Age-related changes in mitochondrial membrane composition of rainbow trout (*Oncorhynchus mykiss*) heart and brain

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Abstract

Membrane composition, particularly of mitochondria, could be a critical factor by determining the propagation of reactions involved in mitochondrial function during periods of high oxidative stress such as rapid growth and aging. Considering that phospholipids not only contribute to the structural and physical properties of biological membranes, but also participate actively in cell signalling and apoptosis, changes affecting either class or fatty acid compositions could affect phospholipid properties and, thus, alter mitochondrial function and cell viability. In the present study, heart and brain mitochondrial membrane phospholipid compositions were analysed in rainbow trout during the four first years of life, a period characterized by rapid growth and a sustained high metabolic rate. Specifically, farmed fish of three ages (1-, 2- and 4-years) were studied, and phospholipid class compositions of heart and brain mitochondria, and fatty acid compositions of individual phospholipid classes were determined. Rainbow trout heart and brain mitochondria showed different phospholipid compositions (class and fatty acid), likely related to tissue-specific functions. Furthermore, changes in phospholipid class and fatty acid compositions with age were also tissue-dependent. Heart mitochondria had lower proportions of cardiolipin (CL), phosphatidylserine (PS) and phosphatidylinositol, and higher levels of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) with age. Heart mitochondrial membranes became more unsaturated with age, with a significative increase of peroxidation index in CL, PS and sphingomyelin (SM). Therefore, heart mitochondria became more susceptible to oxidative damage with age. In contrast, brain mitochondrial PC and PS content decreased in 4-year-old animals while there was an increase in the proportion of SM. The three main phospholipid classes in brain (PC, PE and PS) showed decreased n-3 polyunsaturated fatty acids, docosahexaenoic acid and peroxidation index, which indicate a different response of brain mitochondrial lipids to rapid growth and maturation.

Keywords: Fish, Growth, Mitochondria, Phospholipid, Heart, Brain.

1. Introduction

The combination of a high growth rate and the rapid attainment of a large body size have been reported to produce several negative side-effects in animals and to have important repercussions over a species' life-span (Innes and Metcalfe 2008). These effects are linked to a sustained high metabolic activity, which has been correlated with an increased level of intracellular oxidative stress (Rollo 2002; Alonso-Alvarez *et al.* 2007), along with decreased repair machinery (Almroth *et al.* 2010). In these conditions, mitochondrial molecules have been reported to suffer increasing deterioration that eventually can lead to the impairment of cellular bioenergetics function, increased oxidative stress and attenuation of the ability to respond to stresses (Paradies *et al.* 2011). For all these reasons, mitochondria are considered the key organelle contributing to tissue deterioration during high oxidative stress situations, including rapid growth and aging.

There are different theories for how mitochondrial dysfunction develops and leads to cell and tissue malfunction. It is known that all mitochondrial molecules are affected with age and that mitochondrial gene expression, membrane fluidity and electron transport chain (ETC) complexes are progressively affected (Shigenaga *et al.* 1994; Richter 1995). Although the cause-effect relationship among the observed alterations and, thus, the chain of events leading to mitochondrial decay with age, remains unclear, it could be suggested that mitochondrial membrane lipids may be the pacemakers of such events, determining how they propagate. It has been observed in a wide number of animal species that membrane composition, metabolic rate and lifespan are linked. Increased polyunsaturation of cell membranes results in altered physical properties of bilayers, which can enhance the molecular activity of membrane proteins that, in turn, increases the metabolic activity of cells, tissues and consequently whole animals (Hulbert 2008). At the same time, membranes that have different fatty acid composition will differ dramatically in their susceptibility to oxidative damage and this can affect their lifespan (Hulbert 2005). Therefore, effects of rapid growth on metabolic rate and lifespan could be mediated by lipid composition of membranes, particularly of mitochondria.

Mitochondrial membranes have a particular lipid composition including characteristic phospholipid species in the vicinity of ETC components, which has been suggested to be related with the role of mitochondria in oxygen consumption (Hoch 1992). Besides acylphosphoglycerols, major components of all membranes, mitochondrial membranes uniquely contain cardiolipin (CL), a key molecule for

mitochondrial function, and have small quantities of sphingolipids (Paradies *et al.* 2011). Furthermore, mitochondrial membrane phospholipid composition varies among tissues (Paradies *et al.* 1992; Zabelinskii *et al.* 1999; Modi *et al.* 2008), likely contributing not only to the considerable differences in physical and chemical characteristics of different types of membrane structures, but also determining the functioning of tissue-specific cell signalling systems.

Alterations in mitochondrial lipids have been found in aged mammals and humans, pointing to a key role of mitochondrial membrane composition in several age-related diseases, especially in those affecting to terminally differentiated non-proliferative organs such as brain and heart (Castelluccio *et al.* 1994; Chicco and Sparagna 2007; Pepe 2007; Paradies *et al.* 2011; Bazan *et al.* 2011; Ledesma *et al.* 2012). In these tissues, an increase in the population of dysfunctional mitochondria has been observed (Chaudhary *et al.* 2011). This can trigger removal of damaged cells via apoptosis which would be severely detrimental in these postmitotic tissues by causing tissue degeneration or dysfunction (Trifunovic and Larsson 2008). Aged brain becomes increasingly susceptible to neurodegenerative syndromes and decline of cognitive and motor performance (Ledesma *et al.* 2012), while aged heart has a decreased ability to tolerate stress (Chaudhary *et al.* 2011).

Thus, our hypothesis is that mitochondrial membrane phospholipid composition is the pacemaker of all the processes taking place inside the mitochondria with age, and that eventually lead to mitochondrial dysfunction. The primary aim of the present study was to characterize changes in rainbow trout heart and brain membrane phospholipid with rapid growth and maturation, focusing on alterations to class composition and individual phospholipid fatty acid compositions that may be critical in the modulation of mitochondria function during periods of high oxidative stress. We investigated rainbow trout in their first four years of life, a time during which this species reach their adult size. Rainbow trout is the most common freshwater fish reared in Europe and thus it is a well-known species that has been investigated previously in studies of oxidative stress and mitochondrial function (Otto and Moon 1996; Zabelinskii *et al.* 1999; Kraffe *et al.* 2007; Ostbye *et al.* 2011).

2. Materials and methods

2.1. Experimental fish and sampling

The study was performed on stock rainbow trout (*Oncorhynchus mykiss*) of three ages (1-, 2- and 4-years), all with the same genetic origin and maintained on the same rearing and feeding conditions in the freshwater aquarium facilities at the Institute of Aquaculture, University of Stirling. Fish were kept under natural photoperiod and water temperature conditions (7 ± 1.5ºC) and were sampled at the same time of the year (March 2011). Fish were fed twice a day *ad libitum* with commercial feed formulated to contain 50% protein and 19% or 22% fat for younger (1-2 years) or older (4 year-old) fish, respectively (Skretting, Northwich, UK). Feed fatty acid compositions were essentially similar (Table 1). Fish were anesthetized in 10% benzocaine, killed by a blow to the head, weight and length measured (Table 2), and heart and brain dissected. Whole hearts and brains were pooled and homogenized by blender to produce triplicate samples for biochemical analysis. In order to obtain sufficient mitochondrial material for all the required analyses, 1- and 2-year old trout samples consisted of hearts and brains pooled from 21 (3 pools of 7) and 12 (3 pools of 4) fish, respectively. Samples from 4-year old trout were hearts and brains from three individuals. Lipid extractions were performed on fresh samples of mitochondrial preparations. 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.

2.2. Mitochondria isolation

Approximately 2 g of brain and heart homogenate were further homogenized in 8 ml ice-cold sucrose buffer (0.4M phosphate buffer pH 7.4, 0.25M sucrose, 0.15M KCl, 40mM KF and 1mM N-acetyl-cysteine) using a tissue disrupter (IKA® T25 digital Ultra-Turrax® Fisher Scientific, Loughborough, U.K.). Sucrose buffer homogenates were centrifuged at 600 x *g* for 6 min and the pellet discarded (cell/nuclei debris). Supernatants were then centrifuged at 6,800 x *g* for 10 min. This procedure was sufficient to isolate mitochondria from trout heart but, for brain, further treatment was necessary in

order to disrupt synaptosomal membranes and obtain the maximum yield of mitochondria. With that purpose, resulting brain pellets were resuspended in 8 ml ice-cold sucrose containing 0.02% (w/v) digitonin and placed on ice for 10 min (adapted from Kudin *et al.* 2004). Suspensions were centrifuged at 600 x *g* for 6 min, the pellet discarded and supernatants centrifuged at 6,800 x *g* for 10 min. The resulting pellet constituted the brain mitochondrial fraction used for lipid extraction. To verify that pellets were highly enriched with mitochondria, a portion was fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer overnight at 4ºC, and then processed as specified by Rajapakse *et al.* (2001) prior to analysis by transmission electron microscopy (Tecnai^{TM} G^2 Spirit BioTWIN, FEI Europe, Eindhoven, The Netherlands) (Fig. 1).

2.3. Lipid extraction and phospholipid class composition

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

Phospholipid classes were separated by high-performance thin-layer chromatography (HPTLC) using 10 x 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 ºC 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 1994). Scanned images were recorded automatically and analyzed by computer using winCATS (Planar Chromatography Manager, version 1.2.0).

2.4. Phospholipid fatty acid composition

Individual phospholipid classes of heart and brain mitochondria were separated by preparative-TLC, using silica gel plates (20 x 20 cm) (VWR) and the solvent system as above. Individual phospholipid bands 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 50ºC overnight following addition of 2 ml of 1% (v/v) sulphuric acid in methanol in order to obtain the fatty acid methyl esters (FAME) (Christie 2003). Similarly, FAME were produced by acid-catalyzed transmethylation of samples of total lipid from feeds. FAME were separated and quantified by gas-liquid chromatography (Carlo Erba Vega 8160, Milan, Italy) using a 30 m x 0.32 mm i.d. capillary column (CP Wax 52CB, Chrompak, London, U.K.) and on-column injection at 50 ºC. Hydrogen was used as carrier gas and temperature programming was from 50 ºC to 150 ºC at 40 ºC min^{-1} and then to 230 ºC at 2.0 ºC min^{-1}. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman 1980; Tocher and Harvie 1988). Data were collected and processed using Chromcard for Windows (version 1.19).

2.5. Indexes and statistical analysis

Condition factor (K) was calculated using the formula: $K = [weight/(length)^3] \times 100$. For peroxidation index (PIn) the formula was: PIn = 0.025 × (% monoenoics) +1 × (% dienoics) + 2 × (% trienoics) + 4 × (% tetraenoics) + 6 × (% pentaenoics) + 8 × (% hexaenoics) (Witting and Horwitt 1964). The LC-PUFA index corresponded to the sum of long-chain polyunsaturated fatty acids (LC-PUFA, fatty acids with 20 or more carbons and 2 or more double bonds). Results are presented as mean ± SD (n = 3). Data were checked for homogeneity of variances by the Levene´s test and, where necessary, arc-sin transformed before further statistical analysis. A two-way analysis of variance (ANOVA) was used to assess the differences among groups based on tissue and age. Post hoc comparisons were made using the Bonferroni t-test for multiple comparisons. All statistical analyses were performed using SPSS

Statistical Software System version 15.0 (SPSS Inc, Chicago, USA). Differences were regarded as significant when $P < 0.05$ (Zar 1999).

3. Results

3.1. *Phospholipid class composition of heart and brain mitochondria*

The phospholipid class compositions of heart and brain mitochondria from 1-, 2- and 4-year old rainbow trout are shown in Fig.2. In both tissues, phosphatidylethanolamine (PE) constituted the main phospholipid class representing 37.4 and 42.9% of total phospholipids of 1-year old trout heart and brain, respectively. The second phospholipid in abundance was phosphatidylcholine (PC) which represented around 30% of total phospholipids. Thus, the sum of PE and PC constituted more than 65% of total in both heart and brain mitochondria. The next phospholipid in heart was cardiolipin (CL, 12.3%) followed by phosphatidylserine (PS, 7.4%), phosphatidylinositol (PI, 6.9%) and sphingomyelin (SM, 4.8%), whereas in brain, the third phospholipid in abundance was PS (12.0%) followed by PI (5.9%), CL (4.4%) and SM (0.9%).

Several changes with age were found in mitochondrial phospholipid class composition from trout heart. The proportions of total phospholipid increased significantly (Fig. 2), with the percentages of PC and PE significantly increased while those of CL, PS and PI decreased. Most of the observed changes took place between 2- and 4-year old animals. Some differences with age were also found in brain mitochondrial phospholipids, with decreased proportions of PC and PS, and an increased percentage of SM. Changes in PS and SM occurred mainly between 2- and 4-year old trout. Most of the differences between tissues in 1-year old animals were maintained in the older age groups, with heart having higher levels of SM, PC and CL and brain having higher levels of PS and PE (except 4-year-old). The effect of age on mitochondrial phospholipid composition was tissue-dependent for all classes except PS (Fig. 2).

3.2. *Fatty acid compositions of individual phospholipids* of *heart and brain mitochondria*

Fatty acid compositions of individual phospholipid classes from heart and brain of 1-, 2- and 4- year old rainbow trout are presented in Tables 3-8. Each individual phospholipid class showed a distinctive composition. PC was characterized by high levels of palmitic acid (16:0), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Table 3), PE had high levels of EPA and, particularly DHA, and was characterised by showing dimethyl acetal (DMA) derivatives (Table 4), CL had high levels of 16:0 and linoleic acid (18:2n-6) (Table 5), PS contained high stearic acid (18:0) and DHA (Table 6), PI was characterized by high levels of 18:0 and arachidonic acid (ARA, 20:4n-6) (Table 7), and SM had a high proportion of 16:0 and nervonic acid (24:1n-9) (Table 8).

Several differences were found between brain and heart when 1-year old animals were compared. Brain had higher percentages of monounsaturated fatty acids (MUFA) in most phospholipid classes. Nervonic acid was generally higher in brain phospholipids although it was significantly lower in brain SM (8.2 vs. 31.9%) (Table 8). Brain total n-6 polyunsaturated fatty acids (PUFA) were lower than in heart phospholipids, generally with lower percentages of 18:2n-6 and ARA. Moreover, DHA, total n-3 PUFA and peroxidation index (PIn) were lower in brain PC, PE and CL when compared with heart. Brain PS was the main exception to these differences since it contained higher level of DHA (48.3 vs. 37.0%), n-3 PUFA (53.7 vs. 41.9) and PIn (422.1 vs. 335.7) than heart (Table 6). Brain PE contained higher levels of DMA derivatives than heart (7.4 vs. 4.3) (Table 4).

These differences in PC, PE and CL compositions in 1-year old fish were maintained in the older age groups (Tables 3-5) but several differences were found in the other phospholipid classes. For instance, in 4-year-old trout, brain and heart PS DHA and PIn were similar, whereas PS n-3 PUFA levels were lower in brain than in heart (Table 6). Also in 4-year-old fish, brain and heart PI had similar MUFA and DHA values, but total n-3 PUFA and PIn were higher in brain compared to heart (Table 7). Finally in 2- and 4-year-old animals, SM nervonic acid levels were similar in brain and heart and, in 4-year-old fish, MUFA were higher and total n-3 PUFA and PIn lower in brain compared to heart (Table 8).

The fatty acid composition of individual phospholipid classes from rainbow trout heart and brain mitochondria showed significant changes with age. In heart mitochondria, there was a decrease in the proportions of total saturated fatty acids (SFA) in CL, PS, PI and SM (Tables 5-8). Total n-6 PUFA decreased in PC, PE and SM (Tables 3, 4 and 8). The percentage of DHA in CL and SM increased between 2- and 4-year-old trout, and PIn increased in every phospholipid class (not significant in PE and PI). In brain mitochondria, there was a general decrease in SFA and an increase in MUFA. Total n-6 PUFA increased in PC, PE and PI and n-3 PUFA decreased. The PIn decreased in the three major phospholipids in brain (PC, PE and PS). PI changed in a different way to the other brain phospholipid classes, as MUFA, n-3 PUFA and PIn significantly increased with age (Table 7). Most of the observed changes with age were tissue-dependent, with PI showing lower interaction between age and tissue compared to other phospholipid classes (Tables 3-8).

4. Discussion

Rainbow trout heart and brain mitochondria showed a different phospholipid composition. Although mitochondrial membranes contained similar percentages of total phospholipid, they presented different phospholipid proportions in each tissue. In 1-year-old animals, heart was found to be richer in PC, CL and SM, while brain had higher levels of PE and PS, similar to that observed in rats (Paradies *et al.* 1992; Modi *et al.* 2008). In a previous study, data on lipid compositions of liver mitochondria of rainbow trout were presented (Almaida-Pagán *et al.* 2012). Liver also showed a different phospholipid composition characterized by higher levels of total phospholipid, PC and PI compared with heart and brain mitochondria. Therefore, these data show a tissue-specific distribution of phospholipid classes in trout mitochondrial membranes that would be likely related to the particular tissue-specific properties and functions of the membranes.

The phospholipid class composition of the mitochondrial membranes changed with age in both heart and brain, although not in the same way. Heart mitochondria showed a significant decrease in PI, CL and PS, and an increase in PC and PE, while brain mitochondria had higher SM and lower PC and PS with age. The different effects on mitochondrial membrane composition, also observed in rats (Modi *et al.* 2008), may relate to differential responses of the two tissues to a rapid growth period and

maturation. For instance, changes in the proportions of individual phospholipid classes may lead to altered charge distribution across the membrane, membrane permeability properties, catalytic activities of specific enzymes and ETC function (Daum 1985). Especially interesting was the CL loss observed in heart, which was also reported in rats (Paradies *et al.* 1992; Lee *et al.* 2006). CL is considered a key molecule for mitochondrial viability (Paradies *et al.* 2011) whose proximity to the ETC and high content of PUFA make it highly susceptible to oxidative damage. Changes in CL content with age have been related to mitochondrial dysfunction by promoting the apoptosis cascade (Chicco and Sparagna 2007).

In addition, heart and brain mitochondrial membranes showed different phospholipid compositions that evolved in different ways during the first four years of rainbow trout life-cycle. Heart phospholipids became more unsaturated with age, which would render them more susceptible to peroxidation and, in turn, may promote their degradation and hydrolysis. Castelluccio *et al.* (1994) showed that rat heart mitochondrial membranes were significantly modified during the aging process, showing an increase in PUFA up to 12 months of age, followed by a subsequent decrease. Specially marked was the increase in DHA with age in heart SM with a consequent increase in susceptibility to oxidation. This was interesting as SM is known to retard the lateral propagation of free radicals through the membrane and to be an important mediator of mitochondrial pathways including apoptosis (Hannun and Obeid 1997; Cutler and Mattson 2001). Another interesting result related to dimethyl acetals (DMA) obtained from methylation of PE plasmalogen, and so can be considered as indicators of plasmalogen content. Plasmalogens are rich in some tissues such as heart and brain and have been considered as endogenous antioxidants (Brosche and Platt 1998) and have been shown to decrease with normal ageing in mammals tissues as we found in trout heart mitochondria in the present study.

The changes observed in brain indicate differential modification of mitochondrial membranes in that tissue compared to heart. The three main phospholipid in brain mitochondria, PE, PC and PS, showed decreased DHA and PIn, as observed previously in trout liver associated with oxidative lipid damage (Almaida-Pagán *et al.* 2012). Similar changes were reported previously in mammals (Ledesma *et al.* 2012) and fish brain (total tissue) (Mourente and Tocher 1992). Changes were more marked in older fish, which may reflect that age affects some pathways for phospholipid synthesis in the central nervous system and indicate the presence of compensatory mechanisms to provide a pool of

phospholipid for the maintenance of cellular membrane lipid composition and/or functions during maturation and aging (Ilincheta de Bosquero *et al.* 2000). One of the most affected phospholipids in brain mitochondrial membrane was PS which is known to be very important for nervous tissue function and has been associated with age-related decay and disease (Ulmann *et al.* 2001; Mozzi *et al.* 2003). DMA levels were higher in brain mitochondrial preparations than in heart mitochondria, but this probably reflects PE plasmalogen content in myelin fragments associated with the brain preparation rather than brain mitochondria.

In any case, both heart and brain showed changes with age that affected mitochondrial membrane phospholipid compositions. Membrane composition determines the bilayer physical properties which affect membrane protein activity (Hulbert 2008). More polyunsaturated membrane lipids are correlated with faster turnover rates of individual mitochondrial membrane proteins (Hubert *et al.* 2006). The observed changes in mitochondrial phospholipid would affect membrane reactions, which constitute a quantitatively important component of cellular metabolism. Moreover, individual phospholipids participate actively in cell signalling including apoptosis. An alteration of either phospholipid proportions or individual phospholipid fatty acid compositions in mitochondrial membrane could affect organelle function and thus, cell and tissue viability.

Rainbow trout experience a rapid increase in body size during its early years. Rapid growth has been related with an increase in ROS production by mitochondria, and a diversion of resources into anabolism and away from repairing oxidative damage to cell molecules (Almroth *et al.* 2010). Considering membrane and membrane components as possible pacemakers of the main processes taking place in mitochondria (Hulbert 2007; 2008), phospholipid and fatty acid changes could play a central role by connecting the different processes involved in cumulative damage to cell molecules and dysfunction during periods of high stress. It is conceivable that the primary cumulative damage is to mitochondrial lipids, altering membrane fluidity and ultimately causing defects in ETC and respiration; as a result, the generation of ROS may be accelerated. Eventually, defence mechanisms and repair systems are overwhelmed and damage to mitochondrial DNA becomes permanent. Therefore, by achieving its mature size rainbow trout could be initiating its way into senescence.

In summary, the present study showed differences in mitochondrial membrane composition (phospholipid class and fatty acid compositions) among rainbow trout tissues that points to the

importance of particular phospholipids for tissue-specific functions. Significant changes in heart and brain mitochondrial membranes during the first four years of life in trout were observed. Brain mitochondria had lower levels of DHA and PIn in the major phospholipids while heart phospholipids became more unsaturated, generally associated with higher fluidity, but also with higher susceptibility to damage by high oxidative stress. Considering the importance of phospholipid fatty acid composition and the role of specific phospholipid in mitochondrial function and cell viability, these changes could affect ETC efficiency, ROS production and signalling systems, and be mediators of the processes involved in response to rapid growth and aging in trout.

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441 **Figure legends**

442 Fig. 1. Transmission electron micrograph showing a representative preparation of rainbow trout heart
443 mitochondria. Bar= 2 µm.

444 Fig. 2. Phospholipid content (percentage of weight of total lipid) and phospholipid class composition
445 (percentage of total phospholipids) of mitochondria isolated from heart and brain of 1-, 2- and 4-year
446 old rainbow trout. Results are means ± S.D. (n=3). Different superscript letters represent differences
447 between age groups for each phospholipid class as determined by two-way ANOVA ($P<0.05$). Table
448 represents *P* values for interaction tissue and age for each phospholipid class ($P<0.05$). Asterisks
449 denote statistical differences between tissues for each age group when compared using a Bonferroni
450 test ($P<0.05$). PL, phospholipid; CL, cardiolipin; PC, phosphatidylcholine; PE,
451 phosphatidylethanolamine; PI, phosphatidylinositol; ∑PL, total phospholipids; PS, phosphatidylserine;
452 SM, sphingomyelin.

Dr. Patrick J. Walsh
Editor-in-chief
CBP part B

Dear Dr. Patrick J. Walsh,

We send you our revised manuscript (Ref. No.: 20878).

We greatly appreciate the comments of the reviewers. All comments have been considered and, where appropriate, modifications included in the text. Our responses to the reviewers' comments are detailed below.

Yours sincerely,

Pedro Almaida-Pagán
Institute of Aquaculture
University of Stirling

Reviewer 1:

This manuscript investigates detailed mitochondrial membrane phospholipid compositions of heart and brain in rainbow trout during the four first years of life. This represents an interesting important amount of data on mitochondrial membrane lipid and FA compositions. The set of experiments seem to be well conducted and the data obtained seem to have been carried out using reliable techniques (with some points that need to be clarified: see below).
However, the scientific interest of the study is not obvious. Probably the amount of data deserves the scientific question and interest, and we don't see clearly and understand to which questions authors want to get with this manuscript?

The aim of the present study was to characterize the changes in mitochondrial membrane composition of two important tissues highly relevant to the ageing process. Our hypothesis is that mitochondrial lipid membrane composition can be the pacemaker of all the processes taking place inside the mitochondria with age and that eventually lead to mitochondrial dysfunction. Many diseases, mainly affecting to terminally differentiated tissues, such as heart and brain, have already been related with impairment in mitochondrial function, which has been related with several processes including lipid peroxidation, protein and mtDNA damage. Lipid oxidation is known to be quantitatively the most important process associated with oxidative stress and it produces several highly reactive derivatives that act as propagators of the reaction. Moreover, mitochondrial membrane lipid composition determines its susceptibility to damage by oxidative stress and influences mitochondrial metabolic rate. Degradation of specific phospholipid classes, such as cardiolipin, could partly explain the deterioration observed in mitochondria with age and has been associated with mitochondrial-specific apoptosis. Therefore, by characterizing how the mitochondrial membrane composition changes with age (phospholipid distribution and fatty acid composition) we can contribute to the understanding of the processes which lead to mitochondrial dysfunction associated with high oxidative stress situations and ageing. This has been further emphasised in the Introduction and we have added a clear statement of our working hypothesis.

First example is for the introduction. All concept on importance and roles of membranes lipid composition in mitochondria is focusing on mammals and humans, but nothing is introduced concerning the important plasticity of mitochondria in trout while knowledge on this question is far from being unknown. One important concern is for example about temperature effects on membrane lipid compositions in trout. As no precision was given on temperature regime under which trout were maintained during the all duration of the experiment (e.g. constant temperature or seasonal oscillating temperature), if trout were sampled at the same time of the year and the same reproductive stage, this lead to ask if authors have considered these important questions regarding membrane lipid compositions of mitochondria.

Temperature was controlled. All fish in the different age groups were maintained under identical rearing conditions of temperature and photoperiod. We have added this information to Material and Methods. We are well aware of the influence of external factors (such as temperature, diet composition etc) and, indeed, those issues are in our research programme and are the focus of our current work. For the reported study, we controlled these external factors so we could focus on characterizing the effect of normal ageing on mitochondrial lipids, working in the most similar conditions possible taking into account that 4-year-old animals were much larger and had to be provided with a different pellet size. We are fully aware of the work on the effect of temperature on trout membranes but this was not directly relevant to the results reported. In contrast, there are no

previous studies addressing the changes in mitochondrial membrane composition with age in fish. We have used numerous references to build our hypothesis that mitochondrial phospholipid could play a central role in mitochondrial dysfunction associated with high stress periods. The combined data in previous work suggest that membranes may be the pacemakers of all the processes associated with animal longevity leading the propagation of reactions involved in mtDNA and protein damage, ETC impairment etc. We think that this could be a way to unify all the processes involved in mitochondrial dysfunction during high oxidative stress situations and aging.

About reproductive stage, title of the manuscript speaks of effects of maturation while nothing within the paper is said or specified about maturation stage? So why "maturation" in the title?
"Mis-discussion" and highly hypothetic suggestions based only on lipid compositions should be reconsidered. The discussion should be drastically reduced and much less speculative. One example is on CL content (line 235) while differences are only significative between 2 years and 4 years old trout but not different between 1 and 4 years old.
Another example is found in the conclusion where authors say line 303: "Brain mitochondria had lower levels of DHA and PIn in the main phospholipids while heart phospholipids became more unsaturated and thus, more fluid, but also more prone to be damaged by high oxidative stress". How authors can conclude this based on the data presented?

These comments are welcome and have been taken fully into consideration. We have reworded the Title and revised the Discussion at the points mentioned above in order to be clearer and, hopefully, improve the manuscript.

Paragraph 243-254 are only repeating results.

This paragraph has been substantially revised and all repetition of results removed.

Paragraph 288-298: Nothing linked to the results

This is the last paragraph of the Discussion and, as such, attempts to put the present work into the context of the overall hypothesis, and so does not refer to any particular result. Surely this is an entirely normal approach!

Also it appears that one of the most important modification of FA composition within the different PL classes analysed was found in sphingomyelin with 22:6n-3 changing from 3.9% in heart of mitochondria isolated from one year old trout to 20% in 4 years old, with concomitant and compensatory modifications in total SAT and 24:1n-9. Nothing is discussed within the manuscript? Another example is about DMA (see below). This tends to confirm that authors lost themselves in this large set of data, missing some important and interesting point to discuss without being too speculative on other aspects of their results.

We were not lost in our dataset. While we acknowledge that we did not mention every single change, this was deliberate, and rather we focussed on what we believed were the most important points in the data set. However, we are more than happy to add mention of the points the reviewer highlights. The reader can now judge their importance.

For a study on lipids focusing uniquely on compositions of mitochondria, I would be particularly concerned about non-mitochondrial contamination. This may significantly effects on lipid profiles. That said, I would expect non-mitochondrial contamination by membranes to be relatively minor, but values of 4.8-5.7 % of sphigomyelin in heart mitochondrial preparations make me doubt about it. Indeed, sphingomyelin is known to be at very low levels in mitochondria. Some additional info on the isolation method (e.g., how many washes of

the crude pellet) would be helpful. In addition, as brain and heart mitochondria were isolated using different methods, and that brain mitochondria contain much less sphigomyelin (an therefore, maybe less contaminants), why authors didn't use the same techniques for both tissues?
In addition, authors specified in the Materials and Method section that mitochondrial fraction used for lipid analysis were proceeded for analysis by transmission electron microscopy. No results are given about such analysis which could give important information about any contamination on mitochondrial preparations.

We adapted the standard subcellular fractionation technique for each individual tissue. Heart was generally conventional, but brain required modification likely due to the high content of synaptic/myelin membranes. With the standard procedure we obtained a rather impure fraction with few free mitochondria and so we required detergent treatment to break down the contaminating membranes and release the mitochondria. We have clarified our procedures in the Methods section and have included a representative ETM image (new Fig.1) that demonstrates the purity (highly enriched in mitochondria) of the preparations analysed.

Even if data comparing mitochondrial composition between tissues can be interesting to show to the scientific community, I did not find much in the way of novel results on a scientific question(s) concerning the role and importance of mitochondrial membrane for mitochondrial functions during periods of high oxidative stress such as rapid growth and aging (as stated by the authors in the abstract and the introduction). In addition, this paper appears to be incremental to the paper recently published by the same authors, in the same journal, only showing detailed compositions of mitochondria within two other tissues : "Changes in tissue and mitochondrial membrane composition during rapid growth, maturation and aging in rainbow trout, Oncorhynchus mykiss" – CBP Part B, vol 161, pp 404-412 (2012).

The reviewer suggests the paper is "incremental" to our recent publication. Do they suggest this as a criticism? A more positive description, and the one we argue, is that this paper is complementary to the previous work, advances our understanding and further tests our overall hypothesis as stated above. In any case, the vast majority of scientific papers represent incremental advances. The important word is NOT "incremental" it is "advances" and the present study does advance our hypothesis significantly. This study represents a part of a wider project focused on the characterisation of mitochondrial processes related with ageing in fish. As a first step, it is important to study the changes taking place in mitochondrial membrane lipids during normal ageing.

Ageing is related with impairment of cellular bioenergetics function and high oxidative stress and progressive, cumulative and irreversible damage to all mitochondrial molecules with age has been shown. This damage could be lead by lipid peroxidation as unsaturated fatty acids constituting mitochondrial membrane are the first target of reactive species. By studying different tissues we can have a wider picture of the ageing process and study how mitochondrial membrane composition evolves in a tissue-dependent way so we can focus where the main changes are taking place.

Please find below some other comments/suggestions:

Abstract:
- Line 35: "Heart mitochondria had lower proportions of cardiolipin (CL), phosphatidylserine (PS) and phosphatidylinositol, and higher levels of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) than brain mitochondria" – in table 3, we can see that Heart mitochondria have higher proportions of cardiolipin (CL) than brain mitochondria. PE is higher in brain. For PI, differences are not obvious.

These lines referred to changes in heart mitochondria with age instead of differences between tissues. We have rewritten them in order to clarify.

- Line 38: "Heart mitochondrial membranes became more unsaturated with age, with an increase in n-3 polyunsaturated fatty acids and peroxidation index in the main phospholipid classes (PC, PE, CL and PS)": Not obvious for PIn in PC.

The line has been revised.

Results:
In table 5: authors interestingly specified the presence of DMA in PE. Unfortunately, nothing is said in results, nor discussed in the discussion on levels of DMA between tissues as well as between 1, 2 and 4 years old trout while levels of DMA clearly changed with age (from 7.4% to 14.3% in brain) and show lower levels in heart compare to brain.

See above, we now include discussion of DMA in the Results and Discussion.

Materials and methods:
One concern on the Material and Methods is about phospholipid fatty acid composition. It is specified that the transmethylation was conducted on each phospholipid class by adding sulphuric acid in methanol at 50°C. No time duration of the reaction is specified and if duration was the same for all phospholipid classes. This can be a major concern when considering sphingomyelin due to the relative difficulty to split the amide bonds between the fatty acids and the sphingosine-type bases.

Sorry, the duration of the transmethylation reaction has now been included. Transmethylation with methanolic sulphuric acid is the most robust and consistent method and has been the Standard Operating Procedure in our laboratory for over 25 years. The method requires incubation at 50ºC overnight (16h). This efficiently transmethylates every phospholipid class, including sphingomyelin.

Table and Figure:
- Table 3: - Specify weight % of total lipids. How authors did measured phospholipid contents? Nothing is specified in the Material and Methods section.

Units of phospholipid content and class composition are now clarified in legend to new Fig.2. Phospholipid content was content and compositions were both determined by using high-resolution TLC (HPTLC) followed by quantitative densitometry (Section 2.3). The phospholipid content is simply the sum of all the individual phospholipid classes. This is described in the Materials and Methods.

- Several problems of formatting in Tables 4, 5, 6, 7, 8, 9 should be checked. Table 9 it is written 14:00 for 14:0, same for 16:0, 18:0 and 22:0.

Tables have been revised and corrected.

Reviewer 2:

This is a good and interesting study on the modifications of the fatty acid composition of heart and brain mitochondria in rainbow trout over time. The study was implemented very well and the topic is quite innovative and clearly highly interesting. Additionally the presentation is very elegant (in terms of both: the logical structure of the MS and text readability. Some modifications are suggested:
My primary concern is for the definition of "rapid growth". I do not believe this study was strutted in a way to assess the effects of "rapid growth" and should be much more simply and appropriately refer to effects of "age". By simply rewording all sentences in which "rapid growth" was reported with "age" or "aging", etc.. the problem is solved. My concern is that the actual period of rapid growth in rainbow trout is normally happening between fish of ~100g up to ~700-800g. in this size class fish are really growing quickly, but in the present experimental design this size windows was not assessed as fish of 1st, 2nd and 4th year were assessed. And given the size of these fish, the rapid growth period would have likely happened between year 2 and year 3.

We agree. We have changed the title and revised the manuscript based on this criticism. We consider the rapid growth of rainbow trout as an important period determining their life-span, but we are not considering just that phase in the present paper.

Additionally (but much less importantly), I was a bit surprised by the relatively small size of fish of the 2 year class; in fact, according to my personal experience a 2 year old trout should be much larger.

The experimental animals were stock fish maintained under the standard conditions in our own facilities. It is likely that water temperature could explain the difference between these fish and the fish the reviewer is familiar with. Water temperature in our facility is constant and does not increase in the summer as in, say, trout farms.

My second concern (and I think this is the major point that needs to be addressed) is relative to the statistical test used for data interpretation. Though I am not a statisticians, I believe that with this kind of data the most appropriate statistical test to be used is the two-way ANOVA, assessing for fish class x tissue. Using the two-way ANOVA you will also see if there is any interaction (greatly increasing the information achieved by this study). The use of simple ANOVA and then a t-test is a sort of incomplete two-way ANOVA. (Also, I found it very strange and unjustified that the t-test for comparing tissues was implemented only on 1 year old fish. Why not the others?.) Please take action, and run a proper two-way ANOVA.

We agree. We have performed a two-way ANOVA and included the results in all Tables and Figures.

Last major concern is about the number of tables. However, I have no sensible suggestion as these tables are important and it is not simple to find a way to reduce their number …but if you could find a way to reduce the number of tables, without negatively impacting on the quality of data presented, the MS will be improved.

We have changed Table 3 for a Figure but, like the reviewer, we could see no alternative for Tables 4-9. These fatty acid data are all essential for the paper and are already truncated compositions showing only the quantitatively most important 15 or so fatty acids (out of around 30 in total).

Other comments:
L105 Is it possible to report a sort of feeding history for the 4 year old class? Were they constantly feed the same diet? When they were 1 and 2 year old, were they feed with the exact same diet used for the other two classes of fish analysed for in this experiment? (I do not think so)

We have included further information about the rearing and feeding conditions of the fish used in this study. They were all fed the same commercial feed twice a day ad libitum. As normal for salmonid feed, the

oil content for larger (older) fish was slightly higher than that for the 1- and 2-year-old fish, but the fatty acid compositions were essentially identical.

L118 "ultra turrax" is a registered commercial name for the brand IKA. Please specify/clarify.

This has been clarified in the manuscript.

L136 (more a curiosity than a criticism): how could you quantify gravimetrically such a tiny little amount of lipid?

On average we obtained around 2 mg of lipid in the individual samples and this was quantified gravimetrically. For interest, we simply weigh small glass vials (2 ml), add the chloroform/methanol lipid extract, evaporate the solvent under oxygen free N_2 and finally dry the samples *in vacuo* overnight. We reweigh the vials to 4 or 5 decimal places as required.

L167: the commonly accepted definition of LC-PUFA is 20 or more C, and 2 or more double bonds. This definition is more similar to the, now no longer used, definition of HUFA. Basically the only difference is the inclusion or not of 20:2n-6… please revise.

We have changed this, but it doesn´t affect to our data since we only consider n-3 LC-PUFA.

L186-191: there is no mention relative to comparison between the two tissues.

This is now included.

L211 saturated fatty acid are commonly abbreviated as SFA (not SAFA)… but this is a matter of personal taste…

Agreed. We have revised the abbreviation to SFA.

L221: this is not true for the 4y group! This is a good example of why a two-way ANOVA would have been much more informative, and appropriated with this experimental design.

This is been revised.

L261-269: this section is quite difficult to follow. Please re-write trying to simplify as much as you can.

This section has been revised to clarify.

Age-related changes in mitochondrial membrane composition ~~from~~of rainbow trout~~Effects of rapid growth and maturation on lipid and fatty acid composition of heart and brain mitochondria of rainbow trout,~~ (*Oncorhynchus mykiss*) heart and brain

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Running title: Heart and brain mitochondrial membrane composition in ~~rapid growth of~~ fish

Abstract

Membrane composition, particularly of mitochondria, could be a critical factor by determining the propagation of reactions involved in mitochondrial function during periods of high oxidative stress such as rapid growth and aging. Considering that phospholipids not only contribute to the structural and physical properties of biological membranes, but also participate actively in cell signalling and apoptosis, changes affecting either class or fatty acid compositions could affect phospholipid properties and, thus, alter mitochondrial function and cell viability. In the present study, heart and brain mitochondrial membrane phospholipid compositions were analysed in rainbow trout during the four first years of life, a period characterized by rapid growth and a sustained high metabolic rate. Specifically, farmed fish of three ages (1-, 2- and 4-years) were studied, and phospholipid class compositions of heart and brain mitochondria, and fatty acid compositions of individual phospholipid classes were determined. Rainbow trout heart and brain mitochondria showed different phospholipid compositions (class and fatty acid), likely related to tissue-specific functions. Furthermore, changes in phospholipid class and fatty acid compositions with age were also tissue-dependent. Heart mitochondria had lower proportions of cardiolipin (CL), phosphatidylserine (PS) and phosphatidylinositol, and higher levels of phosphatidylcholine (PC) and phosphatidylethanolamine (PE)~~– with age than brain mitochondria~~. Heart mitochondrial membranes became more unsaturated with age, with a significative~~n~~ increase of~~in n-3 polyunsaturated fatty acids and~~ peroxidation index in ~~the main phospholipid classes (PC, PE,~~ CL~~, and~~ PS and sphingomyelin (SM)~~)~~. Therefore, heart mitochondria became more susceptible to oxidative damage with age. In contrast, brain mitochondrial PC and PS ~~PS~~ content decreased ~~with age~~in 4-year-old animals while there was an increase in the proportion~~s~~ of ~~PC and sphingomyelin~~SM. The three main phospholipid classes in brain (PC, PE and PS) showed decreased n-3 polyunsaturated fatty acids, docosahexaenoic acid and peroxidation index, which indicate a different response of brain mitochondrial lipids to rapid growth and maturation.

Keywords: Fish, Growth, Mitochondria, Phospholipid, Heart, Brain.

1. Introduction

The combination of a high growth rate and the rapid attainment of a large body size have been reported to produce several negative side-effects in animals and to have important repercussions over a species' life-span (Innes and Metcalfe 2008). These effects are linked to a sustained high metabolic activity, which has been correlated with an increased level of intracellular oxidative stress (Rollo 2002; Alonso-Alvarez *et al.* 2007), along with decreased repair machinery (Almroth *et al.* 2010). In these conditions, mitochondrial molecules have been reported to suffer increasing deterioration that eventually can lead to the impairment of cellular bioenergetics function, increased oxidative stress and attenuation of the ability to respond to stresses (Paradies *et al.* 2011). For all these reasons, mitochondria are considered the key organelle contributing to tissue deterioration during high oxidative stress situations, including rapid growth and aging.

There are different theories for how mitochondrial dysfunction develops and leads to cell and tissue malfunction. It is known that all mitochondrial molecules are affected with age and that mitochondrial gene expression, membrane fluidity and electron transport chain (ETC) complexes are progressively affected (Shigenaga *et al.* 1994; Richter 1995). Although the cause-effect relationship among the observed alterations and, thus, the chain of events leading to mitochondrial decay with age, remains unclear, it could be suggested that mitochondrial membrane lipids may be the pacemakers of such events, determining how they propagate. It has been observed in a wide number of animal species that membrane composition, metabolic rate and lifespan are linked. Increased polyunsaturation of cell membranes results in altered physical properties of bilayers, which can enhance the molecular activity of membrane proteins that, in turn, increases the metabolic activity of cells, tissues and consequently whole animals (Hulbert 2008). At the same time, membranes that have different fatty acid composition will differ dramatically in their susceptibility to oxidative damage and this can affect their lifespan (Hulbert 2005). Therefore, effects of rapid growth on metabolic rate and lifespan could be mediated by lipid composition of membranes, particularly of mitochondria.

Mitochondrial membranes have a particular lipid composition including characteristic phospholipid species in the vicinity of ETC components, which has been suggested to be related with the role of

mitochondria in oxygen consumption (Hoch 1992). Besides acylphosphoglycerols, major components of all membranes, mitochondrial membranes uniquely contain cardiolipin (CL), a key molecule for mitochondrial function, and have small quantities of sphingolipids (Paradies *et al.* 2011). Furthermore, mitochondrial membrane phospholipid composition varies among tissues (Paradies *et al.* 1992; Zabelinskii *et al.* 1999; Modi *et al.* 2008), likely contributing not only to the considerable differences in physical and chemical characteristics of different types of membrane structures, but also determining the functioning of tissue-specific cell signalling systems.

Alterations in mitochondrial lipids have been found in aged mammals and humans, pointing to a key role of mitochondrial membrane composition in several age-related diseases, especially in those affecting to terminally differentiated non-proliferative organs such as brain and heart (Castelluccio *et al.* 1994; Chicco and Sparagna 2007; Pepe 2007; Paradies *et al.* 2011; Bazan *et al.* 2011; Ledesma *et al.* 2012). In these tissues, an increase in the population of dysfunctional mitochondria has been observed (Chaudhary *et al.* 2011). This can trigger removal of damaged cells via apoptosis which would be severely detrimental in these postmitotic tissues by causing tissue degeneration or dysfunction (Trifunovic and Larsson 2008). Aged brain becomes increasingly susceptible to neurodegenerative syndromes and decline of cognitive and motor performance (Ledesma *et al.* 2012), while aged heart has a decreased ability to tolerate stress (Chaudhary *et al.* 2011).

Thus, our hypothesis is that mitochondrial membrane phospholipid composition is the pacemaker of all the processes taking place inside the mitochondria with age, and that eventually lead to mitochondrial dysfunction. The primary aim of the present study was to characterize changes in rainbow trout heart and brain membrane phospholipid with rapid growth and ~~age~~maturation, focusing on alterations to class composition and individual phospholipid fatty acid compositions that may be critical in the modulation of mitochondria function during periods of high oxidative stress. We investigated rainbow trout in their first four years of life, a time during which this species reach their adult size~~undergoes rapid growth~~. Rainbow trout is the most common freshwater fish reared in Europe and thus it is a well-known species that has been investigated previously in studies of oxidative stress and mitochondrial function (Otto and Moon 1996; Zabelinskii *et al*. 1999; Kraffe *et al*. 2007; Ostbye *et al*. 2011).

2. Materials and methods

2.1. Experimental fish and sampling

The study was performed on stock rainbow trout (*Oncorhynchus mykiss*) of three ages (1-, 2- and 4-years), all with the same genetic origin and maintained on the same rearing and feeding conditions in the freshwater aquarium facilities at the Institute of Aquaculture, University of Stirling. Fish were kept under natural photoperiod and water temperature conditions (7 ± 1.5ºC) and were sampled at the same time of the year (March 2011). Fish were fed twice a day *ad libitum* with commercial feed ~~twice a day *ad libitum* the diets~~ formulated to contain 50% protein and 19% or 22% fat for younger (1-2 years) or older (4 year-old) fish, respectively (Skretting, Northwich, UK). Feed fatty acid compositions were essentially similar (Table 1). Fish were anesthetized in 10% benzocaine, killed by a blow to the head, weight and length measured (Table 2), and heart and brain dissected. Whole hearts and brains were pooled and homogenized by blender to produce triplicate samples for biochemical analysis. In order to obtain sufficient mitochondrial material for all the required analyses, 1- and 2-year old trout samples consisted of hearts and brains pooled from 21 (3 pools of 7) and 12 (3 pools of 4) fish, respectively. Samples from 4-year old trout were hearts and brains from three individuals. Lipid extractions were performed on fresh samples of mitochondrial preparations. 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.

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2.2. Mitochondria isolation

Approximately 2 g of brain and heart homogenate were further homogenized in 8 ml ice-cold sucrose buffer (0.4M phosphate buffer pH 7.4, 0.25M sucrose, 0.15M KCl, 40mM KF and 1mM N-acetyl-cysteine) using an ~~IKA® T25 digital Ultra-Turrax®~~ tissue disrupter (IKA® T25 digital Ultra-Turrax® Fisher Scientific, Loughborough, U.K.). Sucrose buffer homogenates were centrifuged at 600 x *g* for 6 min and the pellet discarded (cell/nuclei debris). Supernatants were then centrifuged at 6,800 x *g* for 10 min. This procedure was sufficient to isolate mitochondria from trout heart but, for brain, further treatment was necessary in order to disrupt synaptosomal membranes and obtain the maximum~~al~~ yield of ~~total~~ mitochondria~~(adapted from Kudin *et al.* 2004)~~. With that purpose, r~~R~~esulting brain pellets were resuspended in 8 ml ice-cold sucrose containing 0.02% (w/v) digitonin and placed on ice for 10 min (adapted from Kudin *et al.* 2004). Suspensions were centrifuged at 600 x *g* for 6 min, the pellet discarded and supernatants centrifuged at 6,800 x *g* for 10 min. The resulting pellet constituted the brain mitochondrial fraction used for lipid extraction. To verify that pellets were highly enriched with mitochondria, a portion was fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer overnight at 4ºC, and then processed as specified by Rajapakse *et al.* (2001) prior to analysis by transmission electron microscopy (Tecnai^{TM} G^2 Spirit BioTWIN, FEI Europe, Eindhoven, The Netherlands) (Fig.~~ure~~ 1).

2.3. Lipid extraction and phospholipid class composition

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

Phospholipid classes were separated by high-performance thin-layer chromatography (HPTLC) using 10 x 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 ºC 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 1994). Scanned images were recorded automatically and analyzed by computer using winCATS (Planar Chromatography Manager, version 1.2.0).

2.4.Phospholipid fatty acid composition

Individual phospholipid classes of heart and brain mitochondria were separated by preparative-TLC, using silica gel plates (20 x 20 cm) (VWR) and the solvent system as above. Individual phospholipid bands 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 50ºC overnight following addition of 2 ml of 1% (v/v) sulphuric acid in methanol in order to obtain the fatty acid methyl esters (FAME) (Christie 2003). Similarly, FAME were produced by acid-catalyzed transmethylation of samples of total lipid from feeds. FAME were separated and quantified by gas-liquid chromatography (Carlo Erba Vega 8160, Milan, Italy) using a 30 m x 0.32 mm i.d. capillary column (CP Wax 52CB, Chrompak, London, U.K.) and on-column injection at 50 ºC. Hydrogen was used as carrier gas and temperature programming was from 50 ºC to 150 ºC at 40 ºC min^{-1} and then to 230 ºC at 2.0 ºC min^{-1}. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman 1980; Tocher and Harvie 1988). Data were collected and processed using Chromcard for Windows (version 1.19).

2.5. *Indexes and statistical analysis*

Condition factor (K) was calculated using the formula: K = [weight/(length)^3] x 100. For peroxidation index (PIn) the formula was: PIn = 0.025 × (% monoenoics) +1 × (% dienoics) + 2 × (% trienoics) + 4 × (% tetraenoics) + 6 × (% pentaenoics) + 8 × (% hexaenoics) (Witting and Horwitt 1964). The LC-PUFA index corresponded to the sum of long-chain polyunsaturated fatty acids (LC-PUFA, fatty acids with 20 or more carbons and 2~~3~~ or more double bonds). Results are presented as mean ± SD (n = 3). Data were checked for homogeneity of variances by the Levene´s test and, where necessary, arc-sin transformed before further statistical analysis. -A two-way analysis of variance (ANOVA) was used to assess the differences among groups based on tissue and age. Post hoc comparisons were made using the Bonferroni t-test for multiple comparisons. ~~One-way ANOVA was performed to determine statistical significance of differences between age groups for each fatty acid, group of fatty acids or index, and Tukey´s post hoc test was used for multiple comparisons when pertinent. Finally, a t-student test was used for comparisons between heart and brain mitochondria of 1-year old rainbow trout.~~ All statistical analyses were performed using SPSS Statistical Software System version 15.0 (SPSS Inc, Chicago, USA). Differences were regarded as significant when $P < 0.05$ (Zar 1999).

3. Results

3.1. *Phospholipid class composition of heart and brain mitochondria*

~~Figure 2Table 3 shows~~The phospholipid class compositions of heart and brain mitochondria from 1-, 2- and 4-year old rainbow trout are shown in Fig.2. In both tissues, phosphatidylethanolamine (PE) constituted the main phospholipid class representing 37.4 and 42.9% of total phospholipids of 1-year old trout heart and brain, respectively. The second phospholipid in abundance was phosphatidylcholine (PC) which represented around 30% of total phospholipids. Thus, the sum of PE and PC constituted more than 65% of total in both heart and brain mitochondria. The next phospholipid in heart was cardiolipin (CL, 12.3%) followed by phosphatidylserine (PS, 7.4%),

phosphatidylinositol (PI, 6.9%) and sphingomyelin (SM, 4.8%), whereas in brain, the third phospholipid in abundance was PS (12.0%) followed by PI (5.9%), CL (4.4%) and SM (0.9%).

Several changes with age were found in mitochondrial phospholipid class composition from trout heart. The proportions of total phospholipid increased significantly (Fig.~~ure~~ 2~~Table 3~~), with the percentages of PC and PE significantly increased while those of CL, PS and PI decreased~~ (Table 3)~~. Most of the observed changes took place between 2- and 4-year old animals. Some differences with age were also found in brain mitochondrial phospholipids, with decreased proportions of PC and PS, and an increased percentage of SM. Changes in PS and SM occurred mainly between 2- and 4-year old trout.

Most of the differences ~~found~~ between~~ both~~ tissues in 1-year old animals were maintained in the ~~different~~older age groups~~ (Figure 2),~~ with heart having higher levels of SM, PC and CL and brain having higher levels of PS and PE (except~~ing for~~ 4-year-old~~ trout~~). The effect of age on mitochondrial phospholipid composition was tissue-dependent ~~of tissue~~ for all ~~PL~~ classes except~~ing~~ PS (Fig.~~ure~~ 2).

3.2. *Fatty acid compositions of individual phospholipids* of *heart and brain mitochondria*

Fatty acid compositions of individual phospholipid classes from heart and brain of 1-, 2- and 4- year old rainbow trout are presented in Tables ~~4~~3-~~8~~9. Each individual phospholipid class showed a distinctive composition. PC was characterized by high levels of palmitic acid (16:0), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Table 3~~4~~), PE had high levels of EPA and,~~ and~~ particularly ~~of~~ DHA, and ~~it~~ was characterised by ~~having~~showing dimethyl acetal (DMA) derivatives (Table ~~4~~5), CL had high levels of 16:0 and linoleic acid (18:2n-6) (Table 5~~6~~), PS contained high stearic acid (18:0) and DHA (Table 6~~7~~), PI was characterized by high levels of 18:0 and arachidonic acid (ARA, 20:4n-6) (Table 7~~8~~), and SM had a high proportion of 16:0 and nervonic acid (24:1n-9) (Table 8~~9~~).

Several differences were found between brain and heart when 1-year old animals were compared. Brain had higher percentages of monounsaturated fatty acids (MUFA) in most phospholipid classes. Nervonic acid was generally higher in brain phospholipids although it was significantly lower in brain

SM (8.2 vs. 31.9%) (Table 89). Brain total n-6 polyunsaturated fatty acids (PUFA) were lower than in heart phospholipids, generally with lower percentages of 18:2n-6 and ARA. Moreover, DHA, total n-3 PUFA and peroxidation index (PIn) were lower in brain PC, PE and CL when compared with heart. Brain PS was the main exception to these differences since it contained higher level of DHA (48.3 vs. 37.0%), n-3 PUFA (53.7 vs. 41.9) and PIn (422.1 vs. 335.7) than heart (Table 67). Brain PE contained higher levels of DMA derivatives than hHeart (7.4 vs. 4.3) (Table 4).

These differences in PC, PE and CL compositions in 1-year old fish were maintained throughout differentin the older age groups (Tables 3-5) for PC, PE and CL (Tables 3-5) but several changesdifferences were found forin the remainingother phospholipidPL classes (Tables 3-5). For instance, in 4-year-old trout, bBrain and heart PS DHA and PIn were not statistically differentsimilar to those from heart in 4-year-old trout, andwhereas PS n-3 PUFA levels were became lower in brain than in heart (Table 6). Also in 4-year-old fish, bBrain and heart PI had similar monounsaturatedMUFA and DHA values than heart PI for 4-year-old animals, andbut the total n-3 PUFA and PIn were significantly higher in brain compared to heart (Table 7). Finally in 2- and 4-year-old animals, Regarding brain SM, nervonic acid levels were not statisticallysimilar different to that from in brain and heartheart of 2- and 4-year-old trout and, in 4-year-old fish, , monounsaturated fatty acidMUFA level becamewere higher and total n-3 PUFA and PIn became lower in brain compared to heart (Table 8).

The fatty acid composition of individual phospholipid classes from rainbow trout heart and brain mitochondria showed significant changes with age. In heart mitochondria, there was a decrease in the proportions of total saturated fatty acids (SAFASFA) in CL, PS, PI and SM (Tables 56-89). Total n-6 PUFA decreased in the two major phospholipids (PC, and PE) and in SM (Tables 3,4 and 4 and 85). The percentage of DHA in CL and mainly SM DHA increased between 2- and 4-year-old troutin CL and SM, and PIn increased in almost every phospholipid class (not significanttive infor PE and PI). In brain mitochondria, there was a general decrease in SAFASFA and an increase in MUFA. Total n-6 PUFA increased in PC, PE and PI and n-3 PUFA decreased. The PIn decreased in the three major phospholipids in brain (PC, PE and PS). PI changed in a different way to the other brain phospholipid classes, as MUFA, n-3 PUFA and PIn significantly increased with age (Table 78).

Most of the observed changes with age were tissue-dependent, with PI ~~being the PL class with~~showing ~~a~~ lower interaction between age and tissue compared to other phospholipid classes (Tables 3-8).

4. Discussion

Rainbow trout heart and brain mitochondria showed a different phospholipid composition. Although mitochondrial membranes contained similar percentages of total phospholipid, they presented different phospholipid proportions ~~for~~in each tissue. ~~When~~In 1-year-old animal~~s were compared~~, heart was found to be richer in PC, CL and SM, while brain had higher levels of PE and PS, similar to that observed in rats (Paradies *et al.* 1992; Modi *et al.* 2008). In a previous study, data on lipid compositions of liver mitochondria of rainbow trout were presented (Almaida-Pagán *et al.* 2012). Liver also showed a different phospholipid composition characterized by higher levels of total phospholipid, PC and PI compared with heart and brain mitochondria. Therefore, these data show a tissue-specific distribution~~s~~ of phospholipid classes in trout mitochondrial membranes that would be likely related to the particular tissue-specific properties and functions of the membranes.

The phospholipid class composition of the mitochondrial membranes changed with age in both heart and brain, although not in the same way. Heart mitochondria showed a significant decrease in PI, CL and PS, and an increase in PC and PE, while brain mitochondria had higher SM and lower PC and PS with age. The different effects on mitochondrial membrane composition, also observed in rats (Modi *et al.* 2008), may relate to differential responses of the two tissues to a rapid growth period~~s~~ and maturation. For instance, changes in the proportions of individual phospholipid classes may lead to altered charge distribution across the membrane, membrane permeability properties, catalytic activities of specific enzymes and ETC function (Daum 1985). Especially interesting was the CL loss observed in heart, which was also reported in rats (Paradies *et al.* 1992; Lee *et al.* 2006). CL is considered a key molecule for mitochondrial viability (Paradies *et al.* 2011) whose proximity to the ETC and high content of PUFA make it highly susceptible to oxidative damage. Changes in CL content with age

have been related to mitochondrial dysfunction by promoting the apoptosis cascade (Chicco and Sparagna 2007). ~~PS and SM are also interesting as they both are considered as important mediators of mitochondrial pathways and are also related with apoptosis (Hannun and Obeid 1997; Cutler and Mattson 2001; Ulmann *et al.* 2001; Mozzi *et al.* 2003).~~

In addition, ~~fatty acid composition of mitochondrial membrane phospholipid also differed between brain and heart mitochondria. Brain phospholipid classes generally contained higher percentages of SAFA and MUFA, and lower levels of n-6 PUFA, DHA and peroxidation susceptibility (PIn). PS was an exception in this comparison, showing lower SAFA and higher DHA and PIn than heart. Moreover, mitochondrial phospholipid fatty acid composition of both tissues was affected by age although again in different directions. In heart, mitochondrial membranes of older fish had lower SAFA and n-6 PUFA in PC and PE, and higher n-3 PUFA in the main phospholipid classes (PC, PE, CL and PS) with a significant increase in PIn in most phospholipids. Similar to heart, brain mitochondrial membranes in older fish had lower levels of SAFA, although they showed a general increase in MUFA with significantly higher levels of 24:1, an increase in n-6 PUFA in PC, PE and PI, and decreased n-3 PUFA, DHA and PIn in PC, PE and PS. Brain PI changed in a different way to the other phospholipid classes, showing a decrease in MUFA and 24:1, and an increase in DHA, n-3 PUFA and PIn with age. Therefore,~~ heart and brain mitochondrial membranes showed different phospholipid compositions that evolved in different ways during the first four years of rainbow trout life-cycle. Heart phospholipids became more unsaturated with age, which would render them more susceptible to peroxidation and, in turn, may promote their degradation and hydrolysis. Castelluccio *et al.* (1994) showed that rat heart mitochondrial membranes were significantly modified during the aging process, showing an increase in PUFA up to 12 months of age, followed by a subsequent decrease. Specially marked was the increase in DHA with age in heart SM ~~DHA with age (from 3.9 to 20.0%)~~ with a~~the~~ con~~sub~~sequent increase in ~~the molecule~~ susceptibility to ~~be~~ oxidation~~ized~~. This wa~~i~~s interesting ~~since~~as SM is known to retard the lateral propagation of free radicals through the membrane and to be an important mediator of mitochondrial pathways including apoptosis (Hannun and Obeid 1997; Cutler and Mattson 2001).

Another interesting result ~~was that concerning~~related to dimethyl acetals (DMA) ~~derivatives related with PE plasmalogens in tissue mitochondria. DMA derivatives are~~obtained ~~as artefacts of~~ from methylation of PE plasmalogen, ~~methylation~~ and so can be considered as indicators of plasmalogen

content. Plasmalogens are rich in some tissues such as heart and brain and have been considered as endogenous antioxidants~~ defences~~ (Brosche and Platt 1998) and ~~. These defences~~ have been shown to decrease with normal ageing in mammals tissues as we found in trout heart mitochondria in the present study.~~ (4.3 to 2.4%).~~

~~Salmonid heart shows specific characteristics. A significant adaptation for high active aerobic metabolism in adult salmonids, based on the expansion of energy production and an increased myocardial power output, has been proposed (Clark and Farrell 2011). Trout heart grows in the adult stage by a combination of myocite hypertrophy and hyperplasia (Farrell *et al.* 1988). Besides, oxidative potential of trout ventricle appears to be positively scaled with animal size, reflecting a metabolic adaptation for increased oxidation of fatty acids and ventricular performance in larger animals (Rodnick and Williams 1999). That increased oxidative capacity in older animals must be supported by a suitable composition of the mitochondrial membrane where the maintenance of unsaturation, in spite of an increased susceptibility to oxidation, ensures the function.~~

The changes observed in brain ~~appear to~~ indicate ~~a significantly~~ differential~~earlier~~ modification of mitochondrial membranes in that tissue compared to heart. The three main phospholipid in brain mitochondria, PE, PC and PS, showed decreased DHA and PIn, as observed previously in trout liver associated with oxidative lipid damage (Almaida-Pagán *et al.* 2012). Similar changes were reported previously in mammals (Ledesma *et al.* 2012) and fish brain (total tissue) (Mourente and Tocher 1992). Changes were more marked in older fish, which may reflect that age affects some pathways for phospholipid synthesis in the central nervous system and indicate the presence of compensatory mechanisms to provide a pool of phospholipid for the maintenance of cellular membrane lipid composition and/or functions during maturation and aging (Ilincheta de Bosquero *et al.* 2000). One of the most affected phospholipids in brain mitochondrial m~~e~~em~~ne~~brane was PS which is known to be very important for nervous tissue function and has been associated with age-related decay and disease (Ulmann *et al.* 2001; Mozzi *et al.* 2003). ~~Brain~~ DMA levels were ~~PE plasmalogen, significantly~~ higher in brain mitochondrial preparations than in heart mitochondria, but this ~~were found to increase with age.~~probably reflects PE plasmalogen content in myelin fragments associated with the brain preparation rather than brain mitochondria.

In any case, both heart and brain showed changes with age that affected mitochondrial membrane phospholipid compositions. Membrane composition determines the bilayer physical properties which affect membrane protein activity (Hulbert 2008). More polyunsaturated membrane lipids are correlated with faster turnover rates of individual mitochondrial membrane proteins (Hubert *et al.* 2006). The observed changes in mitochondrial phospholipid would affect membrane reactions, which constitute a quantitatively important component of cellular metabolism. Moreover, individual phospholipids participate actively in cell signalling including apoptosis. An alteration of either phospholipid proportions or individual phospholipid fatty acid compositions in mitochondrial membrane could affect organelle function and thus, cell and tissue viability.

Rainbow trout experience a rapid increase in body size during its early years. Rapid growth has been related with an increase in ROS production by mitochondria, and a diversion of resources into anabolism and away from repairing oxidative damage to cell molecules (Almroth *et al.* 2010). Considering membrane and membrane components as possible pacemakers of the main processes taking place in mitochondria (Hulbert 2007; 2008), phospholipid and fatty acid changes could play a central role by connecting the different processes involved in cumulative damage to cell molecules and dysfunction during periods of high stress. It is conceivable that the primary cumulative damage is to mitochondrial lipids, altering membrane fluidity and ultimately causing defects in ETC and respiration; as a result, the generation of ROS may be accelerated. Eventually, defence mechanisms and repair systems are overwhelmed and damage to mitochondrial DNA becomes permanent. Therefore, by achieving its mature size rainbow trout could be initiating its way into senescence.

In summary, the present study showed differences in mitochondrial membrane composition (phospholipid class and fatty acid compositions) among rainbow trout tissues that points to the importance of particular phospholipids for tissue-specific functions. Significant changes in heart and brain mitochondrial membranes during the first four years of life in trout were observed. Brain mitochondria had lower levels of DHA and PIn in the major~~in~~ phospholipids while heart phospholipids became more unsaturated~~ and thus~~, generally ~~this being related~~associated with ~~a~~ higher~~more~~ fluidity, but also with ~~a~~ higher susceptibility ~~more prone~~ to ~~be~~ damage~~d~~ by high oxidative stress. Considering the importance of phospholipid fatty acid composition and the role of specific phospholipid in mitochondrial function and cell viability, these changes could affect ETC efficiency,

ROS production and signalling systems, and be mediators of the processes involved in response to rapid growth and aging in trout.

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Figure legends

Fig.~~ure~~ 1. Transmission electron micrograph showing a representative preparation of rainbow trout ~~tissue~~heart mitochondria. Bar= 2 µm.

Fig.~~ure~~ 2. Phospholipid content (percentage of weight of total lipid) and phospholipid class composition (percentage of total phospholipids) of mitochondria isolated from heart and ~~-~~brain of 1-, 2- and 4-year old rainbow trout. Results are means ± S.D. (n=3). Different superscript letters represent differences between age groups for each ~~PL~~phospholipid class as determined by two-way ANOVA (P<0.05). Table represents *P* values for interaction t~~T~~issue and a~~A~~ge for e~~very~~ach phospholipid class (P<0.05). Asterisks denote statistical differences between tissues for e~~very~~ach age group when compared using a Bonferroni test (P<0.05). PL, phospholipid; CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; ∑PL, total phospho~~olar~~ lipids; PS, phosphatidylserine; SM, sphingomyelin.

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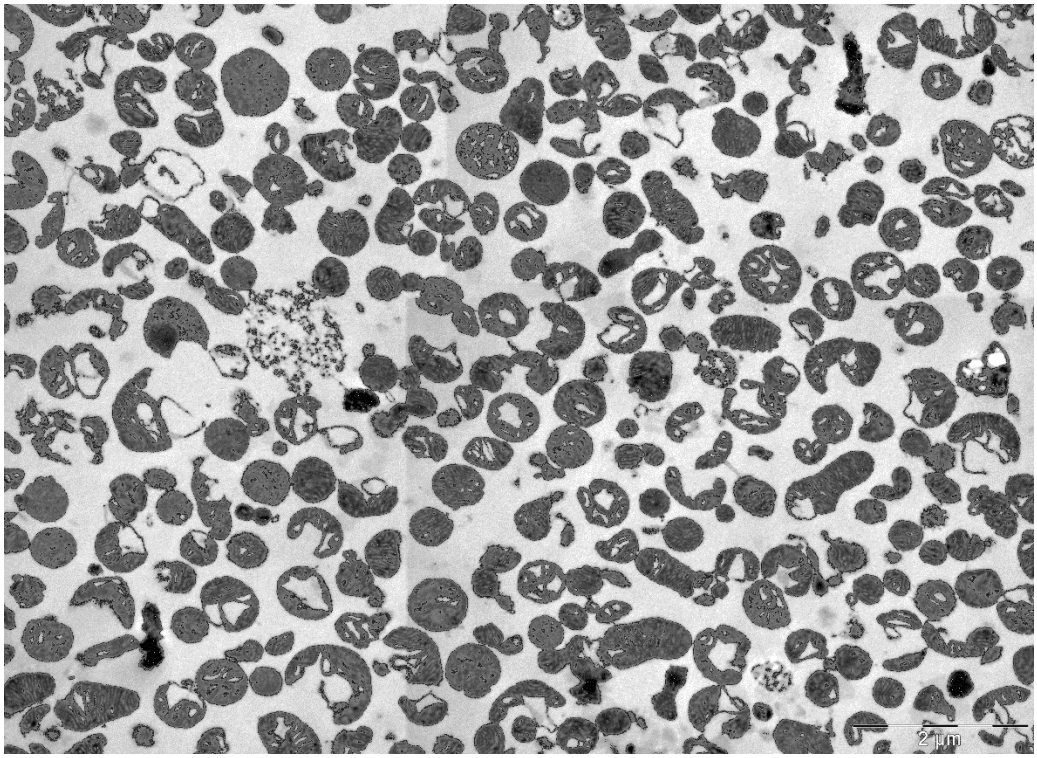
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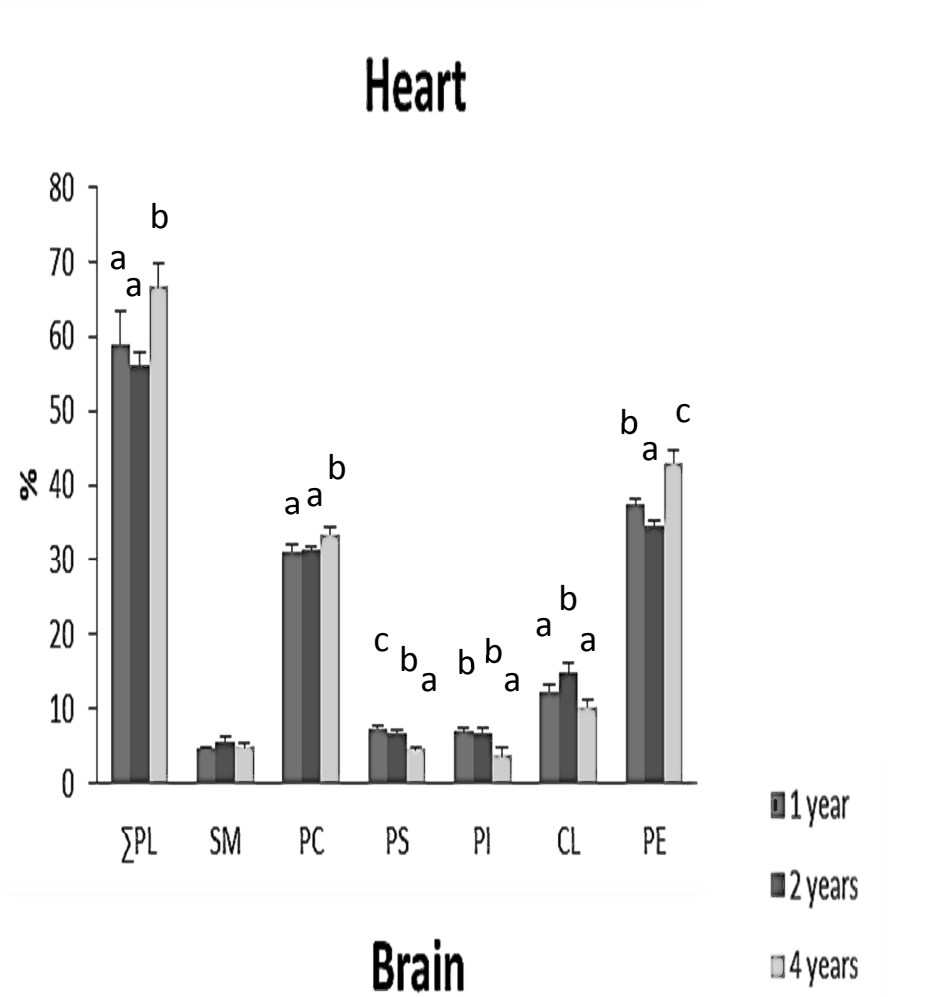

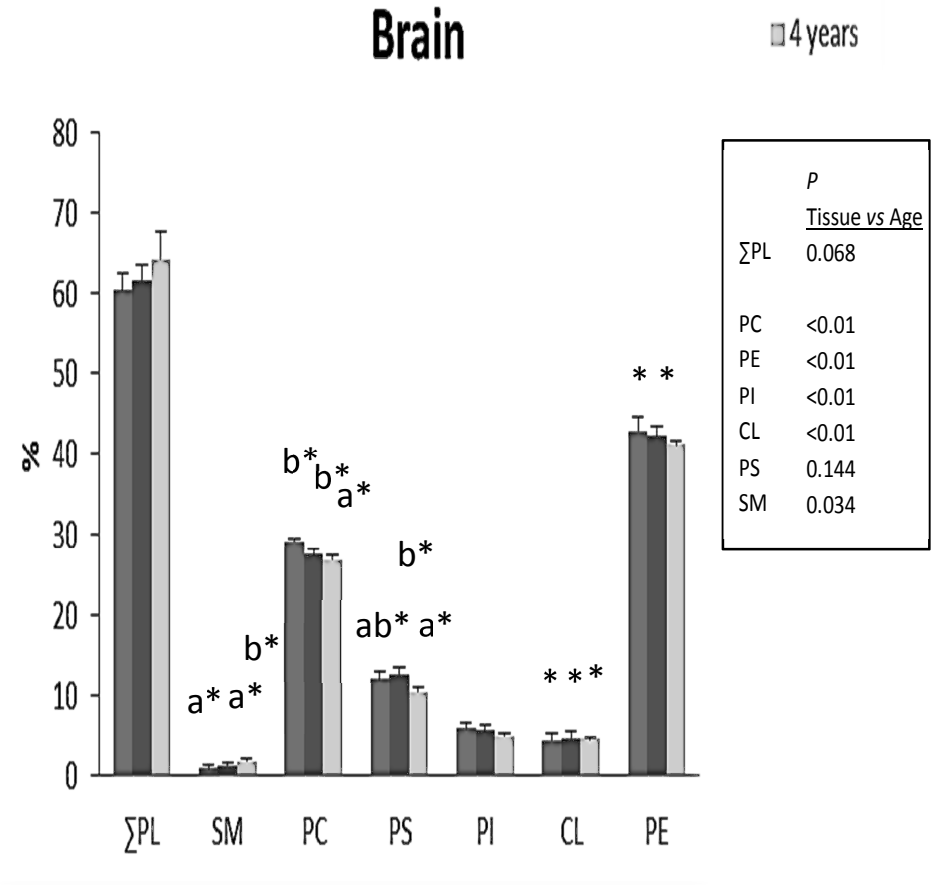

	P
	Tissue *vs* Age
∑PL	0.068
PC	<0.01
PE	<0.01
PI	<0.01
CL	<0.01
PS	0.144
SM	0.034

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Table 1. Fatty acid composition (percentage of total fatty acids) of 1-2 and 4 year-old rainbow trout diets.

Fatty acid	Feeds	
	1-2	4
14:0	6.9	7.6
16:0	19.0	19.0
18:0	3.7	5.4
∑saturated[a]	30.5	33.4
16:1n-7	8.0	8.2
18:1n-7	3.1	3.5
18:1n-9	10.7	8.9
24:1n-9	0.9	0.6
∑monounsaturated[b]	25.8	23.3
18:2n-6	6.6	4.3
20:4n-6	0.9	1.0
∑n-6 PUFA[c]	8.4	6.1
18:3n-3	0.9	1.0
18:4n-3	2.2	2.3
20:4n-3	0.6	0.6
20:5n-3	15.3	16.3
22:5n-3	1.9	2.0
22:6n-3	9.9	9.6
∑n-3 PUFA[d]	30.8	31.9
∑n-3 LC-PUFA	27.7	28.6

LC-PUFA, long-chain PUFA; PUFA, polyunsaturated fatty acids.

[a] Totals include 15:0, 20:0 and 22:0.

[b] Totals include 16:1n-9, 20:1n-9, 20:1n-7, 22:1n-9 and 22:1n-9.

[c] Totals include 18:3n-6, 20:2n-6, 20:3n-6. 22:4n-6 and 22:5n-6.

[d] Totals include 20:3n-3 and 22:4n-3.

Table 2. Biometric data of rainbow trout age groups.

	Age groups		
	1 year (n=21)	2 years (n=12)	4 years (n=3)
Weight (g)	37.9±12.9	115.3±39.6	2986.3±135.9
Length (cm)	14.3±1.9	20.6±2.4	60.0±5.0
K	1.3±0.2	1.3±0.5	1.4±0.3

Data expressed as mean ± SD. n, number of individuals; K, condition factor.

Table 3. Fatty acid composition (percentage of total fatty acids) of phosphatidylcholine of mitochondria isolated from heart and brain of 1-, 2- and 4-year-old rainbow trout.

	Heart			Brain			*P*
Fatty acid	1 year	2 years	4 years	1 year	2 years	4 years	Tissue*Age
14:0	3.1±0.2^{b}	2.6±0.6^{ab}	2.1±0.3^{a}	0.6±0.1*	0.5±0.1*	0.6±0.0*	0.037
16:0	31.2±0.2	31.0±0.9	30.8±1.2	28.4±1.6^{b*}	27.3±1.5^{b*}	24.1±0.3^{a*}	0.014
18:0	2.4±0.2	2.6±0.2	2.8±0.4	3.3±0.4^{a*}	4.0±0.2^{b*}	3.9±0.2^{b*}	0.232
∑saturated^{a}	37.2±0.3	36.6±1.1	36.1±0.7	32.7±1.3^{b*}	32.1±1.5^{b*}	28.8±0.4^{a*}	0.043
16:1n-7	2.8±0.1^{b}	2.2±0.4^{a}	2.0±0.0^{a}	9.0±1.4*	8.5±0.9*	7.5±1.5*	0.677
18:1n-7	1.6±0.1^{a}	1.4±0.2^{a}	2.0±0.2^{b}	4.9±1.2*	5.4±1.2*	5.3±1.5*	0.660
18:1n-9	8.2±0.4^{b}	6.3±0.5^{a}	6.9±0.7^{a}	13.9±1.9^{a*}	17.7±1.8^{b*}	20.9±1.0^{b*}	<0.01
20:1n-9	0.2±0.0	0.2±0.1	0.3±0.1	0.6±0.2^{a*}	0.8±0.2^{a*}	1.2±0.1^{b*}	0.015
24:1n-9	0.4±0.2	0.4±0.1	0.4±0.1	7.4±0.3^{a*}	7.3±0.6^{a*}	9.0±0.2^{b*}	<0.01
∑monounsaturated^{b}	13.1±0.7^{b}	10.6±1.0^{a}	11.8±1.1^{a,b}	36.1±2.5^{a*}	40.2±2.7^{a*}	44.8±0.9^{b*}	<0.01
18:2n-6	0.7±0.0	0.5±0.0	0.7±0.0	0.2±0.0*	0.1±0.0*	0.2±0.0*	0.507
20:4n-6	1.8±0.1^{b}	2.6±0.2^{c}	1.5±0.1^{a}	0.5±0.0^{a*}	0.6±0.0^{a*}	1.0±0.2^{b*}	<0.01
∑n-6 PUFA^{c}	3.4±0.2^{b}	4.1±0.2^{c}	2.9±0.1^{a}	1.1±0.2^{ab}	1.0±0.1^{a}	1.5±0.4^{b}	<0.01
20:5n-3	12.1±0.5	13.6±0.5	12.9±1.4	4.3±0.5*	3.8±0.7*	3.8±0.6*	0.051
22:5n-3	1.5±0.1^{a}	1.7±0.2^{a}	3.4±0.3^{b}	1.3±0.2	1.1±0.2*	1.2±0.1*	<0.01
22:6n-3	31.5±0.3	32.5±1.0	32.0±0.9	24.2±2.5^{b*}	21.6±1.3^{ab*}	19.5±1.4^{a*}	<0.01
∑n-3 PUFA^{d}	45.8±0.6^{a}	48.4±1.5^{b}	49.0±1.0^{b}	29.9±1.9^{b*}	26.6±1.2^{a*}	24.7±0.6^{a*}	<0.01
∑PUFA	49.6±0.8^{a}	52.9±1.8^{b}	52.1±0.9^{b}	31.2±1.9^{b*}	27.8±1.3^{a*}	26.4±0.5^{a*}	<0.01
∑n-3 LC-PUFA	45.4±0.6^{a}	48.0±1.5^{b}	48.7±0.9^{b}	29.9±1.9^{b*}	26.6±1.2^{a*}	24.7±0.6^{a*}	<0.01
n-3/n-6	13.4±0.7^{b}	11.8±0.5^{a}	17.2±0.8^{c}	27.5±5.1^{b*}	26.6±2.5^{b*}	16.8±4.0^{a*}	<0.01
PIn	349.5±4.7^{a}	370.0±12.1^{b}	365.9±6.4^{ab}	233.3±16.5^{b*}	207.8±9.5^{a*}	193.5±6.3^{a*}	<0.01

Data expressed as mean ± S.D. (n = 3). Different superscript letters within a row and for each sample type (heart or brain) represent significant differences between age groups as determined by two-way ANOVA (P<0.05). Asterisks denote statistical differences between tissues for 1-, 2- or 4-year-old trout when compared using a Bonferroni test (P<0.05). Right column represents signification values for the interaction between Tissue and Age (P<0.05). LC-PUFA, long-chain polyunsaturated fatty acids; PIn, peroxidation index; PUFA, polyunsaturated fatty acids.

[a] Totals include 15:0, 20:0 and 22:0 present up to 0.6%.

[b] Totals include 20:1n-7, 22:1n-11 and 22:1n-9 present up to 0.6%.
[c] Totals include 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6 and 22:5n-6 present up to 0.7%.
[d] Totals include 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3 and 22:4n-3 present up to 0.3%.

Table 4. Fatty acid composition (percentage of total fatty acids) of phosphatidylethanolamine of mitochondria isolated from heart and brain of 1-, 2- and 4-year-old rainbow trout.

	Heart			Brain			*P*
Fatty acid	1 year	2 years	4 years	1 year	2 years	4 years	*Tissue*Age*
16:0 DMA	1.5±0.0^{b}	2.5±0.2^{c}	1.1±0.2^{a}	1.1±0.2^{a}	1.5±0.5^{ab*}	2.0±0.3^{b*}	<0.01
16:0	11.3±0.7	12.3±0.9	11.5±0.7	10.6±0.8^{b}	10.0±1.0^{b*}	7.2±0.1^{a*}	<0.01
18:0 DMA	0.4±0.1	0.3±0.1	0.1±0.1	2.9±0.6*	3.6±1.3*	4.7±0.3*	0.049
18:0	7.2±0.2^{a}	8.2±0.5^{b}	6.9±0.3^{a}	8.1±0.4^{a}	11.2±1.2^{b*}	6.6±0.5^{a}	<0.01
∑saturated[a]	21.3±0.7^{a}	24.1±1.4^{b}	20.1±0.3^{a}	23.6±0.4^{b*}	26.8±0.6^{c*}	20.9±0.4^{a}	0.119
16:1n-7	1.1±0.1^{ab}	1.3±0.4^{b}	0.6±0.1^{a}	2.1±0.3^{a*}	1.9±0.4^{a*}	2.9±0.2^{b*}	<0.01
18:1n-9 DMA	1.8±0.1^{b}	1.7±0.1^{b}	1.0±0.3^{a}	1.7±0.3^{a}	2.5±0.7^{a*}	4.3±0.2^{b*}	<0.01
18:1n-7 DMA	0.6±0.0	0.6±0.0	0.2±0.1	1.7±0.3^{a*}	2.0±0.6^{a*}	3.3±0.5^{b*}	<0.01
18:1n-7	5.4±0.3^{b}	4.1±0.3^{a}	4.6±0.5^{a}	4.8±0.2^{b*}	3.2±0.2^{a*}	3.5±0.2^{a*}	0.353
18:1n-9	5.4±0.4^{a}	4.7±0.3^{a}	6.1±0.4^{b}	13.0±2.0*	12.7±3.6*	17.7±1.0*	0.141
20:1n-9	0.8±0.0	0.9±0.0	1.0±0.2	1.1±0.2^{a*}	1.0±0.3^{a}	1.6±0.2^{b*}	0.082
24:1n-9	0.8±0.0	0.9±0.0	1.0±0.2	0.4±0.1	0.3±0.1	0.8±0.2	0.154
∑monounsaturated[b]	15.7±1.0	14.3±1.1	14.0±1.5	25.1±3.5^{a*}	23.8±5.9^{a*}	34.6±2.3^{b*}	<0.01
18:2n-6	1.8±0.1^{c}	1.1±0.2^{a}	1.5±0.0^{b}	0.6±0.1*	0.2±0.0*	0.5±0.0*	0.016
20:4n-6	2.2±0.0	2.2±0.1	2.0±0.2	0.9±0.1^{a*}	0.9±0.1^{a*}	2.0±0.2^{b}	<0.01
∑n-6 PUFA[c]	5.7±0.2^{b}	4.8±0.4^{a}	4.7±0.3^{a}	2.0±0.3^{a*}	1.4±0.3^{a*}	3.4±0.2^{b*}	<0.01
20:5n-3	5.9±0.1^{b}	4.9±0.5^{a}	7.8±0.8^{c}	5.7±0.2^{b}	3.8±0.1^{a*}	5.5±0.4^{b*}	<0.01
22:5n-3	3.9±0.1^{a}	4.6±0.2^{b}	6.8±0.3^{c}	3.1±0.1*	2.8±0.2*	3.1±0.1*	<0.01
22:6n-3	45.8±0.7	45.8±2.0	45.2±0.5	39.1±3.4^{b*}	40.5±5.6^{b*}	31.6±2.0^{a*}	0.059
∑n-3 PUFA[d]	56.6±1.0^{a}	56.1±2.3^{a}	60.9±1.6^{b}	48.5±3.1^{b*}	47.6±5.7^{b*}	41.0±2.2^{a*}	<0.01
∑PUFA	63.0±0.9^{ab}	61.6±2.5^{a}	66.0±1.4^{b}	51.3±3.4^{b*}	49.4±5.7^{ab*}	44.6±2.2^{a*}	0.028
∑n-3 LC-PUFA	56.3±1.0^{a}	55.8±2.2^{a}	60.5±1.6^{b}	48.1±3.1^{b*}	47.3±5.7^{ab*}	40.7±2.2^{a*}	<0.01
n-3/n-6	9.8±0.4^{a}	11.7±0.7^{b}	12.9±1.1^{b}	24.7±2.1^{b*}	34.0±6.7^{b*}	12.3±1.2^{a}	<0.01

PIn	446.3±7.3	443.6±18.1	467.0±9.9	374.9±26.4[b*]	371.3±45.3[ab*]	319.2±17.0[a*]	0.023

Data expressed as mean ± S.D. (n = 3). Different superscript letters within a row and for each sample type (heart or brain) represent significant differences between age groups as determined by two-way ANOVA (P<0.05). Asterisks denote statistical differences between tissues for 1-, 2- or 4-year-old trout when compared using a Bonferroni test (P<0.05). Right column represents signification values for the interaction between Tissue and Age (P<0.05). DMA, dimethyl acetal; LC-PUFA, long-chain polyunsaturated fatty acids; PIn, peroxidation index; PUFA, polyunsaturated fatty acids.

[a] Totals include 15:0, 20:0 and 22:0 present up to 0.6%.

[b] Totals include 20:1n-7 and 22:1n-9 present up to 0.3%.

[c] Totals include 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6 and 22:5n-6 present up to 0.6%.

[d] Totals include 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3 and 22:4n-3 present up to 0.7%.

Table 5. Fatty acid composition (percentage of total fatty acids) of cardiolipin of mitochondria isolated from heart and brain of 1-, 2- and 4-year-old rainbow trout.

	Heart			Brain			*P*
Fatty acid	1 year	2 years	4 years	1 year	2 years	4 years	*Tissue*Age*
14:0	1.6±0.1[b]	3.2±0.4[c]	0.8±0.1[a]	1.7±0.5[b]	1.0±0.2[a*]	0.7±0.0[a]	<0.01
15:0	0.7±0.1	0.6±0.2	0.2±0.0	1.1±0.3[*]	0.4±0.1	0.3±0.1	<0.01
16:0	17.6±1.1[c]	9.0±0.6[a]	14.4±0.7[b]	22.8±0.1[b*]	16.9±0.7[a*]	16.3±0.1[a]	<0.01
18:0	3.9±0.2[b]	2.4±0.3[a]	2.8±0.3[a]	9.3±2.8[*]	7.1±1.1[*]	6.6±0.4[*]	0.328
∑saturated[a]	23.8±1.4[c]	15.4±0.2[a]	18.2±1.0[b]	35.5±2.4[b*]	25.6±1.7[a*]	24.1±0.4[a*]	<0.01
16:1n-7	2.6±0.1[a]	7.0±1.0[b]	1.6±0.1[a]	4.4±0.7[b*]	3.9±1.0[ab*]	2.3±0.2[a*]	<0.01
18:1n-7	6.4±0.4[b]	3.5±0.3[a]	8.7±0.1[c]	6.6±0.3	7.2±1.1[*]	8.1±0.6	<0.01
18:1n-9	10.0±1.2[a]	13.2±2.0[b]	9.6±0.9[a]	10.1±0.4[a]	18.3±2.7[b*]	17.2±0.3[b*]	<0.01
20:1n-9	0.4±0.0[a]	0.8±0.1[b]	0.7±0.1[b]	0.9±0.3[a]	1.2±0.5[ab*]	1.8±0.3[b*]	0.088
24:1n-9	0.7±0.3	1.0±0.6	0.7±0.4	3.4±1.9[*]	5.0±2.3[*]	6.4±0.6[*]	0172
∑monounsaturated[b]	20.2±1.3[a]	25.9±1.2[b]	21.8±1.4[a]	25.8±2.5[a*]	36.3±2.1[b*]	37.0±0.6[b*]	<0.01
18:2n-6	5.4±0.5	5.4±1.0	4.2±0.3	1.3±0.0[*]	0.9±0.3[*]	1.0±0.1[*]	0.104
20:4n-6	1.0±0.1[ab]	1.3±0.2[b]	1.0±0.1[a]	1.8±1.0	1.7±0.5	2.7±0.4[*]	0.029
∑n-6 PUFA[c]	8.2±0.5[*]	8.7±1.0	7.4±0.4	4.9±1.3[*]	3.6±0.3[*]	4.9±0.6[*]	<0.01
18:3n-3	1.3±0.1[a]	2.1±0.1[c]	1.6±0.1[b]	0.3±0.1[*]	0.2±0.1[*]	0.3±0.0[*]	<0.01
20:4n-3	0.8±0.0[a]	1.0±0.0[b]	1.1±0.1[b]	0.2±0.0[*]	0.3±0.0[*]	0.3±0.0[*]	0.099
20:5n-3	2.5±0.2	2.9±0.5	2.5±0.3	6.8±0.5[*]	6.8±1.7[*]	7.2±0.3[*]	0.670
22:5n-3	2.0±0.2[a]	1.6±0.1[a]	2.4±0.2[b]	2.6±0.1[*]	2.3±0.4[*]	2.5±0.1	0.096
22:6n-3	39.9±0.6[a]	40.2±2.2[a]	43.8±1.4[b]	21.9±1.6[*]	23.9±3.6[*]	22.8±0.5[*]	0.149
∑n-3 PUFA[d]	46.8±0.6[a]	48.3±2.3[a]	52.1±1.3[b]	32.7±0.9[*]	33.8±2.0[*]	33.3±0.7[*]	0.040
∑PUFA	56.0±0.3[a]	58.7±1.4[b]	60.0±0.9[b]	38.7±0.1[*]	38.1±1.9[*]	38.9±0.6*	0.028
∑n-3 LC-PUFA	45.2±0.5[a]	45.8±2.4[a]	50.1±1.3[b]	31.7±1.1[*]	33.4±2.1[*]	33.0±0.7[*]	0.049
n-3/n-6	5.7±0.4[a]	5.6±0.9[a]	7.0±0.6[b]	6.9±2.0	9.6±1.1[*]	6.9±1.0	<0.01
PIn	370.3±3.7[a]	378.3±17.2[a]	404.1±9.9[b]	253.8±3.8[*]	261.3±17.4[*]	260.5±4.2[*]	0.118

Data expressed as mean ± S.D. (n = 3). Different superscript letters within a row and for each sample type (heart or brain) represent significant differences between age groups as determined by two-way ANOVA ($P<0.05$). Asterisks denote statistical differences between tissues for 1-, 2- or 4-year-old trout when compared using a Bonferroni test ($P<0.05$). Right column represents signification values for the interaction between Tissue and Age ($P<0.05$). LC-PUFA, long-chain polyunsaturated fatty acids; PIn, peroxidation index; PUFA, polyunsaturated fatty acids.

[a] Totals include 20:0 and 22:0 present up to 0.4%.

[b] Totals include 20:1n-7, 22:1n-11 and 22:1n-9 present up to 0.7%.

[c] Totals include 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6 and 22:5n-6 present up to 0.9%.

[d] Totals include 18:4n-3, 20:3n-3 and 22:4n-3 present up to 0.7%.

Table 6. Fatty acid composition (percentage of total fatty acids) of phosphatidylserine of mitochondria isolated from heart and brain of 1-, 2- and 4-year-old rainbow trout.

	Heart			Brain			*P*
Fatty acid	1 year	2 years	4 years	1 year	2 years	4 years	*Tissue*Age*
14:0	1.4±0.6	1.1±0.4	0.7±0.1	0.5±0.1*	0.3±0.0*	0.2±0.1	0.433
16:0	16.8±2.0	14.8±2.4	13.2±0.6	10.8±2.0^{b*}	5.7±0.4^{a*}	5.4±0.1^{a*}	0.285
18:0	20.9±0.3^{b}	24.8±1.5^{c}	16.4±1.0^{a}	16.4±1.5^{a*}	20.5±0.2^{b*}	17.3±0.4^{a}	<0.01
20:0	1.0±0.0^{b}	0.7±0.1^{a}	0.6±0.0^{a}	n.d.	n.d.	n.d.	
∑saturated^{a}	41.3±2.6^{b}	42.3±3.2^{b}	31.2±1.1^{a}	28.1±3.4^{b*}	26.7±0.3^{ab*}	23.2±0.3^{a*}	0.031
16:1n-7	1.5±0.3^{ab}	1.8±0.7^{b}	0.7±0.2^{a}	2.0±0.4	1.3±0.3	1.5±0.1*	0.038
18:1n-7	4.3±0.3^{c}	2.5±0.1^{a}	3.5±0.1^{b}	2.6±0.1^{a*}	1.8±1.2^{a}	5.2±0.4^{b*}	<0.01
18:1n-9	4.3±0.8	5.1±1.2	5.1±0.5	7.1±0.4^{a*}	9.0±0.5^{b*}	13.4±0.8^{c*}	<0.01
20:1n-9	1.0±0.1^{a}	0.9±0.1^{a}	1.7±0.2^{b}	0.4±0.1^{a*}	0.4±0.1^{a*}	0.9±0.1^{b*}	0.037
24:1n-9	1.0±0.3	0.8±0.5	1.0±0.4	4.3±0.9^{a*}	5.6±0.3^{b*}	5.9±0.0^{b*}	0.017
∑monounsaturated^{b}	12.7±1.3	11.4±1.5	12.8±0.2	16.8±1.8^{a*}	18.5±0.9^{a*}	27.4±1.5^{b*}	<0.01
18:2n-6	0.7±0.1	0.9±0.5	0.8±0.1	0.3±0.0^{b}	0.1±0.1^{a*}	0.3±0.0^{b*}	0.447
20:4n-6	0.6±0.0	0.7±0.1	0.9±0.3	0.2±0.0^{a*}	0.2±0.0^{a*}	0.7±0.2^{b*}	0.135
∑n-6 PUFA^{c}	3.1±0.3	3.2±0.2	2.9±0.4	1.0±0.0*	0.5±0.3*	1.2±0.2*	0.023
20:5n-3	1.4±0.0^{a}	1.5±0.5^{a}	3.2±0.3^{b}	1.4±0.2^{a}	1.3±0.1^{a}	2.2±0.1^{b*}	0.028
22:5n-3	2.8±0.1^{a}	2.7±0.3^{a}	6.1±0.1^{b}	3.7±0.3^{a*}	4.6±0.4^{ab*}	5.1±0.3^{b*}	<0.01
22:6n-3	37.0±4.1	37.3±3.8	42.9±0.2	48.3±0.9^{b*}	47.9±0.7^{b*}	40.4±1.5^{a}	<0.01
∑n-3 PUFA^{d}	41.9±3.9^{a}	42.4±4.0^{a}	52.8±0.6^{b}	53.7±1.5^{b*}	53.9±0.8^{b*}	48.0±1.2^{a*}	<0.01
∑PUFA	46.0±3.8^{a}	46.3±3.9^{a}	56.1±1.0^{b}	55.1±1.6^{b*}	54.8±1.2^{b*}	49.4±1.2^{a*}	<0.01
∑n-3 LC-PUFA	41.2±4.0^{a}	42.0±4.1^{a}	52.4±0.5^{b}	53.5±1.4^{b*}	53.9±0.7^{b*}	48.0±1.2^{a*}	<0.01
n-3/n-6	13.7±1.9^{a}	13.4±2.0^{a}	18.3±2.4^{b}	51.4±0.1*	159.5±98.6*	39.8±8.2*	0.035

PIn	335.7±31.8[a]	340.4±32.6[a]	410.2±5.2[b]	422.1±10.8[b*]	421.7±6.3[b*]	373.4±9.9[a]	<0.01

Data expressed as mean ± S.D. (n = 3). Different superscript letters within a row and for each sample type (heart or brain) represent significant differences between age groups as determined by two-way ANOVA ($P<0.05$). Asterisks denote statistical differences between tissues for 1-, 2- or 4-year-old trout when compared using a Bonferroni test ($P<0.05$). Right column represents signification values for the interaction between Tissue and Age ($P<0.05$). LC-PUFA, long-chain polyunsaturated fatty acids; PIn, peroxidation index; PUFA, polyunsaturated fatty acids; n.d., non-detectable.

[a] Totals include 15:0 and 22:0 present up to 0.9%.

[b] Totals include 20:1n-7 and 22:1n-9 present up to 0.5%.

[c] Totals include 18:3n-6, 20:2n-6, 22:3n-6, 22:4n-6 and 22:5n-6 present up to 0.7%.[d] Totals include 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3 and 22:4n-3 present up to 0.5%.

Table 7. Fatty acid composition (percentage of total fatty acids) of phosphatidylinositol of mitochondria isolated from heart and brain of 1-, 2- and 4-year-old rainbow trout.

	Heart			Brain			*P*
Fatty acid	1 year	2 years	4 years	1 year	2 years	4 years	*Tissue*Age*
14:0	1.6±0.2[b]	1.2±0.5[a,b]	0.7±0.0[a]	1.3±0.8	0.8±0.2	0.7±0.2	0.518
16:0	15.6±0.7[b]	14.5±2.3[b]	11.1±0.5[a]	19.5±4.8[b*]	16.1±1.4[ab]	10.7±0.8[a]	0.220
18:0	24.9±1.1	25.1±2.3	24.7±0.7	17.1±1.2[a*]	23.9±1.2[b]	23.0±1.4[b]	<0.01
∑saturated[a]	43.7±1.0[b]	41.8±1.1[b]	36.9±0.5[a]	40.2±3.8	41.4±2.4	34.7±2.4	0.427
16:1n-7	2.0±0.3	1.9±1.0	0.9±0.1	5.6±1.7[b*]	1.6±0.4[a]	1.3±0.2[a]	<0.01
18:1n-7	5.0±1.7	1.4±0.8	3.5±0.2	4.1±0.7[b]	2.5±0.3[a]	2.3±0.0[a]	0.085
18:1n-9	4.7±0.4	7.6±2.5	7.5±0.6	10.4±0.5[b*]	9.2±1.5[ab]	7.0±0.1[a]	<0.01
24:1n-9	1.8±0.6	1.0±0.4	1.5±0.8	2.7±0.3[b]	1.4±0.2[a]	1.2±0.3[a]	0.142
∑monounsaturated[b]	13.9±1.0	12.7±2.9	14.8±1.1	23.8±0.9[c*]	16.0±1.6[b*]	12.9±0.1[a]	<0.01
18:2n-6	0.8±0.2	0.9±0.4	0.9±0.1	0.9±0.6	0.4±0.1[*]	0.6±0.1	0.158
20:4n-6	14.5±1.0	18.1±3.3	18.3±1.5	6.3±1.0[a*]	8.0±0.7[a*]	11.5±1.2[b*]	0.313
∑n-6 PUFA[c]	17.5±1.7	20.5±2.9	20.1±1.3	8.1±0.4[a*]	9.2±0.9[a*]	13.2±1.5[b*]	0.129
20:5n-3	4.1±0.2[a]	3.6±0.1[a]	6.2±1.1[b]	14.8±2.8[a*]	18.1±0.9[ab*]	19.0±0.6[b*]	0.040
22:5n-3	2.2±0.4[ab]	1.6±0.3[a]	2.9±0.2[b]	1.3±0.5[*]	1.6±0.2	1.8±0.3[*]	0.061
22:6n-3	15.5±4.5	17.4±1.5	17.1±0.9	9.6±1.6[a*]	12.2±1.4[ab*]	17.1±3.0[b]	0.200
∑n-3 PUFA[d]	22.9±3.8	23.6±1.4	27.5±2.0	26.8±4.8[a]	32.6±0.8[ab*]	38.4±3.0[b*]	0.152
∑PUFA	41.9±1.4[a]	45.5±4.0[ab]	48.3±1.1[b]	36.1±4.7[a*]	42.6±1.6[a]	52.3±2.4[b*]	0.029
∑n-3 LC-PUFA	22.2±3.7	22.8±1.6	27.1±1.9	26.3±5.1[a]	32.1±0.8[ab*]	38.1±3.1[b*]	0.164
n-3/n-6	1.3±0.4	1.2±0.1	1.4±0.2	3.3±0.4[*]	3.6±0.3[*]	2.9±0.5[*]	0.124
PIn	232.5±27.6	255.8±23.1	277.0±9.2	208.2±34.5[a]	255.3±9.4[a]	315.7±21.5[b*]	0.090

Data expressed as mean ± S.D. (n = 3). Different superscript letters within a row and for each sample type (heart or brain) represent significant differences between age groups as determined by two-way ANOVA ($P<0.05$). Asterisks denote statistical differences between tissues for 1-, 2- or 4-year-old trout when compared using a Bonferroni test ($P<0.05$). Right column represents signification values for the interaction between Tissue and Age ($P<0.05$). LC-PUFA, long-chain polyunsaturated fatty acids; PIn, peroxidation index; PUFA, polyunsaturated fatty acids.

[a] Totals include 15:0, 20:0 and 22:0 present up to 0.9%.

[b] Totals include 20:1n-7, 20:1n-9 and 22:1n-9 present up to 0.9%.

[c] Totals include 18:3n-6, 20:2n-6, 22:3n-6, 22:4n-6 and 22:5n-6 present up to 0.6%.

[d] Totals include 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3 and 22:4n-3 present up to 0.7%.

Table 8. Fatty acid composition (percentage of total fatty acids) of sphingomyelin of mitochondria isolated from heart and brain of 1-, 2- and 4-year-old rainbow trout.

	Heart			Brain			*P*
Fatty acid	1 year	2 years	4 years	1 year	2 years	4 years	*Tissue*Age*
14:0	9.3±1.6[b]	13.2±2.0[c]	4.6±0.5[a]	3.0±0.8[b*]	1.4±0.2[a*]	2.5±0.2[ab*]	<0.01
15:0	1.8±0.4	1.5±0.2	0.6±0.1	1.8±0.9	0.8±0.6	0.7±0.1	0.350
16:0	23.7±2.8[a]	36.1±1.9[b]	18.8±3.6[a]	33.4±1.9[c*]	28.1±1.4[b*]	20.0±1.9[a]	<0.01
18:0	7.2±1.2[b]	4.3±0.4[a]	6.5±0.7[a]	8.6±2.1	7.9±2.5[*]	6.3±0.9	0.171
22:0	1.0±0.3[b]	0.6±0.2[ab]	0.4±0.1[a]	0.4±0.3	0.1±0.0	0.2±0.1	0.762
∑saturated[a]	43.1±3.8[b]	55.9±2.7[c]	30.9±2.9[a]	47.5±4.0[c]	38.3±4.6[b*]	29.7±2.8[a]	<0.01
16:1n-7	2.9±0.8[a]	5.2±0.3[b]	2.1±0.5[a]	11.0±0.9[*]	9.6±2.5[*]	6.6±1.1[*]	0.033
18:1n-7	2.0±1.3[a]	2.0±0.2[b]	1.1±0.2[a]	2.8±0.5	8.0±3.6[*]	3.9±0.3	0.068
18:1n-9	5.2±1.2[a]	12.6±2.9[b]	8.4±3.3[ab]	14.2±1.4[*]	23.7±8.0[*]	13.6±4.8	0.511
20:1n-9	0.2±0.3	0.3±0.1	0.4±0.0	0.9±0.3[a]	1.2±0.5[ab*]	1.8±0.3[b*]	0.114
24:1n-9	31.9±3.7[b]	13.6±4.5[a]	24.0±6.8[ab]	8.2±0.5[a*]	10.8±2.3[b]	24.3±8.2[c]	<0.01
∑monounsaturated[b]	42.1±1.8[b]	34.3±1.3[a]	37.4±3.6[ab]	36.5±2.0[a*]	52.8±9.3[b*]	49.4±12.1[ab*]	<0.01
18:2n-6	0.9±0.3	1.1±0.8	1.0±0.1	1.7±0.3	0.6±0.6	1.6±0.8	0.090
18:3n-6	1.0±0.2[b]	0.4±0.1[a]	0.2±0.0[a]	0.4±0.0[b*]	0.2±0.0[a*]	0.1±0.0[a]	0.101
∑n-6 PUFA[c]	4.7±1.4[b]	3.6±0.8[b]	2.8±0.1[a]	2.8±0.3[*]	1.2±0.2[*]	3.9±1.3	0.017
18:4n-3	1.5±0.2[b]	0.6±0.1[a]	0.2±0.2[a]	n.d.	n.d.	0.5±0.1	<0.01
20:5n-3	1.4±0.9[a]	1.7±1.6[a]	6.4±0.9[b]	1.9±1.6	0.7±0.5	0.8±0.7[*]	<0.01
22:5n-3	0.6±0.5[a]	0.5±0.2[a]	1.6±0.2[b]	0.4±0.4	0.1±0.2	0.9±0.5	0.415
22:6n-3	3.9±1.3[a]	1.9±0.8[a]	20.0±1.2[b]	8.8±3.5[*]	4.7±2.5	10.7±6.2[*]	<0.01
∑n-3 PUFA[d]	7.9±3.1[a]	5.1±2.7[a]	28.4±2.0[b]	11.4±5.1	6.1±3.4	14.9±8.8[*]	0.012
∑PUFA	14.8±4.2[a]	9.8±3.4[a]	31.8±2.1[b]	16.0±4.6	8.9±5.1	20.9±9.3[*]	0.120
∑n-3 LC-PUFA	6.4±2.8[a]	4.4±2.7[a]	28.0±1.9[b]	10.7±8.8	5.6±3.0	14.3±8.7[*]	<0.01
n-3/n-6	13.4±0.7[a]	11.8±0.5[a]	17.2±0.8[b]	3.2±1.1	5.3±2.9[*]	3.7±1.0[*]	<0.01
PIn	69.3±21.7[a]	47.2±19.2[a]	219.0±14.8[b]	97.6±3.8	49.4±27.0	112.9±49.8[*]	<0.01

Data expressed as mean ± S.D. (n = 3). Different superscript letters within a row and for each sample type (heart or brain) represent significant differences between age groups as determined by two-way ANOVA ($P<0.05$). Asterisks denote statistical differences between tissues for 1-, 2- or 4-year-old trout when compared using a Bonferroni test ($P<0.05$). Right column represents signification values for the interaction between Tissue and Age ($P<0.05$). LC-PUFA, long-chain polyunsaturated fatty acids; PIn, peroxidation index; PUFA, polyunsaturated fatty acids; n.d., non-detectable.

[a] Totals include 20:0 present up to 0.2%.

[b] Totals include 20:1n-7, 22:1n-9 and 22:1n-11 present up to 0.7%.

[c] Totals include 20:2n-6, 20:3n-6, 22:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6 present up to 0.9%.

[d] Totals include 18:3n-3, 20:3n-3, 20:4n-3 and 22:4n-3 present up to 0.5%.