

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

M. B. Betancor · P. F. Almaida-Pagán ·
A. Hernández · D. R. Tocher

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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 to 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

M. B. Betancor and P. F. Almaida-Pagán have contributed equally to this work.

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M. B. Betancor (✉) · P. F. Almaida-Pagán ·
A. Hernández · D. R. Tocher
School of Natural Sciences, Institute of Aquaculture,
University of Stirling, Stirling FK9 4LA, Scotland, UK
e-mail: m.b.betancor@stir.ac.uk

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
PI _n	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	Standard 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
T _m	Melting temperature

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 has a high content of PUFA that, along with its proximity to the site of ROS production, makes it particularly prone to peroxidation. Reduced CL content and/or composition could lead to

mitochondrial dysfunction and cell decay. Mitochondrial membranes also contain small amounts of sphingomyelin (SM), which has membrane-rigidifying properties (due to its low content in PUFA) and may retard the lateral propagation of free radicals (Subbaiah et al. 1999; Cutler and Mattson 2001). Other PL species such as phosphatidylserine (PS) and phosphatidylinositol (PI) are also important as they are precursors for signaling molecules, some associated with apoptosis (Hannum and Obeid 1994; Ulmann et al. 2001). In addition, fish mitochondrial activity also appears to be highly modulated by nutritional stressors (Enyu and Shu-Chien 2011) or PUFA levels (Kjaer et al. 2008; Pérez-Sánchez et al. 2013; Almaida-Pagán et al. 2015).

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

In summary, mitochondria are among the first responders to various stressors that challenge the

homeostasis of cells and organisms (Manoli et al. 2007) and are deeply involved in the aging process. Mitochondrial decay is generally associated with impairment in mitochondrial bioenergetics function and increased oxidative stress (Paradies et al. 2011), and it seems clear that deterioration of mitochondrial inner membrane PL, particularly of CL, and accumulation of mtDNA mutations are mechanisms involved in this process. Diet FA 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 FA 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 signaling, 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 (eight tanks in total). One hundred and twelve zebrafish of 0.21 ± 0.04 g (8 months) and 32 fish of 0.61 ± 0.12 g (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 ± 1 °C and under a 12-h light: 12-h 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 2 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 given 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 to 0.5 mm and stored at -20°C until use. The FA compositions of the diets are given 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 [three fish per replicate for 8-month-old ($n = 6$) 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 RNAlater[®] (Life technologies, Paisley, UK) following the manufacturer's instructions and stored at -20°C for molecular analysis ($n = 6$ and 4 for 8- and 21-month-old fish, respectively). The purified mitochondrial isolates were analyzed 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 centrifuged twice at 600g for 6 min. After the first centrifugation, the pellet was discarded (cell/nuclei debris) and the

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

	RO	DHA
<i>Ingredients (g per 100 g dry weight)</i>		
Vit-free casein ^a	480	480
Potato starch ^b	150	150
Fishmeal ^c	50	50
Mineral mix ^d	47	47
Vitamin mix ^e	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 ^f	0	44
Rapeseed oil ^g	110	66
Antioxidant mix ^h	0.4	0.4
<i>Proximate analysis (% 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

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

^a Vitamin-free micropulverised (ICN Biomedical Ltd., High Wycombe, UK)

^b Passeli WA4 (Avebe Ltd., Ulceby, South Humberside, UK)

^c Norse-LT94 (Norsidmel AS, Fyllingsdalen, Norway)

^d Supplied (per kg diet): KH_2PO_4 , 22 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.13 g; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 52.8 mg; $\text{Cu} \cdot \text{SO}_4 \cdot 5\text{H}_2\text{O}$, 12 mg; $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg

^e 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

^f CRODA International Plc (East Yorkshire, UK)

^g The Cooperative Food (UK)

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

supernatant recentrifuged at 600g. Resulting supernatants were then centrifuged twice at 6800g for 10 min. After the first centrifugation, the pellet was resuspended in sucrose buffer and recentrifuged at 6800g. The final pellet was resuspended in 3 ml of

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

100 mM potassium phosphate buffer pH 7.8, with 0.1 mM EDTA. A 250- μ l 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 (Almáida-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 and mixing, and layers were 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 FA 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) sulfuric acid in methanol in order to prepare FA methyl esters (FAME) (Christie 2003). FAME were separated and quantified by gas-liquid chromatography (Carlo Erba Vega 8160, Milan, Italy) using a $30 \text{ m} \times 0.32 \text{ mm}$ 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 programming was from 50 to 150 at $40^{\circ}\text{C min}^{-1}$ and then to 230 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 of 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 2000g, 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 adrenochrome 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 was 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 adrenochrome 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 microcuvette, 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 were 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 was 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 (Untergasser 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 aging (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 the 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); 1 µl 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 °C for 15 min, followed by 35 cycles: 15 s at 95 °C, 30 s at the annealing T_m and 30 s at 72 °C.

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 (PI_n) was used as an estimate of PL susceptibility to oxidation and was calculated using the formula: $PI_n = 0.025 \times (\text{percentage of monoenoics}) + 1 \times (\text{percentage of dienoics}) + 2 \times (\text{percentage of trienoics}) + 4 \times (\text{percentage of tetraenoics}) + 6 \times (\text{percentage of pentaenoics}) + 8 \times (\text{percentage of hexaenoics})$ (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 (Jayaram et al. 2008). Results from mitochondrial lipid analyses, lipid peroxidation (TBARS) and superoxide dismutase (SOD) are presented as mean \pm 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's 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 analyzed 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 \pm 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

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

No significant mortalities were found for any of the experimental groups, and no differences in food intake between groups during the experiment were observed. The 8-month-old zebrafish increased in weight during the feeding trial from 0.21 ± 0.04 to 0.40 ± 0.07 g for the RO group and to 0.30 ± 0.11 g for the DHA group, while no significant growth 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 the DHA group). Younger fish fed the RO diet showed a higher specific growth rate (SGR) than those fed the DHA diet ($t = -3.187$; $p = 0.001$), but there was no difference in SGR between diets in older animals ($t = -1.550$; $p = 0.152$) (Fig. 1). No significant interaction between fish age and diet was observed for SGR ($F = 0.630$; $p = 0.432$).

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

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

Dietary FA composition affected phospholipid content and composition in whole zebrafish. Total phospholipid content was higher in fish fed the DHA diet than in fish fed the RO diet in both 8- (72.2 vs.

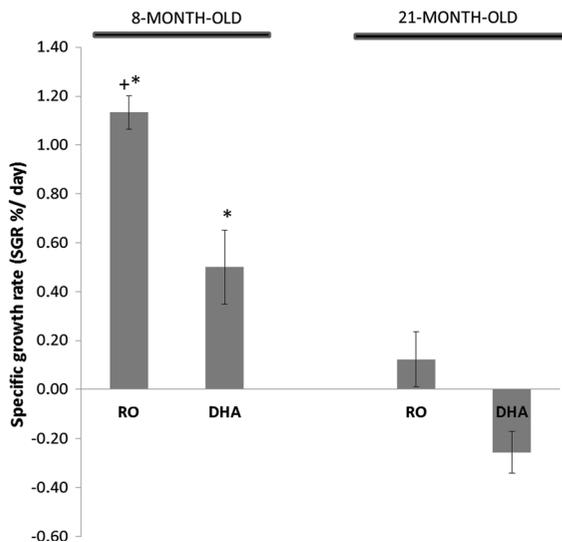


Fig. 1 Specific growth rate (SGR % day⁻¹) of 8- and 21-month-old zebrafish fed the two experimental diets for 2 months. Results are mean \pm SEM ($n = 56$ for 8- and $n = 16$ for 21-month-old fish). Plus symbols represent differences between feeding groups, and asterisks denote significant differences between age groups as determined by Student's t test ($p < 0.05$)

60.6 %) ($t = 3.076$; $p = 0.053$) and 21-month-old animals (61.5 vs. 48.4 %) ($t = 2.680$; $p = 0.037$) although it was only significant for the older fish. Mitochondria from 8-month-old fish fed the RO diet had less proportions of sphingomyelin (SM) ($t = 2.530$; $p = 0.045$) and phosphatidylserine (PS) ($t = 3.770$; $p = 0.009$) than those fed the DHA diet. In older fish, mitochondria from fish fed the DHA diet had a lower proportion of CL compared to that of fish fed the RO diet (10.5 vs. 12.8 %) ($t = -4.288$; $p = 0.005$).

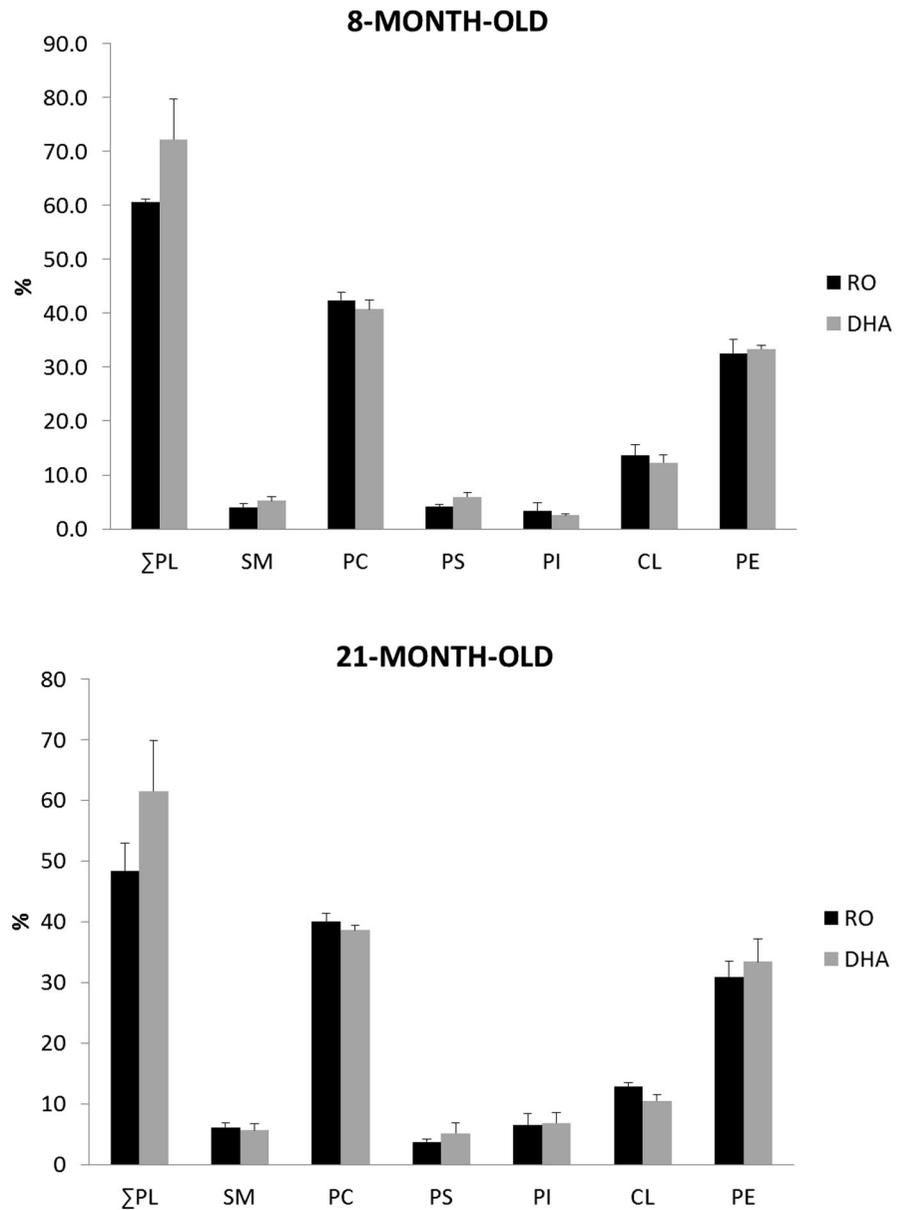
Mitochondria from 8-month-old zebrafish fed the RO diet showed some significant differences in phospholipid composition compared to that of 21-month-old fish, with higher total phospholipid content ($t = 5.229$; $p = 0.002$) and lower proportions of SM ($t = -4.079$; $p = 0.007$) and PI ($t = -2.657$; $p = 0.0038$). No significant differences were found between age groups for animals fed the DHA diet. Fish age and dietary FA composition did not show a significant interaction (Age*Diet) on mitochondrial phospholipid content and composition (F values for \sum 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 Fig. 2).

Effects of age and diet on phospholipid FA compositions of mitochondria of whole zebrafish

There were some significant differences in the FA profiles of mitochondrial PC, PE and CL from whole zebrafish when the two ages were compared (Tables 3, 4, 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 (PI_n) ($t = 6.501$; $p = 0.001$) (Table 4), and CL had higher SFA ($t = -3.259$; $p = 0.047$) and lower n-6 PUFA ($t = 4.343$; $p = 0.035$) (Table 5). Regarding the minor phospholipid classes, PS showed no differences in the main FA 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 PI_n ($t = -8.773$ 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 FA profiles of individual phospholipid species from 8-month-old fish mitochondria, and these effects were different for each phospholipid class. The FA composition of PS and SM from mitochondria of 8-month-old zebrafish was largely unaffected by diet (S. Tables 2 and 4). The three main phospholipid classes in zebrafish mitochondria responded to dietary FA 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 PI_n value (only significant for PE) ($t = 1.839$, 5.890 and 1.314 ; $p = 0.125$, 0.001 and 0.010 , respectively) (Tables 3, 4, 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 PI_n ($t = -4.578$; $p = 0.010$) than PI

Fig. 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 mean \pm SEM. ($n = 4$). Plus symbols represent differences between feeding groups for each phospholipid class as determined by a Student's t test ($p < 0.05$). Asterisks indicate significant differences between age groups for each phospholipid class as determined by a Student's 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, ΣPL total phospholipids, PS phosphatidylserine, SM sphingomyelin



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 FA composition of any single phospholipid class when the two feeding groups were compared.

When the interaction between fish age and diet (Age*Diet) on FA composition was analyzed, some significant differences were found in specific PL 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 FA composition than those from 21-month-old fish ($F = 24.359$ and

Table 3 Fatty acid composition (percentage of total fatty acids) of phosphatidylcholine of mitochondria isolated from whole zebrafish fed with one of the 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
<i>PIn</i>	193.4 ± 10.6	214.8 ± 20.4	175.7 ± 12.4	190.5 ± 17.4	0.684

Data expressed as mean ± SD (*n* = 4)

Fatty acids representing less than 1 % of total fatty acids are not shown

‘+’ 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 denotes 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 represents 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

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 (Fig. 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 FA composition on mitochondrial lipid peroxidation was influenced by age as determined by a general linear model (*F* = 9.700; *p* = 0.036).

Table 4 Fatty acid composition (percentage of total fatty acids) of phosphatidylethanolamine of mitochondria isolated from whole zebrafish fed with one of the 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
∑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
PI _n	330.6 ± 11.8+	386.8 ± 15.0*	289.8 ± 38.2	269.9 ± 33.5	0.031

Data expressed as mean ± SD (*n* = 4)

Fatty acids representing less than 1 % of total fatty acids are not shown

‘+’ 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 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 represents significance values for the interaction between diet and age as indicated by a general linear model (*p* < 0.05)

DMA dimethyl acetal, RO rapeseed oil group, DHA DHA500 TG oil group, LC-PUFA long-chain polyunsaturated fatty acids, PI_n peroxidation index, PUFA polyunsaturated fatty acids

Mitochondrial superoxide dismutase

Mn-SOD or SOD2 represented the main SOD activity in zebrafish mitochondria accounting more than 95 % of total SOD in all the experimental groups. SOD2 activity was higher in fish fed the DHA diet, but these differences were not statistically significant in either of the age groups (*t* = 1.615 and 1.697; *p* = 0.304 and 0.332 for 8- and 21-month-old fish, respectively) (Fig. 4). SOD2 activity remained stable between 8- and 21-month-old animals (*t* = 0.343 and -0.537;

p = 0.771 and 0.656 for RO and DHA group, respectively), and no interaction between age and diet was found (*F* = 0.363; *p* = 0.579).

Mitochondrial gene expression

The 8-month-old zebrafish fed the two diets showed significant differences in mitochondrial gene expression in muscle and liver (Fig. 5). Muscle from younger fish fed the DHA diet had lower expression of all the analyzed genes COX3, ND3, ND4, ND4L and ND5,

Table 5 Fatty acid composition (percentage of total fatty acids) of cardiolipin of mitochondria isolated from whole zebrafish fed with one of the two experimental diets

	8-month-old		21-month-old		Age × Diet <i>p</i>
	RO	DHA	RO	DHA	
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

Fatty acids representing less than 1 % of total fatty acids are not shown

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 represents 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, PI_n peroxidation index, PUFA polyunsaturated fatty acids

than zebrafish fed the RO diet (*t* = 2.387, 2.644, 4.407, 2.625 and 3.317; *p* = 0.038, 0.025, 0.004, 0.03 and 0.008 for COX3, ND3, ND4L, ND4 and ND5, respectively). The same trend was found in 8-month-old fish liver although the differences were only significant for COX3 (*t* = 3.099; *p* = 0.013) and ND3 (*t* = 2.570; *p* = 0.030) (Fig. 5). Diet had no significant effect on mitochondrial gene expression in 21-month-old zebrafish. Muscle from 21-month-old zebrafish showed relatively stable mitochondrial gene expression, particularly of ND genes, with no differences between dietary groups. Data from liver of older

zebrafish showed a high variation, and although there was a consistent trend of lower mitochondrial gene expression in the DHA group compared to the RO group, the differences were not statistically significant.

When mitochondrial gene expression of muscle from the two age groups was compared, older fish fed the DHA diet showed a higher expression of all the ND genes, although it was only significant for ND3 (*t* = 3.180; *p* = 0.013) and ND5 (*t* = 4.965; *p* = 0.001). Mitochondrial gene expression was found to be higher in liver of 21-month-old zebrafish than in

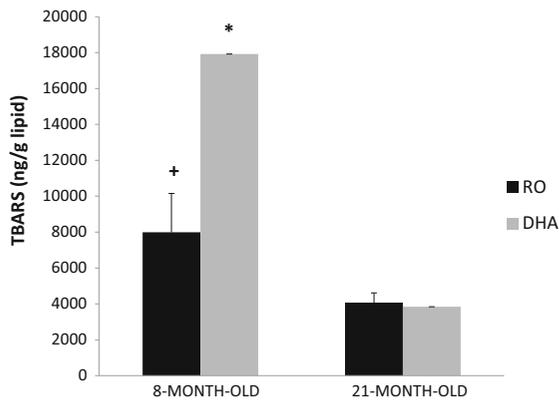


Fig. 3 TBARS contents (ng g lipid^{-1}) of 8- and 21-month-old zebrafish fed the two experimental diets. Data expressed as mean \pm SEM ($n = 4$). Plus symbols represent differences between feeding groups, and asterisks denote significant differences between age groups as determined by a Student's t test ($p < 0.05$)

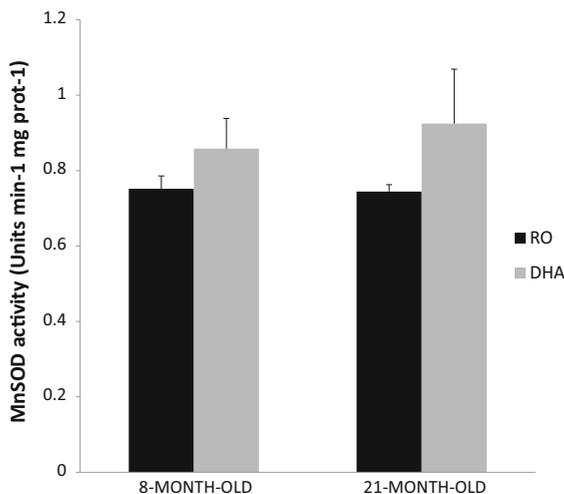


Fig. 4 Mitochondrial superoxide dismutase (Mn-SOD) activity ($\text{units min}^{-1} \text{mg prot}^{-1}$) of 8- and 21-month-old zebrafish fed the two experimental diets. Data are expressed as mean \pm SEM ($n = 4$)

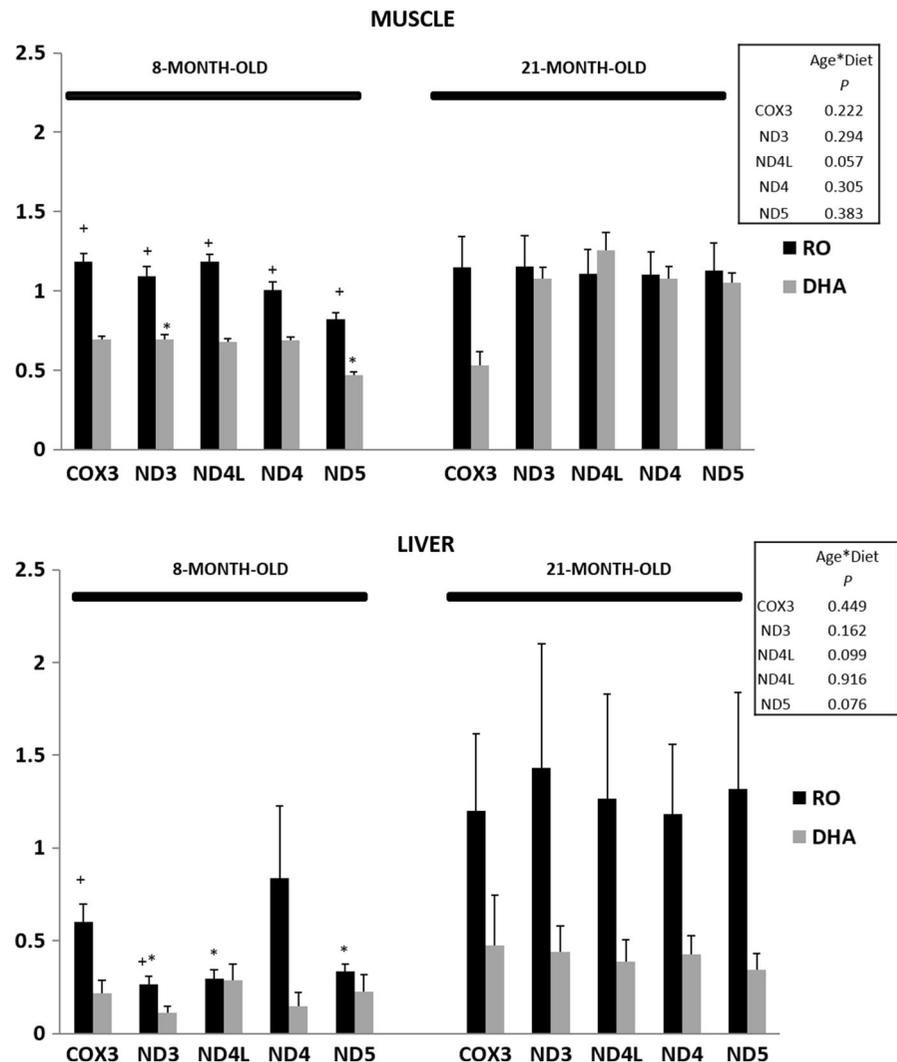
younger fish, particularly for the RO group, these differences being significant for ND3 ($t = -2.405$; $p = 0.043$), ND4L ($t = -2.159$; $p = 0.049$) and ND5 ($t = -2.377$; $p = 0.045$). No interaction between age and diet was found for any of the studied genes in either muscle or liver ($F = 1.617, 1.175, 4.217, 1.123$

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 Fig. 5).

Discussion

Dietary FA 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 FA 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 PL 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 (*Oncorhynchus mykiss*; Almáida-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

Fig. 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 \pm SEM ($n = 6$ for younger and $n = 4$ for older fish). *Plus symbols* represent differences between feeding groups, and *asterisks* denote significant differences between age groups as determined by a Student's *t* test ($p < 0.05$). The *inset Table* presents significant values for the interaction between age and diet for each gene as calculated by a general linear model ($p < 0.05$)



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.

The present data showed that mitochondria from 8-month-old zebrafish fed the DHA diet had higher levels of n-3 LC-PUFA and higher peroxidation index (PI_n) value in the three main phospholipid classes, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and cardiolipin (CL) (changes in PI_n only significant for PE). These data are consistent with those showing considerable dietary modifications of FA composition of mitochondrial PL in rats (Lemieux

et al. 2008; Abbott et al. 2010) and fish (Ushio et al. 1997; Robin et al. 2003; Guderley et al. 2008; Ostbye et al. 2011; Martin et al. 2013; Almáida-Pagán et al. 2015). However, they also indicate the existence of regulatory mechanisms of specific fatty acids within mitochondrial membranes as the observed changes did not clearly reflect the dietary compositions. Different mitochondrial phospholipid classes were affected by diet in different ways, suggesting the importance of FA composition at the level of specific PL, as suggested for mammals (Carrie et al. 2000; Cha and Jones 2000, Hulbert et al. 2005). Besides, phospholipid FA compositions did not entirely reflect the diet fatty composition. Interestingly, DHA incorporation in the main mitochondrial PL was significantly lower

than expected, considering the differences in this FA between the experimental diets. This can be in part explained by preferential retention of DHA in fish tissues when this FA is very low in the diet, as found in Atlantic salmon and other fish species (Bell et al. 2004; NRC 2011) and/or increased endogenous synthesis of DHA from linolenic acid (18:3n-3) in zebrafish when dietary levels of n-3 LC-PUFA are reduced (Tocher et al. 2002). This could also be due to the increase in lipid peroxidation observed in 8-month-old zebrafish fed the DHA diet, which could reduce membrane PUFA as found in senescent zebrafish (Almaida-Pagán et al. 2014). The observed increase in SM content in 8-month-old zebrafish mitochondria could also suggest higher lipid peroxidation (Lucas-Sánchez et al. 2013; Almaida-Pagán et al. 2014) and may represent an adaptive response in the organelle to mitigate propagation of oxidative reactions through the membrane. On the other hand, high levels of dietary DHA up-regulate uncoupling proteins (UCP), MIM transporters that uncouple oxidative phosphorylation, in mammals (Lee et al. 2013). In fish, major changes in UCP mRNA expression have been associated with switches in energy demand and oxidative capacities (Bermejo-Nogales et al. 2010), which may explain the increased oxidation observed in 8-month-old zebrafish.

Assay of mtDNA gene expression showed several differences in muscle and liver between feeding groups for 8-month-old zebrafish. Muscle of young fish fed the DHA diet 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 FA 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 lifetime 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 FA 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 FA compositions between age groups. Mitochondrial lipids of 21-month-old zebrafish were affected differently by dietary FA 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 homeostasis of mitochondrial membranes.

In conclusion, the present results suggested that 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 FA composition, these processes appear to be overridden by the change in dietary FA 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 signaling systems. The effects of dietary FA 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 FA 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 changes in the animal's capacity

for maintaining the structural homeostasis of mitochondrial membranes.

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Ethical standard The authors confirm that there is no potential conflict of interests. Fish were treated in accordance with British national ethical requirements and the experiments conducted under the UK Government Home Office project Licence number PPL 60/03969 in accordance with the amended Animals Scientific Procedures Act 1986 implementing EU directive 2010/63.

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