

SHORT COMMUNICATION

Membrane peroxidation index and maximum lifespan are negatively correlated in fish of the genus *Nothobranchius*

Jorge de Costa^{1,2,*}, Gustavo Barja³ and Pedro F. Almada-Pagan^{1,2}

ABSTRACT

The lipid composition of cell membranes is linked to metabolic rate and lifespan in mammals and birds but very little information is available for fish. In this study, three fish species of the short-lived annual genus *Nothobranchius* with different maximum lifespan potential (MLSP) and the longer-lived outgroup species *Aphyosemion australe* were studied to test whether they conform to the predictions of the longevity–homeoviscous adaptation (LHA) theory of ageing. Lipid analyses were performed in whole-fish samples and the peroxidation index (PLn) for every phospholipid (PL) class and for the whole membrane was calculated. Total PL content was significantly lower in *A. australe* and *N. korthausae*, the two species with the highest MLSP, and a negative correlation between membrane total PLn and fish MLSP was found, meaning that the longer-lived fish species have more saturated membranes and, therefore, a lower susceptibility to oxidative damage, as the LHA theory posits.

KEY WORDS: Ageing, Annual fish, Longevity–homeoviscous theory, Lipids, Phospholipids

INTRODUCTION

Annual fish of the genus *Nothobranchius* have proved to be a remarkable system for gerontological research (Lucas-Sánchez et al., 2014a,b; Tozzini et al., 2013). These small teleost fishes from East Africa adapted to live in ephemeral habitats, so they are forced to complete their life cycle in very short periods (3–18 months, depending on the species). If the duration of the habitat (i.e. aridity) strictly limits the natural lifespan of *Nothobranchius* fishes in the wild (Tozzini et al., 2013), this short lifespan is retained under captive conditions and is coupled to rapid expression of a host of conserved age-associated phenotypes (Cellerino et al., 2016). In addition, the genus *Nothobranchius* evolved from a non-annual (therefore, longer-lived) ancestor, the sister genus *Aphyosemion* (Sahm et al., 2019) and the two taxa provide a sharp phenotypic contrast.

The longevity–homeoviscous adaptation (LHA) theory of ageing states that lipid composition of cell membranes (particularly that of mitochondria) is linked to metabolic rate and lifespan, which has been shown in a wide number of animal species (Pamplona et al., 1998, 2000). The LHA theory of ageing rests upon the mitochondrial oxygen free-radical theory of ageing and the fact that short-lived

mammals and birds have species-specific high mitochondrial reactive oxygen species (ROS) production rates at complex I (Barja, 2013). Although ROS damage affects all cell macromolecules, lipid peroxidation is quantitatively the main oxidative process in tissues because of the high sensitivity to oxidation of polyunsaturated fatty acids (PUFA), which are essential constituents of cell membrane phospholipids (PLs) (Bielski et al., 1983).

In comparative studies, performed on various species of mammals and birds, it has been found that species with a shorter lifespan have more unsaturated membranes than species with a longer life expectancy (Pamplona et al., 2002). 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 (Pamplona et al., 1998).

In this study, three species of the genus *Nothobranchius* [*N. korthausae*, *N. rachovii* and *N. guentheri*, with a maximum lifespan potential (MLSP) of 80, 63 and 53 weeks, respectively] (Genade and Lang, 2013; Lucas-Sánchez et al., 2014a,b; Tozzini et al., 2013) and *Aphyosemion australe*, which lives in permanent habitats (MLSP of ~3 years) (<https://en.aqua-fish.net/fish/lyretail-killifish>), were chosen to check the LHA theory of ageing in fish as very little information on this vertebrate group is available.

MATERIALS AND METHODS


Animal housing and sampling

For this study, young adults (taken just after attaining adult size and sexual maturation) of the following fish species were used: *Nothobranchius korthausae* Meinken 1973 (total length, L_T , 3.0 ± 0.4 mm; total mass, M_T , 0.3 ± 0.1 g; $n=8$), *Nothobranchius rachovii* Ahl 1926 (L_T , 3.5 ± 0.3 mm; M_T , 0.6 ± 0.1 g; $n=8$), *Nothobranchius guentheri* (Pfeffer 1893) (L_T , 3.0 ± 0.5 mm; M_T , 0.4 ± 0.2 g; $n=8$) (*Cyprinodontiformes*, *Nothobranchiidae*) and *Aphyosemion australe* (Rachow 1921) (L_T , 2.9 ± 0.2 mm; M_T , 0.3 ± 0.1 g; $n=8$) (*Cyprinodontiformes*, *Aplocleilidae*). *Aphyosemion australe* share with *Nothobranchius* general traits linked to their life in nature and predation/mortality rates and, thus, they represent a well-suited outgroup species for our analyses (Sahm et al., 2017). Fish were acquired from local dealers and subjected to acclimation for 1 month at the facilities of the Fish Chronobiology Laboratory at the University of Murcia. Fish were kept in groups under exactly the same conditions (water temperature, $26 \pm 2^\circ\text{C}$; flow, 41 h^{-1} ; photoperiod, 12 h light:12 h dark with lights on at 20:00 h; hardness $<6 \text{ dKH}$; $\text{NO}_3^- <0.1 \text{ mg l}^{-1}$; $\text{NO}_2^- <0.1 \text{ mg l}^{-1}$; $\text{NH}_3 <0.5 \text{ mg l}^{-1}$; pH 7.4) and fed *ad libitum* red mosquito larvae manually delivered twice per day.

Fish were killed by exposure to the anaesthetic MS222 (200 mg l^{-1}) for 10 min following the cessation of gill movement. The whole bodies of fish were used for analyses.

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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 for Animal Experimentation of the University of Murcia (A13160603, from the Consejería de Agua, Agricultura, Ganadería y Pesca, Comunidad Autónoma de la Región de Murcia, Spain).

Lipid extraction and PL class composition

Total lipid from whole animals 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, fish samples were homogenized in 20 ml of ice-cold chloroform/methanol followed by the addition of 5 ml of 0.88% (w/v) KCl and mixing, then 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 10×10 cm silica gel plates (VWR, Lutterworth, UK) 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). Scanned images were recorded automatically and analysed by computer using IQ-Image Quant TL 8.1 software (GE Healthcare Bio-Sciences AB).

PL fatty acid composition

Individual PL classes from fish total lipid extract were separated by preparative thin layer chromatography (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 PL class was scraped from the plate into a test tube and subjected directly (on silica) to acid-catalysed 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 using a Hewlett-Packard 5890 gas chromatograph with a capillary column (SPTH-2560, SUPELCO, $100\text{ m}\times 0.25\text{ mm}$ i.d., $0.20\text{ }\mu\text{m}$ 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 minute to 230°C , then further increased at a rate of 1°C per minute 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 the carrier gas. Peaks were identified by comparing their retention times with appropriate FAME standards purchased from Sigma Chemical Company (St Louis, MO, USA). Individual fatty acid concentrations were expressed as percentages of the total content.

Lipid profile and lifespan

Lipid profiles from *Nothobranchius* species and *A. australe* kept under the same feeding and housing conditions were correlated with the maximum lifespan of each species. A maximum lifespan of 80, 63 and 53 weeks, respectively, for *N. korthausae* (Baumgart et al., 2016; Lucas-Sánchez et al., 2011), *N. rachovii* (Lucas-Sánchez et al., 2014a,b; Tozzini et al., 2013) and *N. guentheri* (Genade and Lang, 2013; Wang et al., 2017; Zhou et al., 2019), and of 156 weeks

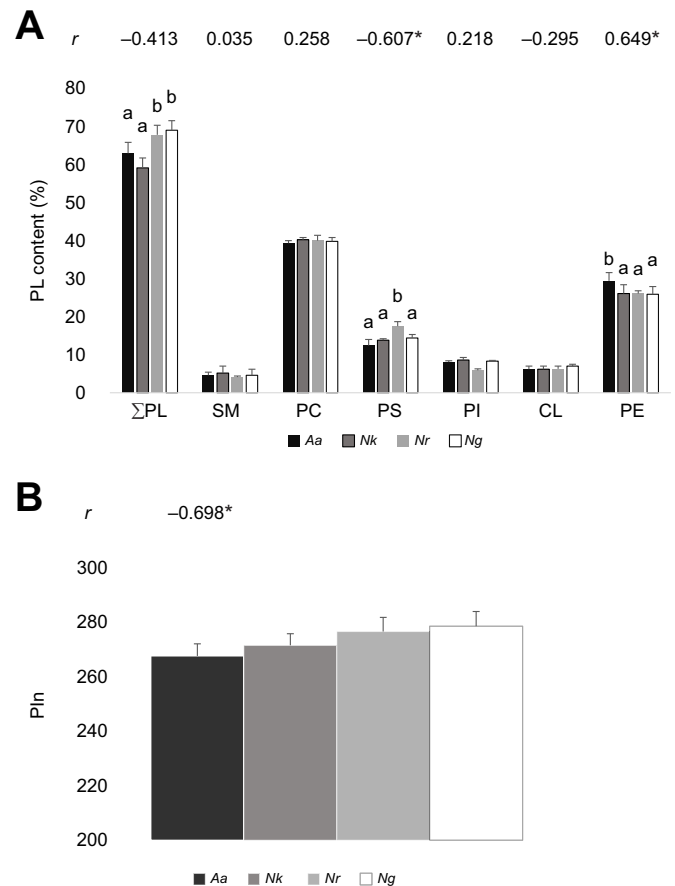


Fig. 1. Phospholipid (PL) composition and peroxidation index (Pln) from whole fish membranes. (A) Total phospholipid content (ΣPL , percentage of total lipid by mass) and PL class composition (percentage of total PLs) of cell membranes from whole *Aphyosemion australe* (Aa), *Nothobranchius korthausae* (Nk), *Nothobranchius rachovii* (Nr) and *Nothobranchius guentheri* (Ng). Results are expressed as means \pm s.d. ($n=4$). Different superscript letters denote significant differences among fish species for PL content and individual PL class as determined by Tukey's *post hoc* test (b indicates a statically higher value than a for the same PL class; $P<0.05$). SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; CL, cardiolipin; PE, phosphatidylethanolamine. (B) Pln values of total PLs from each of the four fish species. Results are means \pm s.d. ($n=4$). No statistical differences between fish species (compared one to one) were obtained when a Tukey's *post hoc* test was used ($P<0.05$). Pearson correlation values (r) between fish maximum lifespan potential (MLSP) and PL content and class composition (A), and whole membrane Pln (B) are presented in the upper boxes (* $P<0.05$, ** $P<0.01$).

for *A. australe* (<https://en.aqua-fish.net/fish/lyretail-killifish>) has been reported.

Indexes and statistical analysis

The peroxidation index (Pln) was used as an estimate of PL susceptibility to oxidation and was calculated using the formula: $\text{Pln} = 0.025 \times (\text{percentage of monoenoics}) + 1 \times (\text{percentage of dienoics}) + 2 \times (\text{percentage of trienoics}) + 4 \times (\text{percentage of tetraenoics}) + 6 \times (\text{percentage of pentaenoics}) + 8 \times (\text{percentage of hexaenoics})$ (Witting and Horwitt, 1964). The results are presented as means \pm s.d. ($n=4$). Data were checked for homogeneity of variances by 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 total PL content (ΣPL), individual PL class, single fatty acids, and fatty acid group and index, and Tukey's *post hoc* test was

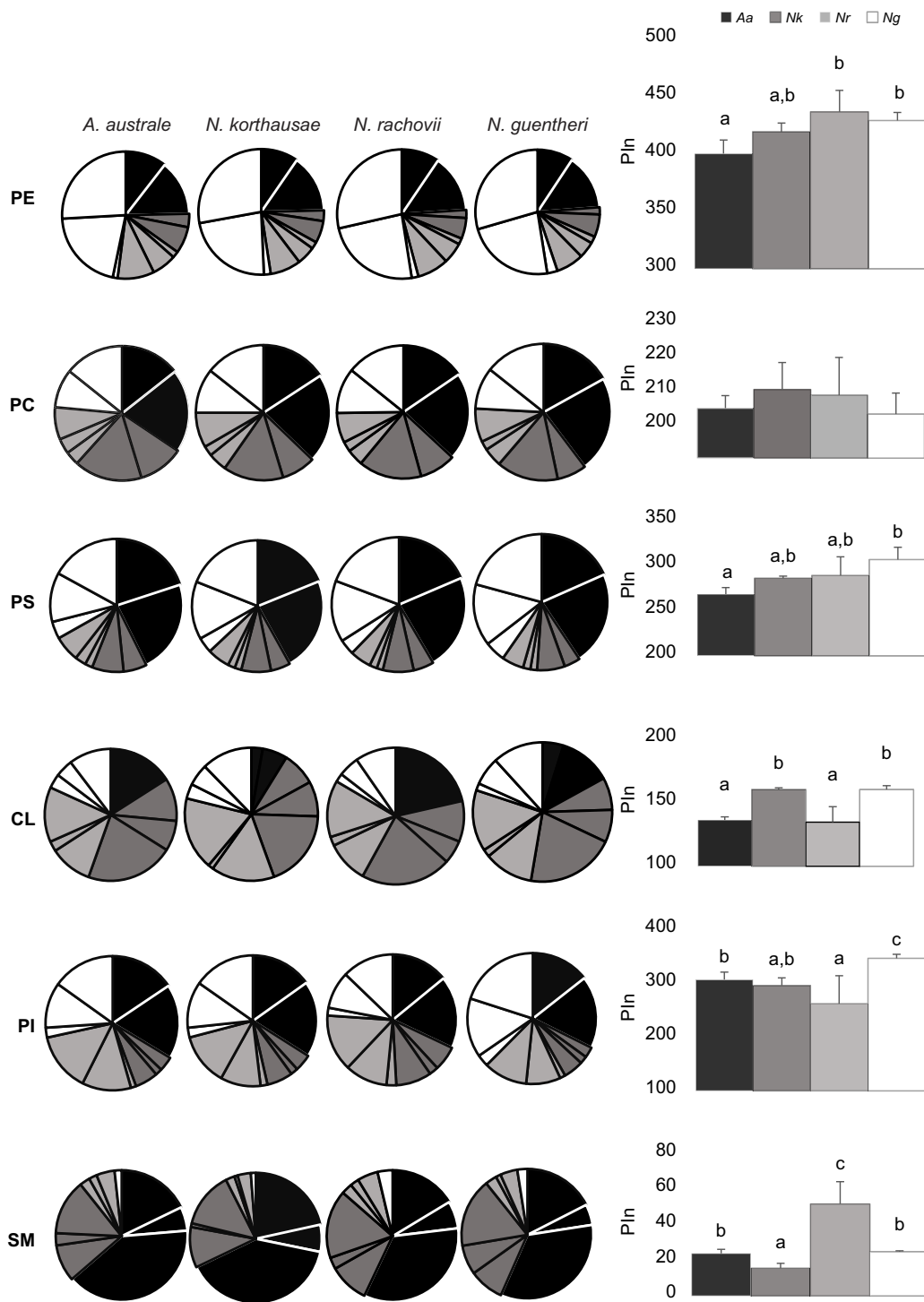


Fig. 2. Phospholipid fatty acid composition and peroxidation indexes of whole fish membranes. Each segment of the pie chart represents the following fatty acids (clockwise order): PE, saturated (black: 18:0 and Σ saturated), monounsaturated (dark grey: 18:1n-9 and Σ monounsaturated), n-6 polyunsaturated (light grey: 18:2 n-6, 20:4 n-6 and Σ n-6) and n-3 polyunsaturated (white: 20:5 n-3, 22:6 n-3 and Σ n-3); PC, saturated (black: 16:0, Σ saturated), monounsaturated (dark grey: 18:1 n-9, Σ monounsaturated), n-6 polyunsaturated (light grey: 18:2 n-6, 20:4 n-6, Σ n-6) and n-3 polyunsaturated (white: 22:6 n-3 and Σ n-3); PS, saturated (black: 18:0, Σ saturated), monounsaturated (dark grey: 18:1 n-9, Σ monounsaturated), n-6 polyunsaturated (light grey: 20:4 n-6, 22:4 n-6, Σ n-6) and n-3 polyunsaturated (white: 22:6 n-3 and Σ n-3); CL, saturated (black: 18:0, Σ saturated), monounsaturated (dark grey: 18:1 n-9, 18:7 n-7, Σ monounsaturated), n-6 polyunsaturated (light grey: 18:2 n-6, 20:4 n-6, Σ n-6) and n-3 polyunsaturated (white: 18:3 n-3, 22:6 n-3 and Σ n-3); PI, saturated (black: 18:0, Σ saturated), monounsaturated (dark grey: 18:1 n-9, 18:1 n-7, Σ monounsaturated), n-6 polyunsaturated (light grey: 18:2 n-6, 20:4 n-6, Σ n-6) and n-3 polyunsaturated (white: 22:5 n-3, 22:6 n-3 and Σ n-3); SM, saturated (black: 16:0, 18:0, saturated), monounsaturated (dark grey: 18:1 n-9, 24:1 n-9, Σ monounsaturated), n-6 polyunsaturated (light grey: 18:2 n-6, 20:4 n-6, Σ n-6) and n-3 polyunsaturated (white: Σ n-3). Right column graphs present peroxidation index (PIn) values of each PL class for the four fish species. Results are means \pm s.d. ($n=4$). Different superscript letters denote significant differences in PIn values between fish species (compared one to one) for each PL class as determined by a one-way ANOVA and Tukey's *post hoc* test ($P<0.05$). Pearson correlation (r) values between maximum lifespan potential and PIn values for each PL were: PE, -0.743^{**} ; PC, -0.030 ; PS, -0.779^{**} ; CL, -0.454 ; PI, -0.002 ; and SM, -0.290 ($*P<0.05$, $**P<0.01$).

used for multiple comparisons when pertinent. $P < 0.05$ was considered to be statistically significant. A Pearson correlation test was performed for Σ PL, individual PL percentages and every PL fatty acid and index with fish maximum lifespan. 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, USA).

RESULTS AND DISCUSSION

Fig. 1A shows total PL content and percentages of the main PLs that integrate whole fish membranes from *Nothobranchius* species and *A. australe*. Total PL content was significantly lower in the two species with the highest MLSP (*A. australe* and *N. korthausae*), which is in accordance with previous data in mammals (Ma and Gladyshev, 2017; Mitchell et al., 2007) and points to low membrane PL content as a potential predictor of longevity also in fish. The three main PL classes in fish membranes were phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS), representing 80.1–83.6% of total PL (Fig. 1A). Considering that PLs are not randomly distributed among biological membranes but rather are highly specific and characteristic, influencing their shape, structure and function (Naudí et al., 2013), observed differences in PL composition among fish species can denote distinct adaptations of biological membranes as dynamic structural defence against reactive species. In the present study, PE content was higher in whole *A. australe* membranes as compared with those of the *Nothobranchius* species while PS content of whole *N. rachovii* membranes was significantly higher than that in the other three species. A negative correlation between fish MLSP and PS content was found ($r = -0.607$, $P < 0.05$) while maximum lifespan and PE content were positively correlated ($r = 0.649$, $P < 0.05$). This is interesting as the abundance of PE positively regulates autophagy, regarded as one of the major cytoprotective mechanisms during ageing (Feng et al., 2014). Intracellular levels of PE can rise as a result of the action of phosphatidylserine decarboxylases on PS, and this increase has been associated with a reduction in the ageing-associated production of ROS and with an extension of longevity in yeast (*Saccharomyces cerevisiae*), mammalian cell cultures and flies (*Drosophila melanogaster*) (Rockenfeller et al., 2015). This mechanism could also be operating in *Nothobranchius* species as a *psid* gene, which is transduced into a mitochondrial phosphatidylserine decarboxylase proenzyme, has been identified in the genome of at least three species of the genus (*N. furzeri*, *N. rachovii* and *N. korthausae*) (www.uniprot.org).

Phospholipid fatty acid composition of whole-fish membranes was also analysed (Fig. 2; Table S1) and PIn for every PL class (Fig. 2) and for the whole membrane (Fig. 1B) were calculated. The PIn of PE and PS negatively correlated with fish maximum lifespan. This is also interesting because, as has been mentioned above, these are two of the three most abundant PL classes in fish membranes and PE and PS have a high content of docosahexaenoic acid (DHA, 22:6n-3; 22–27% for PS and 37–44% for PE) (Table S1). Therefore, PE and PS will greatly contribute to a membrane's susceptibility to oxidative damage, as the LHA theory of ageing states (Pamplona et al., 1998, 2000). Cardiolipin (CL) is a key PL for mitochondrial function that is almost exclusively located close to the site of ROS production in the electron transport chain (ETC). Additionally, CL contains high levels of linoleic acid (LA, 18:2n-6; 16–28% of CL fatty acids; Table S1), which makes it highly prone to oxidative damage. All these properties make CL a potential regulator of the processes connecting ageing and membrane lipid composition (Paradies et al., 2011). Nevertheless, although the PIn of CL was

generally higher in *Nothobranchius* species (higher susceptibility to peroxidation) than in *A. australe* (Fig. 2), there was no significant correlation between CL PIn and MLSP.

Finally, a negative correlation between membrane total PIn and fish MLSP was found (Fig. 1B), meaning that the most long-lived fish species have a lower susceptibility to oxidative damage, which is in accordance with the LHA theory of ageing. Longer-lived fish have a lower degree of fatty acid unsaturation in cell membranes as a result of decreases in highly unsaturated fatty acids like DHA (Fig. 2; Table S1) as has been widely shown in many mammals and birds (Naudí et al., 2013; Pamplona et al., 1996, 1998, 2002). The magnitude of the observed differences in these fishes, however, was much smaller than that of the inter-species differences in longevity. When an ANOVA and a Tukey's *post hoc* analysis were performed, no statistical differences in membrane total PIn values between fish species (compared one to one) were found (Fig. 1B). This suggests that the LHA theory of ageing alone is not sufficient to explain those differences and other ageing effectors, such as mitochondrial ROS production and autophagy, may be operating in an integrated way inside cells to determine longevity.

Acknowledgements

We want to thank Dr Alessandro Cellerino for his advice and support in preparing the manuscript.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.d.C., P.F.A.-P.; Methodology: P.F.A.-P.; Formal analysis: P.F.A.-P.; Investigation: P.F.A.-P.; Resources: P.F.A.-P.; Writing - original draft: P.F.A.-P.; Writing - review & editing: J.d.C., G.B.; Supervision: J.d.C., G.B.; Funding acquisition: J.d.C., P.F.A.-P.

Funding

This work was supported by Fundación ONCE (research grant to P.F.A.-P as part of the 'Oportunidad al Talento' programme for disabled scientists, co-financed by the European Social Fund), the Spanish Ministerio de Economía y Competitividad, the Instituto de Salud Carlos III through CIBERFES (CB16/10/00239), the Séneca Foundation through grant 19899/GERM/15 and the Spanish Ministerio de Ciencia, Innovación y Universidades (RTI2018-093528-B-I00), all of them co-financed by FEDER.

Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.224063.supplemental>

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