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Age-related changes in mitochondrial membrane composition of *Nothobranchius furzeri.* Comparison with a longer-living *Nothobranchius* species.

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Abstract

Membrane compositions, particularly of mitochondria, could be critical factors in the mechanisms of growth and aging, especially during phases of high oxidative stress that result in molecular damage. Changes affecting lipid class or fatty acid (FA) compositions could affect phospholipid (PL) properties and alter mitochondrial function. In the present study, mitochondrial membrane PL compositions were analysed throughout the life-cycle of *Nothobranchius furzeri*, a species with explosive growth and one of the shortest-lived vertebrates. Mitochondrial PLs showed several changes with age. Proportions of total PLs and PC were reduced while an increase in PS, CL and PE was observed, mainly between the 2.5 and 5 months of fish age, the time during which animals doubled their weight. FA compositions of individual PLs in mitochondria were also significantly affected with age suggesting the existence of increasing damage to mitochondrial lipids during the life-cycle of *N. furzeri* that could be one of the main contributors to degraded mitochondrial function associated with aging. The peroxidation index values from *N. furzeri* mitochondrial PLs were significantly lower than those reported in *N. rachovii*, a species with a 2-fold longer life span than *N. furzeri*, which seems to contradict the membrane pacemaker theory of animal metabolism.

Keywords: Biology of aging; Mitochondria; Fish; Lipids

1. Introduction

Nothobranchius furzeri, also known as African turquoise killifish, inhabits seasonal pools which are formed during the monsoon season in the Eastern Africa and their maximum natural lifespan is limited to several months, making them among the shortest-lived vertebrates (Genade et al. 2005). The short life-cycle is due to the adaption to the ephemeral and unpredictable conditions of the natural habitat (Terzibasi et al. 2008) although this fish maintains its compressed life span and short generation time in the laboratory, when water is in constant supply (D´Angelo 2017). Accelerated maturation is observed in captivity as well; growth and maturation are accelerated even when compared with other, longer-living, species of the genus which originate from more humid climates with longer rain seasons.

Several studies have reported positive correlations between growth rate and levels of oxidative stress in animals [(Alonso-Alvarez et al. 2007)) and references therein]. The combination of a high growth rate and the rapid attainment of the adult body size has several negative side-effects in animals including reduced immunological competence, depletion of energy reserves and decreased lifespan (Inness 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; Ademowo et al. 2017). For all these reasons, mitochondria is considered the key organelle contributing to tissue deterioration during high oxidative stress situations, including rapid growth and aging.

Although ROS damage affects all cell macromolecules, lipid peroxidation is quantitatively the main oxidative process in tissues due to the high sensitivity to oxidation of polyunsaturated fatty acids (PUFA), which are essential constituents of cell membrane phospholipids (PLs) (Bielski et al. 1983). Lipid peroxidation produces several oxidized fatty acid derivatives that propagate oxidative damage by attacking other membrane components, lipids, proteins and nucleic acids (Sanz et al. 2006), and it could therefore be suggested that lipid peroxidation, mainly that of mitochondrial membranes, may be the primary process associated with periods of high oxidative stress. Moreover, it has been suggested that lipid peroxidation derivatives could also have specific signalling roles inducing adaptive responses driven to decrease oxidative damage and improve antioxidant defences (Pamplona and Barja 2011), membrane composition acting as a pacemaker of processes related with oxidative damage accumulation and aging.

Membrane lipid composition has been shown to be linked to metabolic rate and lifespan in a wide number of animal species (Hulbert 2008). In comparative studies, performed on various species of mammals, birds and reptiles, it has been found that species with a shorter life span have more unsaturated membranes (plasma and mitochondrial) than species with a longer life expectancy (Pamplona et al. 2002; Hulbert et al. 2007). Membranes with high levels of PUFA are more fluid and this can enable or promote higher molecular activity of membrane proteins and, in turn, increase the metabolic activity of cells, tissues and, consequently, whole animals. At the same time, susceptibility to oxidative damage increases with the proportion of PUFA in membranes (Hulbert 2005).

Thus, our hypothesis is that mitochondrial membrane PL composition plays a central role in mitochondrial processes related with aging and situations of high oxidative

stress. The main goal of the present study was to characterize changes in mitochondrial membrane PLs with age over the whole life-cycle of *Nothobranchius furzeri*, a species that shows many molecular, cellular and physiological aging phenotypes, shared with many other organisms, including humans. Besides, we wanted to compare results in *N. furzeri* with those obtained in a previous work on a longer-living species of the genus *Nothobranchius* in order to analyse the relationship between mitochondrial membrane composition and life span.

2. Methods

2.1. Animal housing and sampling

Nothobranchius furzeri (Jubb 1971) (*Actinopterygii, Cyprinodontiformes, Nothobranchiidae*) of the MZM-04/10 strain (longer-living phenotype, ~7 months) (Terzibasi et al. 2008) belonging to a resident population established in the Fish Chronobiology Laboratory at the University of Murcia were used. Fish were kept under constant temperature (26 ± 2 °C) in 70-L tanks equipped with a recirculating freshwater system (4 L/h flow) and biological and mechanical filtration. The water parameters were controlled to conform to the following values: water hardness < 6 dKH, NO_3^- < 0.1 mg/L, NO_2^- < 0.1 mg/L, NH_3 < 0.5 mg/L and pH = 7.4. The photoperiod was set at 12L:12D (lights on at 08:00) and light intensity at the surface of the water during the photophase was 200 lx. Fish were fed red mosquito larvae (Petra-Aqua, Praque-West, Czech Republic) manually delivered twice per day. The fatty acid composition of the diet is presented in Supplementary Table 1.

In order to obtain isolated mitochondria from *N. furzeri* at different ages, fish of 2.5, 5 and 7 months were euthanized by exposure to the anesthetic MS222 (200 mg/L), weight measured, and whole animals homogenized in a blender to produce a pate that served as

a source of material for analyses. Four replicate samples of each age group were collected for lipid and fatty acid analysis. In order to obtain sufficient material for all the required analyses, fish samples were pools of two whole bodies.

Fish were treated in accordance with the current Spanish law regarding animal experiments, and the experimental protocol performed for this work was approved by the Bioethics Committee of the University of Murcia.

2.2. Mitochondria isolation

~~Approximately~~ An amount of 0.6 to 1.4~~1~~ g of fresh (non-frozen) whole fish pate was homogenized in 8-mL ice-cold sucrose buffer (0.4 M phosphate buffer pH 7.4, 0.25 M sucrose, 0.15 M KCl, 40 mM KF, and 1 mM *N*-acetyl-cysteine) using a tissue disrupter (IKA T25 digital Ultra-Turrax Fisher Scientific, Loughborough, UK). Sucrose buffer homogenates were then centrifuged at 600*g* twice for 6 minutes and the pellet discarded (cell/nuclei debris). Supernatants were then centrifuged at 6,800*g* twice for 10 minutes and the resulting pellet was used for lipid extraction, basically as described in previous works (Almaida-Pagan et al. 2014; Lucas-Sanchez et al. 2014). To verify that pellets were highly enriched in mitochondria, a portion of our isolates was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4 ºC, and then processed prior to analysis by transmission electron microscopy (Tecnai™ G2 Spirit BioTWIN, FEI Europe, Eindhoven, The Netherlands) as described in a previous work (Almaida-Pagan et al. 2014). Purity of preparations was also tested by measuring total superoxide dismutase (SOD) and SOD2 (mitochondria specific) in our mitochondria isolates [as described in (Tocher et al. (2003)]. SOD2 activity in mitochondria pellets always represented more than 95% of total SOD activity.

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2.3. Lipid extraction and PL class composition

Total lipid from whole-animal (males and females) mitochondria was obtained by extraction with chloroform/methanol (2:1, v/v) containing 0.01% (w/v) butylated hydroxytoluene as antioxidant, basically according to Folch et al. (1957). Briefly, mitochondrial pellets were homogenized in 5 mL of ice-cold chloroform/methanol followed by the 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. All lipids extracts were stored at −20°C under a N_2 atmosphere prior to analysis. PL classes were separated by high-performance thin-layer chromatography 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 volume) as solvent system (Olsen and Henderson 1989). The lipid classes were visualized by charring at 160 °C for 15 min after spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid and quantified by visible densitometry using Image Scanner II (Amersham Biosciences, UK). Scanned images were recorded automatically and analyzed by computer using IQ-Image Quant TL 8.1 software (GE Healthcare Bio-Sciences AB, Sweden).

2.4. PL fatty acid composition

Individual phospholipid classes from tissue´s total lipid extract were separated by preparative-TLC, using silica gel plates (20 × 20 cm) (VWR) and the solvent system as above. Individual PL classes were identified by comparison with known standards after spraying with 1% (w/v) 2′,7′-dichlorofluorescein in 97% (v/v) methanol containing 0.05% (w/v) BHT, and visualization under UV light. Each phospholipid class was scraped from the plate into a test tube and subjected directly (on silica) to acid-catalyzed

transmethylation at 50 °C overnight following addition of 2 ml of 1% (v/v) sulphuric acid in methanol in order to prepare fatty acid methyl esters (FAME) (Christie 2003). FAME were separated and quantified by gas–liquid chromatography a Hewlett-Packard 5890 gas chromatograph with a capillary column (SPTH-2560, SUPELCO, 100 m×0.25 mm I.D., 0.20 μm film thickness). The oven temperature, held at an initial value of 140 °C for 5 min, was increased at a rate of 4 °C per min to 230 °C, then further increased at a rate of 1 °C per min to 240 °C, and finally held at that temperature for 6 min. The injector and flame ionization detector were set at 250 °C. Helium at a pressure of 290 kPa was used as carrier gas. Peaks were identified by comparing their retention times with appropriate FAME standards purchased from Sigma Chemical Company (St. Louis, MO, USA). Individual FA concentrations were expressed as percentages of the total content.

2.5. Lipid profile and life span.

Results from *N. furzeri* were represented along with those obtained under the same feeding and housing conditions from *Nothobranchius rachovii* (Lucas-Sanchez et al. 2014) to correlate the changes in mitochondrial membrane lipid class composition and the peroxidation index (PIn) of individual PLs with the stage of the fish life-cycle. With this aim, age groups of the two *Nothobranchius* species were unified by using the corrected age (cA = Actual age/Maximum life span) as correction factor. Considering a maximum life span of 7 and 14 months for *N. furzeri* and *N. rachovii*, respectively, cA values for *N. furzeri* of 2.5, 5 and 7 months were 0.36, 0.71 and 1 while cA values for *N. rachovii* of 3, 7, 11 and 14 months were 0.21, 0.50, 0.79 and 1.

2.6. Indexes and statistical analysis

The peroxidation index (PIn) was used as an estimate of PL susceptibility to oxidation and was calculated using the formula: PIn= 0.025 × (percentage of monoenoics) + 1 × (percentage of dienoics) + 2 × (percentage of trienoics) + 4 × (percentage of tetraenoics) + 6 × (percentage of pentaenoics) + 8 × (percentage of hexaenoics) (Witting and Horwitt 1964). Results are presented as mean ± SD (n = 4). Data were checked for homogeneity of variances by the Levene's test and, where necessary, arc-sin transformed before further statistical analysis. One-way analysis of variance (ANOVA) was performed to determine statistical significance of differences between fish species and tissues for individual PL class, single fatty acid, group of fatty acids and index and Tukey's post-hoc test was used for multiple comparisons when pertinent. $p < 0.05$ was considered to be statistically different. A Pearson correlation test was performed for total PL content (∑PL), each PL percentage and every PL fatty acid and index with fish age or cA. Two levels of statistical significance of differences, $*p< 0.05$ and $**p< 0.01$, were considered. Statistical analyses were performed using SPSS, version 22.0 (SPSS Inc., Chicago, IL).

3. Results

3.1. Biometric measurements

The biometric data of the fish used in the study are presented in Figure 1. The increase in weight with age was around 2.0-fold from 2.5 to 5 months, while no significant changes in weight were observed from 5 to 7 months. The increase in length with increasing age was 0.3-fold from 2.5 to 5 months, and no changes were shown from 5 to 7 months. Condition factor (K) was significantly higher in 7-month-old *N. furzeri* (1.0±0.0) than in 2.5-month-old fish (1.2±0.1).

3.2. PL class composition of whole-fish mitochondria

Data from male and female fish were combined in the present study as they did not differ in their lipid profiles. The PL class composition of whole-fish mitochondria from 2.5-, 5- and 7-month-old *N. furzeri* is shown in Figure 2. Phosphatidylcholine (PC) was the predominant PL class, representing 70.1% of total PL in 2.5-month-old fish. The next PL in abundance was phosphatidylethanolamine (PE), which constituted 14.1% of the total PL. Thus, the sum of both PC and PE represented nearly 85% of total PL in 2.5-month-old *N. furzeri*. The remaining 15% of total PLs was, in rank order, phosphatidylserine (PS), sphingomyelin (SM), cardiolipin (CL) and phosphatidylinositol (PI), representing 5.4%, 4.8%, 3.6% and 2.0%, respectively.

Several changes in *N. furzeri* mitochondrial PL class composition were observed with age (Figure 2). Total PL content significantly ($p<0.05$) decreased in fish mitochondria with age as reflected by the Pearson correlation test ($r = -0.789$), which mainly took place from 2.5 to 5 months (from 80.4% to 51.3%). PC content was drastically reduced to 22.7% and 20.1% in 5- and 7-month-old fish, respectively and also correlated with age ($r = -0.883$, $p<0.01$). Regarding PE content, a significant increase in mitochondria from 5-month-old *N. furzeri* was obtained when compared to younger animals although r value (0.565) was not significant. Proportions of PS and CL increased with age (r = 0.857 and 0.761, $p<0.01$ and $p<0.05$, respectively), particularly from 2.5 to 5 months (5.4% vs 27.3% for PS and 3.6% vs 12.4% for CL) while no statistical changes with age for SM and PI were obtained (r =−0.425 and 0.549, respectively).

3.3. Fatty acid compositions of individual PL of whole-fish mitochondria

Fatty acid compositions of individual PL classes from 2.5-, 5- and 7-month-old *N. furzeri* are summarised in Table 1 (for full fatty acid compositions, see Supplemental

Tables 2−7). Each PL class had a characteristic FA composition. PC was characterized by high levels of palmitic acid (16:0) and docosahexaenoic acid (DHA, 22;6n-3; S. Table 2). Both, PE and PS showed high levels of stearic acid (18:0) and DHA (S. Tables 3 and 4). SM had a highly saturated profile with high proportions of 16:0 and 18:0 and the monounsaturated FA, nervonic acid (24:1n-9; S. Table 5). CL had high levels of linoleic acid (LA, 18:2n-6; S. Table 6). The PIn was also different among individual PL from 2.5-month-old fish, with PE and PS having higher PIn values, whereas SM was the PL with the lowest PIn.

The FA composition of individual PL from whole-fish mitochondria showed several significant changes with age. There was a general increase in total saturated fatty acid (SFA) content in all PLs, manifested by a positive Pearson correlation coefficient in all the lipid classes. This increase occurred mainly between 2.5- and 5-month-old *N. furzeri* in PE, PS, CL and PI (Table 1). Proportions of monounsaturated fatty acid (MUFA) decreased in all PL classes, mainly between the 2.5 and 5 months of their life-cycle (in PC, PE, PS, CL and PI; Table 1). This decrease in MUFA content was particularly influenced by the change in oleic acid content (OA, 18:1n-9; S. Tables 2, 3, 4, 6 and 7). In contrast, total n-6 PUFA decreased significantly in PS, SM and CL (Table 1), mainly due to a reduction in LA (especially relevant in SM; S. Table 5). No statistical differences in total n-3 fatty acids and PIn values were found for any PL class except for SM. SM PIn value decreased, particularly between 5- and 7-month-old animals (Table 1).

3.4. Lipid profile and life span

Figure 3 represents percentages of every single PL against corrected age (cA) for *N. furzeri* and *N. rachovii*. PL composition was significantly different in the two species.

2.5-month-old *N. furzeri* had lower content in SM, PI and PE and higher proportion of PC than 3-month-old *N. rachovii*. With age, both species showed alike changes for some mitochondrial PLs. When age groups of the two species were represented in the same scale (cA), a significant correlation between percentage and cA was found for PC (negative correlation) and CL (positive correlation).

Regarding PIn values for single PLs (Figure 4), *N. furzeri* showed lower PIn values for every single PL and cA value than *N. rachovii*. The two *Nothobranchius* species showed different trends. While *N. rachovii* showed a significant decrease in the PIn value of the three most abundant PLs (PC, PE and PS) with age, no significant changes were observed in *N. furzeri* except for SM, which significantly decreased with age (Table 1). There was no significant correlation between PIn and cA for any PL class.

4. Discussion

Nothobranchius furzeri doubled their weight between the 2.5 and 5 months of their life-cycle ~~(from 0.3 to 0.7 g)~~, a time during which fish reached their adult size. The life-cycle of *N. furzeri*´s would be "tied with explosive growth and accelerated with sexual maturation", as it was described by Terzibasi et al. ~~(Terzibasi et al. (~~2007). The rapid attainment of the adult body size has been linked to a high metabolic rate and oxidative stress accumulation in animals (Alonso-Alvarez et al. 2007; Monaghan et al. 2009). Regarding fish, it has been reported a rapid increase in mitochondrial aconitase, TBARS and superoxide dismutase (SOD2) during the first months of the life-cycle of whole zebrafish (Almaida-Pagan et al. 2014), a progressive increase in oxidative stress linked with a decrease in the response of antioxidant systems in aged *N. rachovii* (Hsu et al.

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2008) and *N. guentheri* (Liu et al. 2012) and an increase in MDA content in *N. furzeri* liver and muscle with age (Milinkovitch et al. 2018).

Mitochondria isolated from whole *N. furzeri* showed a distinctive PL composition in which, PC and PE predominated~~, representing more than 80% of total PLs in 2.5-month-old fish~~, followed in rank order by PS ~~(5.4%)~~, SM ~~(4.8%)~~, CL ~~(3.6%)~~ and PI ~~(2.0%)~~. Similar to that found in 3-month-old *N. rachovii* (Lucas-Sanchez et al. 2014), CL represented nearly a 3% of total PLs, which is surprising if we consider the critical role that CL plays in the mitochondrial inner membrane as regulator of those processes related to oxidative phosphorylation and mitochondrial integrity (Paradies et al. 2002). The proportions of mitochondrial PLs showed several changes with age with a significant reduction in total PLs and PC and an increase in PS, CL and PE, mainly between the 2.5 and 5 month of fish age, that it is when animals doubled their weight. This is pivotal for mitochondrial function as individual PLs are not randomly distributed among biological membranes but rather are highly specific and characteristic for each organelle, influencing their shape, structure and function (Dowhan 1997; Sargent et al. 2002). Therefore, disruption of lipid homeostasis by altering PL composition of membranes or the PL fatty acid composition, can promote pathological changes that contribute towards biological ageing and age-related diseases (Ademowo et al. 2017). Several age-related diseases have been associated with altered lipid metabolism and an elevation in highly damaging lipid peroxidation products; the latter has been ascribed, at least in part, to mitochondrial dysfunction and elevated ROS formation. Under conditions of high metabolism, higher CL would be necessary and so the levels of CL in mitochondrial inner membrane are consisting with the existing paradigm. Changes observed in PS of *N. furzeri* with age could also be important as this PL is precursor for signalling molecules, some of which are associated with apoptotic processes (Ulmann et

al. 2001; Mozzi et al. 2003). Besides, PC loss has been reported in a mouse model of Alzheimer's disease (Hong et al. 2016).

Individual PLs from whole *N. furzeri* mitochondrial membranes showed characteristic fatty acid (FA) compositions that are likely related to their specific roles in membrane fluidity and function (Zabelinskii et al. 1999; Tocher et al. 2008) and thus, asit we stated above, ~~can be deduced~~ thecat effects of oxidative stress on mitochondrial and cell membranes at the level of ~~both PL content and~~ individual PL fatty acid~~FA~~ composition will also ~~modify~~ alter PL properties and therefore, their efficacy in membrane functions. Fatty acid compositions of individual PLs of *N. furzeri*'s mitochondria were influenced significantly by age. Saturated fatty acid (SFA) content was generally increased in all PL classes while the sum of monounsaturated fatty acids (MUFA) significantly decreased. These changes occurred mainly between the 2.5 and 5 months of the fish life-cycle ~~(in PE, PS, CL and PI for SFA; in PC, PE, PS, CL and PI for MUFA)~~. Regarding polyunsaturated fatty acids (PUFA), n-6 PUFA mainly decreased ~~in PE, PS, SM and CL~~ while no significant changes in n-3 PUFA were found with age ~~for any PL class except for SM, in which a significant reduction between 5 and 7 months of the fish age was found~~. Some of these results were similar to those reported for *N. rachovii* in a previous study (Lucas-Sanchez et al. 2014). However ~~although~~, *N. furzeri* did not show any significant change in PIn values with age (except for SM) as ~~it was in~~ *N. rachovii* did. Interestingly, mitochondrial membranes from *N. furzeri* showed lower PIn values for all PL classes than *N. rachovii*, a species with a 2-fold longer life span. This data would contradict the results from comparative studies that indicate that animal species with a shorter life span have more unsaturated membranes than species with a longer life expectancy (Pamplona et al. 2002; Hulbert et al. 2007). However, this should be taken with caution and studies on a wider number of *Nothobranchius* species should be

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done in order to elucidate whether *N. furzeri*´s represents (or not) an outlier within the genus.

Summarizing, although the mitochondrial isolation from whole fish performed in the present study could constitute a limitation to conclude about specific tissues, the present results, along with other data showing an increase in lipid and protein oxidation and a reduction in antioxidant activities in whole *N. rachovii* (Hsu et al. 2008; Lucas-Sanchez et al. 2014) and *N. furzeri* (Milinkovitch et al. 2018) with age, suggest increasing oxidative stress and damage to mitochondrial lipids during the first months of this species´ life-cycle, which could likely determine their short life span. Following the membrane pacemaker theory of animal metabolism (Sanz et al. 2006; Hulbert et al. 2007), lipids would be among the first molecules affected by mitochondrial ROS, and lipid peroxidation could be the propagator of oxidative damage reactions. Both mitochondrial membrane PL class and PL FA compositions changed in *N. furzeri* with age, which could considerably alter their properties as the major constituents of mitochondrial membranes. Observed results, however, significantly differed from those reported on *N. rachovii*, a species with double the life expectancy of *N. furzeri*. Strikingly, the PIn of every single PL from *N. furzeri* mitochondrial membranes was lower than that from *N. rachovii* which contradicts the membrane pacemaker theory. These findings make necessary to study other species of genus *Nothobranchius* in order to characterize the relationship between mitochondrial membrane lipids and life span.

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References

Ademowo OS, Dias HKI, Burton DGA, Griffiths HR (2017) Lipid (per) oxidation in mitochondria: an emerging target in the ageing process? Biogerontology 18:859–879. doi: 10.1007/s10522-017-9710-z

Almaida-Pagan PF, Lucas-Sanchez A, Tocher DR (2014) Changes in mitochondrial membrane composition and oxidative status during rapid growth, maturation and aging in zebrafish, Danio rerio. Biochim Biophys Acta - Mol Cell Biol Lipids 1841:1003–1011. doi: 10.1016/j.bbalip.2014.04.004

Almaida-pagán PF, Lucas-sánchez A, Tocher DR Author ' s personal copy Biochimica et Biophysica Acta Changes in mitochondrial membrane composition and oxidative status during rapid growth , maturation and aging in zebra fi sh , Danio rerio

Almroth B, Johansson A, Förlin L, Sturve J (2010) Early-age changes in oxidative stress in brown trout, Salmo trutta. Comp Biochem Physiol - B Biochem Mol Biol 155:442–448. doi: 10.1016/j.cbpb.2010.01.012

Alonso-Alvarez C, Bertrand S, Faivre B, Sorci G (2007) Increased susceptibility to oxidative damage as a cost of accelerated somatic growth in zebra finches. Funct Ecol 21:873–879. doi: 10.1111/j.1365-2435.2007.01300.x

Bielski, B.J.H.; Arudi, R.L.; Sutherland MW (1983) A Study of the Reactivity of H02/02- with Unsaturated Fatty Acids. J Biol Chem 258:4789–4761

Christie WW (2003) Lipid Analysis: Isolation, Separation, Identification and Structural Analysis of Lipids, 3rd editio. Oily Press, Somerset, UK

D´Angelo LD (2017) An aquatic organism as time machine : Nothobranchius furzeri. J Gerontol Geriatr 66:307–310

Dowhan W (1997) Molecular basisi for membrane phospholipid diversity:Why are there so many lipids? Annu Rev Biochem 66:199–232. doi: 10.1146/annurev.biochem.66.1.199

Folch, J.; Lees, M.; Stanley S (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497–509

Genade T, Benedetti M, Terzibasi E, et al (2005) Annual fishes of the genus Nothobarnchius as a model system for aging research. Aging Cell 4:223–233. doi: 10.1111/j.1474-9726.2005.00165.x

Hong JH, Kang JW, Kim DK, et al (2016) Global changes of phospholipids identified by MALDI imaging mass spectrometry in a mouse model of Alzheimer's disease. J Lipid Res 57:36–45. doi: 10.1194/jlr.M057869

Hsu CY, Chiu YC, Hsu WL, Chan YP (2008) Age-related markers assayed at different developmental stages of the annual fish nothobranchius rachovii. Journals Gerontol - Ser A Biol Sci Med Sci 63:1267–1276. doi: 10.1093/gerona/63.12.1267

Hulbert AJ (2008) The links between membrane composition, metabolic rate and lifespan. Comp Biochem Physiol - A Mol Integr Physiol 150:196–203. doi: 10.1016/j.cbpa.2006.05.014

Hulbert AJ (2005) On the importance of fatty acid composition of membranes for aging. J Theor Biol 234:277–288. doi: 10.1016/j.jtbi.2004.11.024

Hulbert AJ, Pamplona R, Buffenstein R, Buttemer WA (2007) Life and Death: Metabolic Rate, Membrane Composition, and Life Span of Animals. Physiol Rev 87:1175–1213. doi: 10.1152/physrev.00047.2006

Inness CL., Metcalfe NB (2008) The impact of dietary restriction, intermittent feeding

and compensatory growth on reproductive investment and lifespan in a short-lived fish. Proc R Soc B Biol Sci 275:1703–1708. doi: 10.1098/rspb.2008.0357

Jubb A (1971) New Nothobranchius species (Pisces, Cyprinodontidae) from Southeastern Rhodesia. J Am Kill Assoc 8:12–19

Liu C, Wang X, Feng W, et al (2012) Differential expression of aging biomarkers at different life stages of the annual fish Nothobranchius guentheri. Biogerontology 13:501–510. doi: 10.1007/s10522-012-9395-2

Lucas-Sanchez A, Almaida-Pagan PF, Tocher DR, et al (2014) Age-Related Changes in Mitochondrial Membrane Composition of Nothobranchius rachovii. Journals Gerontol Ser A Biol Sci Med Sci 69A:142–151. doi: 10.1093/gerona/glt066

Milinkovitch T, Lefrançois C, Durollet M, Thomas-Guyon H (2018) Influence of temperature on age-related lipid peroxidation in a short-lived vertebrate (Nothobranchius furzeri). Fish Physiol Biochem 44:343–347. doi: 10.1007/s10695-017-0439-z

Monaghan P, Metcalfe NB, Torres R (2009) Oxidative stress as a mediator of life history trade-offs: Mechanisms, measurements and interpretation. Ecol Lett 12:75–92. doi: 10.1111/j.1461-0248.2008.01258.x

Mozzi R, Buratta S, Goracci G (2003) Metabolism and functions of phosphatidylserine in mammalian brain. Neurochem Res 28:195–214. doi: 10.1023/A:1022412831330

Olsen RE, Henderson RJ (1989) The rapid analysis of neutral and polar marine lipids using double-development HPTLC and scanning densitometry. J Exp Mar Bio Ecol 129:189–197. doi: 10.1016/0022-0981(89)90056-7

Pamplona R, Barja G (2011) An evolutionary comparative scan for longevity-related oxidative stress resistance mechanisms in homeotherms. Biogerontology 12:409–435. doi: 10.1007/s10522-011-9348-1

Pamplona R, Barja G, Portero-Otin M (2002) Membrane fatty acid unsaturation, protection against oxidative stress, and maximum life span: a homeoviscous-longevity adaptation? Ann N Y Acad Sci 959:475–490. doi: 10.1111/j.1749-6632.2002.tb02118.x

Paradies G, Petrosillo G, Paradies V, Ruggiero FM (2011) Mitochondrial dysfunction in brain aging: Role of oxidative stress and cardiolipin. Neurochem Int 58:447–457. doi: 10.1016/j.neuint.2010.12.016

Paradies G, Petrosillo G, Pistolese M, Ruggiero FM (2002) Reactive oxygen species affect mitochondrial electron transport complex I activity through oxidative cardiolipin damage. Gene 286:135–141. doi: S0378111901008149

Rollo CD (2002) Growth negatively impacts the life span of mammals. Evol Dev 4:55–61. doi: 10.1046/j.1525-142x.2002.01053.x

Sanz A, Pamplona R, Barja G (2006) Is the mitochondrial free radical theory of aging intact? Antioxid Redox Signal 8:582–99. doi: 10.1089/ars.2006.8.582

Sargent, J.R.; Tocher, D.R.; Bell JG (2002) The lipids. In: Halver, J. E., Hardy RW (ed) Fish Nutrition, 3rd edition. Elsevier (Academic Press), San Diego, California, pp 181–257

Terzibasi E, Valenzano DR, Benedetti M, et al (2008) Large differences in aging phenotype between strains of the short-lived annual fish Nothobranchius furzeri. PLoS One 3:. doi: 10.1371/journal.pone.0003866

Terzibasi E, Valenzano DR, Cellerino A (2007) The short-lived fish Nothobranchius furzeri as a new model system for aging studies. Exp Gerontol 42:81–89. doi: 10.1016/j.exger.2006.06.039

Tocher DR, Bendiksen EÅ, Campbell PJ, Bell JG (2008) The role of phospholipids in nutrition and metabolism of teleost fish. Aquaculture 280:21–34. doi:

10.1016/j.aquaculture.2008.04.034

Tocher DR, Mourente G, Eecken AVANDER, et al (2003) Comparative study of antioxidant defence mechanisms in marine fish fed variable levels of oxidised oil and V E.pdf. Aquac Int 11:195–216

Ulmann L, Mimouni V, Roux S, et al (2001) Brain and hippocampus fatty acid composition in phospholipid classes of aged-relative cognitive deficit rats. Prostaglandins Leukot Essent Fat Acids 64:189–195. doi: 10.1054/plef.2001.0260

Witting L, Horwitt M (1964) Effect of degree of fatty acid unsaturation in tocopherol deficiency-induced creatinuria. J Nutr 82:19–33

Zabelinskii SA, Chebotareva MA, Kostkin VB, Krivchenko AI (1999) Phospholipids and their fatty acids in mitochondria, synaptosomes and myelin from the liver and brain of trout and rat: A new view on the role of fatty acids in membranes. Comp Biochem Physiol - B Biochem Mol Biol 124:187–193. doi: 10.1016/S0305-0491(99)00113-3

Figure legends

Figure 1. Average weight, total length and condition factor (K) (mean ± SEM) of 2.5, 5 and 7 month-old *Nothobranchius furzeri* (n=8). Different superscript letters represent significant differences between age groups as determined by one-way analysis of variance and Tukey's post-hoc test ($p < 0.05$).

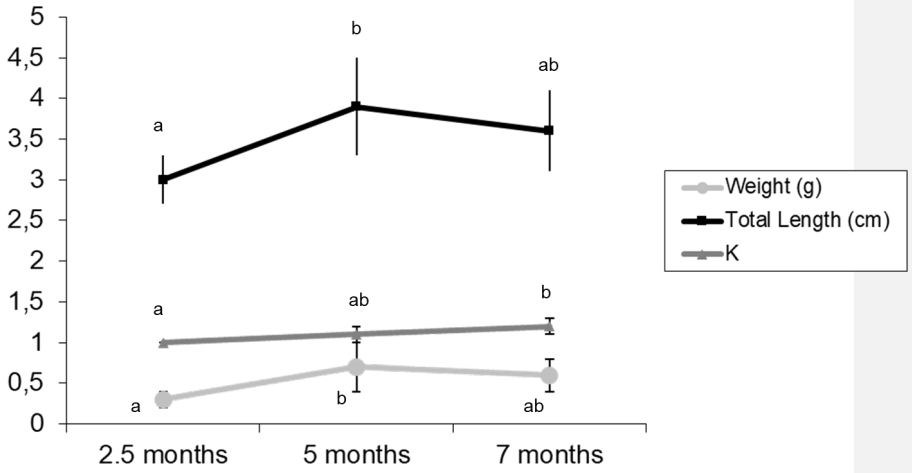
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
2.5 months
5 months
7 months
a
b
ab
a
ab
b
a
b
ab
Weight (g)
Total Length (cm)
K

Figure 2. Phospholipid (PL) content (percentage of weight of total lipid) and PL class composition (percentage of total PL) of 2.5-, 5- and 7-month-old *Nothobranchius furzeri*. Results are expressed as mean ± SD (n=4). Different superscript letters denote significant differences between age groups for each PL class as determined by one-way analysis of variance and Tukey's post-hoc test (p <0.05). ΣPL, total phospholipids; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; CL, cardiolipin; PE, phosphatidylethanolamine.

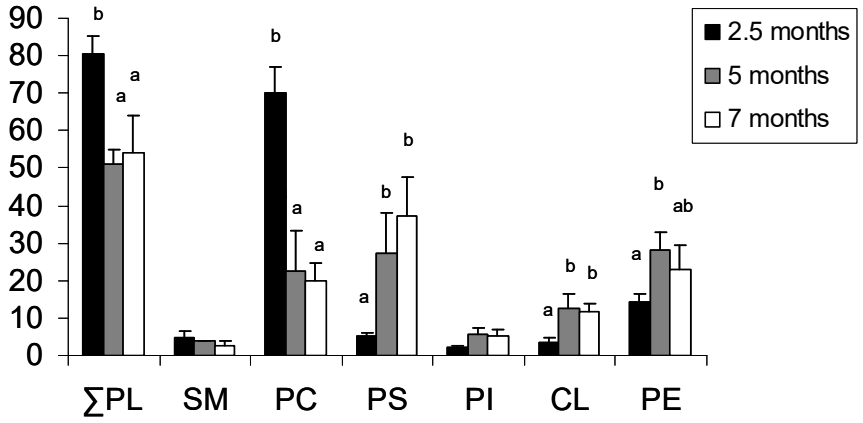

Figure 3. Phospholipid (PL) class composition (percentage of total PLs) against corrected age (cA= age/maximum life span) of *Nothobranchius furzeri* (NF) and *Nothobranchius rachovii* (NR) at different ages. Legend indicates cA values for every fish species and age (NR =0.21 (3 months), NF= 0.36 (2.5), NR= 0.50 (7), NF=0.71 (5), NR =0.79 (11), NF =1 (7) and NR =1 (14)). Results are expressed as mean ± SD (n=4). Different superscript letters denote significant differences between age groups for each PL class as determined by one-way analysis of variance and Tukey's post-hoc test ($p<0.05$). Pearson correlation values between cA and PL class (*$p<0.05$, **$p<0.01$) − SM=0.294; PC= −0.618**; PS= 0.492*; PI= −0.170; CL= 0.456*; PE= 0.091. CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

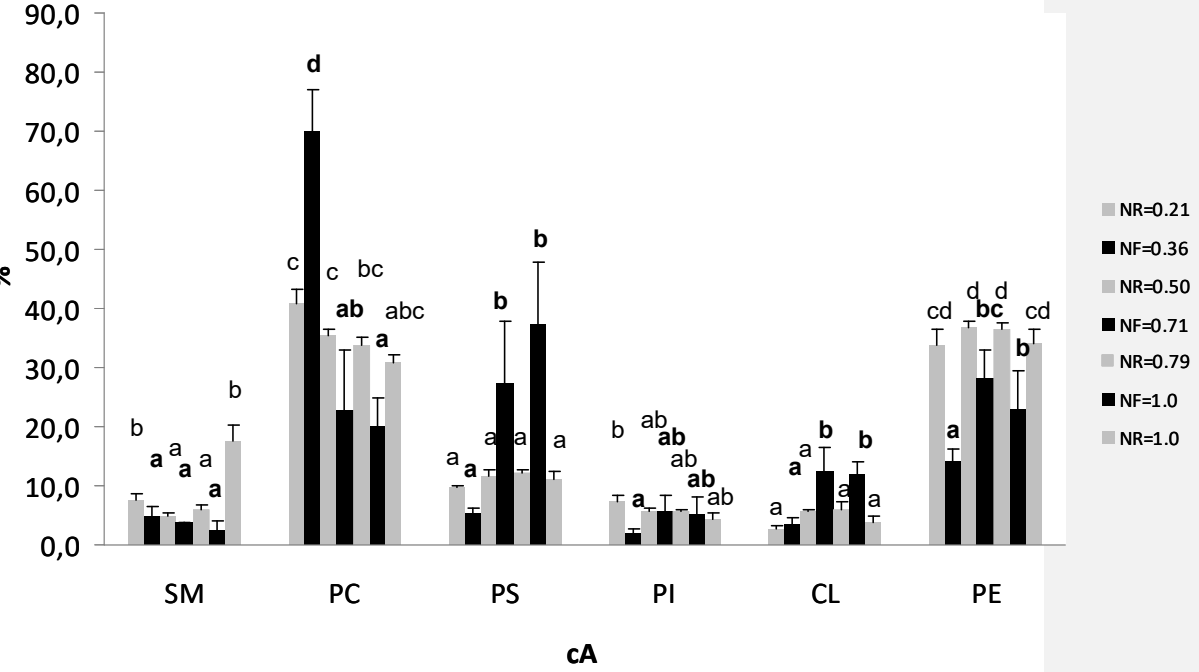

Figure 4. Peroxidation Index (PIn) values of single PLs against corrected age (cA) of *Nothobranchius furzeri* (NF) and *Nothobranchius rachovii* (NR) at different ages. Legend indicates cA values for every fish species and age (NR =0.21 (3 months), NF= 0.36 (2.5), NR= 0.50 (7), NF=0.71 (5), NR =0.79 (11), NF =1 (7) and NR =1 (14)). Results are expressed as mean ± SD (n=4). Different superscript letters denote significant differences between age groups for each PL class as determined by one-way analysis of variance and Tukey's post-hoc test ($p<0.05$), Pearson correlation values between cA and PL class (*$p<0.05$, **$p<0.01$) − SM=−0.334; PC= −0.333; PS= −0.343; PI= −0.058; CL= −0.068; PE= −0.374. CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

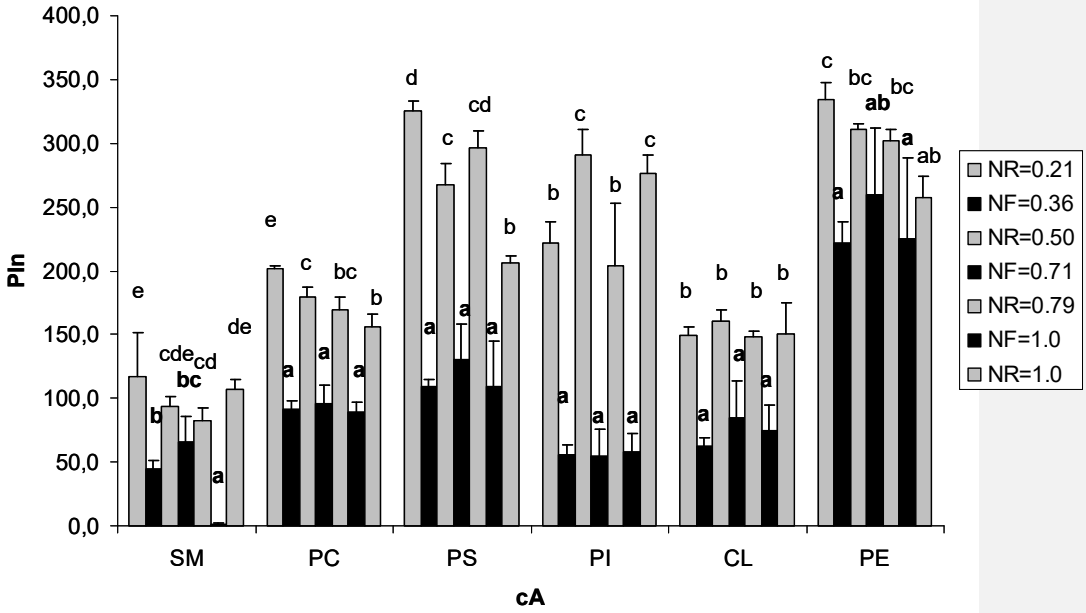

Table 1. Fatty Acid Composition and Peroxidation Index Value for Every Phospholipid Class of 2.5-, 5- and 7-Month-Old *Nothobranchius furzeri*.

PL	2.5 months	5 months	7 months	*r*
PC				
∑Saturated	47.1±0.5[a]	52.8±4.7[a]	60.1±0.6[b]	0.893**
∑Monounsaturated	32.7±0.2[b]	22.0±4.6[a]	18.5±2.9[a]	-0.867**
∑n-6 PUFA	10.7±0.3	10.7±2.3	9.3±0.2	-0.395
∑n-3 PUFA	9.5±0.0	13.0±2.2	11.4±3.3	0.261
PIn	91.0±1.2	95.7±14.4	91.2±12.1	-0.072
PE				
∑Saturated	33.0±1.0[a]	46.1±5.3[b]	54.2±8.6[b]	0.886**
∑Monounsaturated	27.8±3.1[b]	10.0±2.1[a]	7.5±0.8[a]	-0.899**
∑n-6 PUFA	13.1±1.3[b]	14.1±1.5[b]	11.1±0.7[a]	-0.561
∑n-3 PUFA	26.0±0.9	29.8±6.5	27.2±8.5	-0.003
PIn	222.0±10.5	259.1±53.3	236.0±72.6	0.280
PS				
∑Saturated	32.6±1.8[a]	61.1±3.9[b]	68.3±4.8[b]	0.914**
∑Monounsaturated	39.6±1.7[b]	11.1±1.4[a]	9.1±0.6[a]	-0.873**
∑n-6 PUFA	16.6±1.8[b]	12.7±1.1[a]	10.3±1.4[a]	-0.906**
∑n-3 PUFA	11.2±2.0	15.1±2.7	12.2±3.4	0.095
PIn	109.3±6.7	130.9±27.4	109.0±36.3	-0.040
SM				
∑Saturated	37.2±6.4[a]	64.3±8.2[b]	98.4±1.1[c]	0.983**
∑Monounsaturated	40.0±3.2[c]	12.5±1.9[b]	5.5±1.2[a]	-0.968**
∑n-6 PUFA	16.7±3.2[c]	6.7±1.3[b]	0.7±0.5[a]	-0.974**
∑n-3 PUFA	6.1±0.0	16.5±10.3	n.d.	-0.360
PIn	44.1±12.2[b]	65.4±25.7[b]	1.1±1.0[a]	-0.645*
CL				
∑Saturated	34.5±1.3[a]	47.5±2.0[b]	64.6±7.0[b]	0.873**
∑Monounsaturated	42.1±1.4[b]	19.7±2.5[a]	13.7±4.4[a]	-0.892**
∑n-6 PUFA	17.8±0.7[b]	13.8±1.4[a]	12.0±2.2[a]	-0.791**
∑n-3 PUFA	5.7±0.8	20.0±0.8	9.1±3.3	0.199
PIn	62.2±4.7	97.4±21.8	74.4±20.3	0.228
PI				
∑Saturated	37.0±3.8[a]	57.9±9.6[b]	69.3±6.0[b]	0.888**
∑Monounsaturated	42.5±2.5[c]	20.1±4.8[b]	11.6±2.5[a]	-0.936**
∑n-6 PUFA	15.5±0.4	17.1±3.0	13.9±2.5	-0.302
∑n-3 PUFA	5.0±1.0	4.9±2.8	5.3±1.0	0.074
PIn	55.4±13.1	54.9±20.5	57.8±14.5	0.072

Notes: Results are presented as mean ± *SD* ($n = 4$). Different superscript letters represent significant differences between age groups as determined by one-way analysis of variance ($p < 0.05$). Right column includes values for Pearson correlation coefficient (r) calculated for each group of fatty acids or peroxidation index and age. Significant values are indicated with asterisks (*$p < 0.05$, **$p < 0.01$). CL= Cardiolipin; PC= Phosphatidylcholine; PE; Phosphatidylethanolamine; PI= Phosphatidylinositol; PIn =

peroxidation index; PS= Phosphatidylserine; PUFA = polyunsaturated fatty acids; SM= Sphingomyelin. Fatty acids representing less than 1% of total fatty acids are not shown.