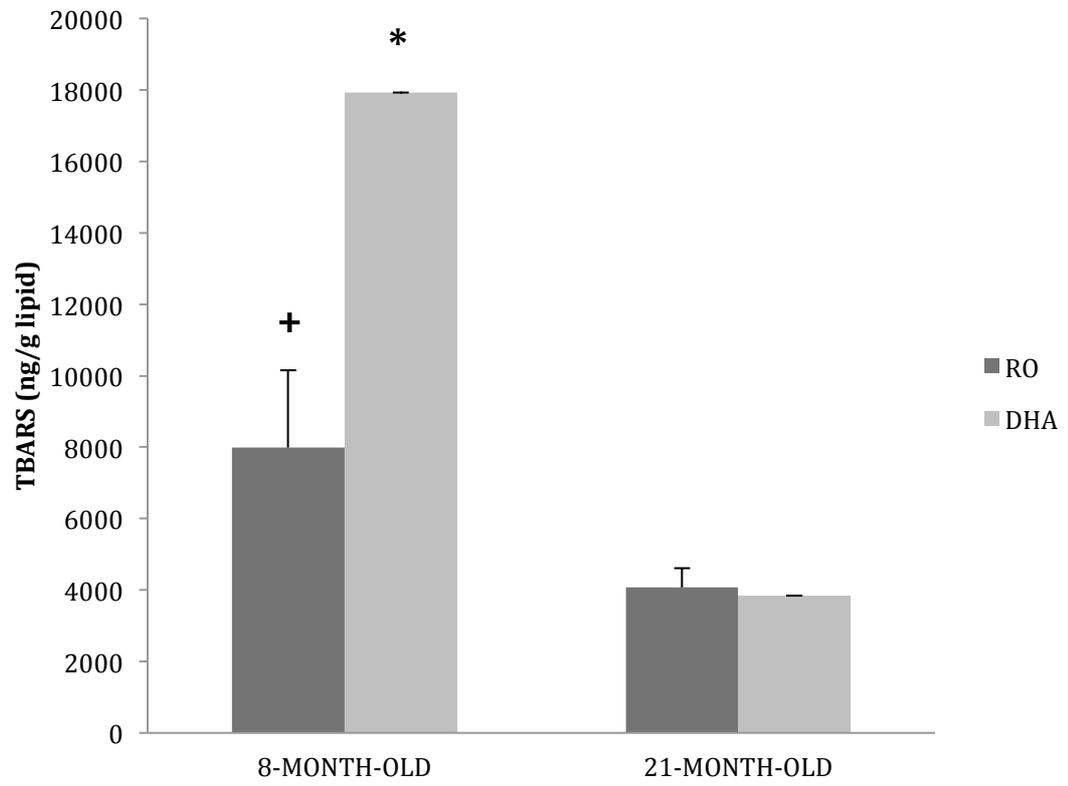
8-MONTH-OLD



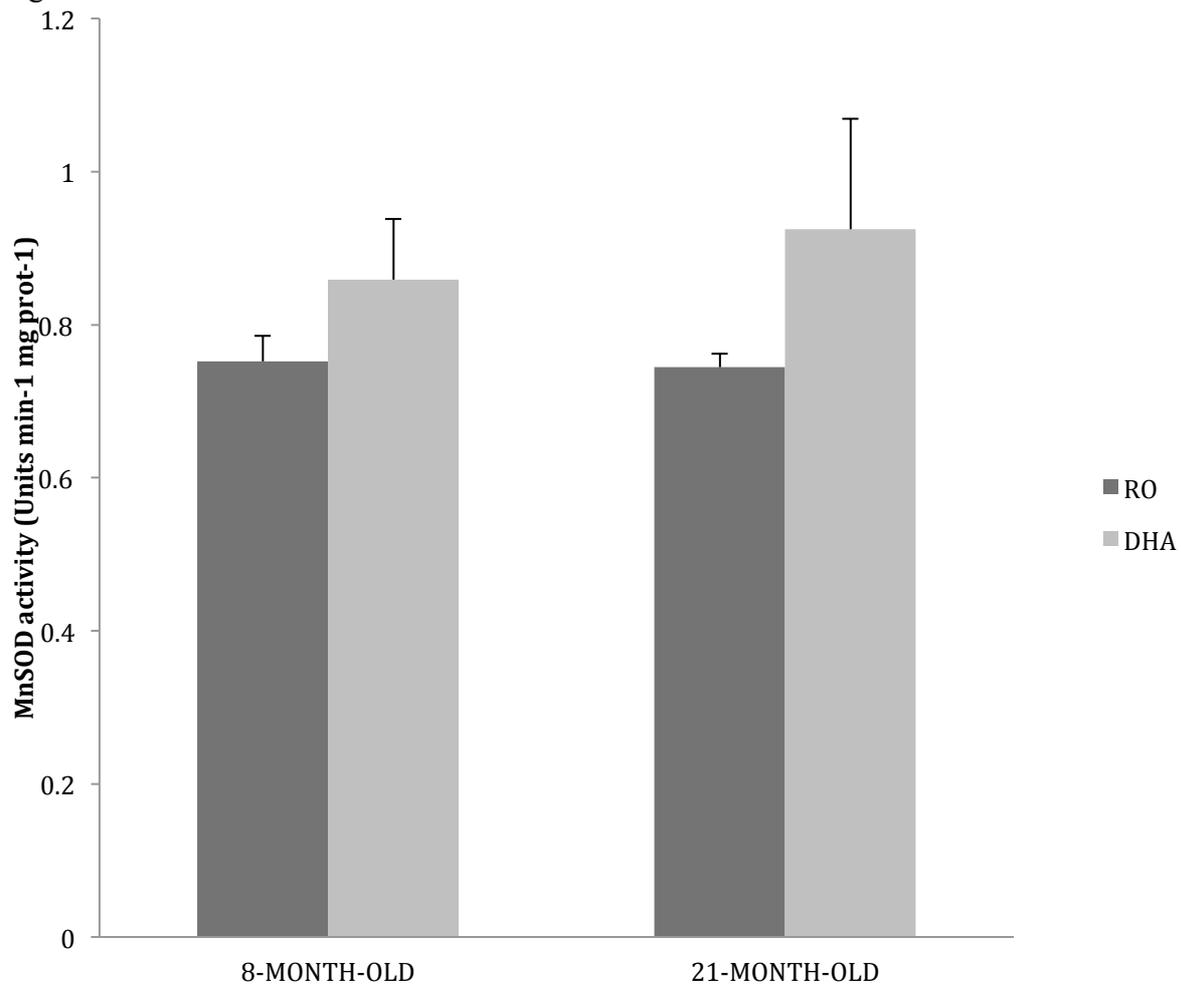
21-MONTH-OLD



836 Fig.3
837



838 Fig.4



839

