SHORT COMMUNICATION

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

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Abstract

Lipid composition of cell membranes is linked to metabolic rate and lifespan in mammals and birds but very little information is available for fishes. In this study, three fish species of the short-lived annual genus *Nothobranchius* with different maximum lifespan potentials (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 aging. Lipid analyses were performed in whole fish samples and peroxidation indexes (PIn) for every PL class and for the whole membrane, were 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 PIn and fish MLSP was found, this meaning that the longer-lived fish species have more saturated membranes and therefore, a lower susceptibility to oxidative damage, as the LHA theory posits.

Keywords: Membrane, Lifespan, *Nothobranchius*, Fish, Lipids, Peroxidation.

Summary statement: Membrane lipid analyses were performed and peroxidation indexes calculated in fishes of genus *Nothobranchius* to test if cell membrane compositions are linked to the aging rate and lifespan.

Introduction

Annual fishes of genus *Nothobranchius* have proved to be a remarkable system for gerontological research (Lucas-Sanchez et al., 2014; 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 duration of the habitat (aridity) strictly limits 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 aging 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 aging rests upon the mitochondrial oxygen free radical theory of aging and the fact that short-lived mammals and birds have species-specific high mitochondrial ROS production (mitROSp) rates at complex I (Barja, 2013). 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 (PL) (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 maximum lifespan potentials [MLSP] of 80, 63 and 53 weeks, respectively) (Genade and Lang, 2013; Lucas-Sanchez et al., 2014; Tozzini et al., 2013) and *Aphyosemion australe* that lives in permanent habitats (MLSP of ~3 years) (https://en.aqua-fish.net/fish/lyretail-killifish) were chosen to check the LHA theory of aging in fish since very little information on this vertebrate group is available.

Materials and Methods

1. Animal housing and sampling

For this study, young adults (taken just after attaining adult size and sexual maturation) of three *Nothobranchius* fish species: *Nothobranchius korthausae* (total length, 3.0 ± 0.4 mm; total weight, 0.3 ± 0.1 g; n=8), *N. rachovii* (L_T, 3.5 ± 0.3 mm; W_T, 0.6 ± 0.1 g; n=8) and *N. guentheri* (L_T, 3.0 ± 0.5 mm; W_T, 0.4 ± 0.2 g; n=8) (*Cyprinodontiformes*, *Nothobranchiidae*), and of *Aphyosemion australe* (L_T, 2.9 ± 0.2 mm; Wt, 0.3 ± 0.1 g; n=8) (*Cyprinodontiformes*, *Aplocleilidae*) were used. *A. 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). Fishes were acquired from local dealers and subjected to acclimation during 1 month in 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 ± 2ºC; Flow, 4L/h; photoperiod, 12L12D with lights on at 8pm; hardness < 6dKH; NO_3^- <0.1mgL; NO_2^-<0.1mg/L; NH_3<0.5mg/L; pH= 7.4) and fed ad libitum red mosquito larvae manually delivered twice per day.

Fishes were euthanized by exposure to the anaesthetic MS222 (200 mg/L) for 10 min following the cessation of gill movement. The whole bodies of fish were used for analyses.

Fish were treated in accordance with the current Spanish law regarding animal's 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 Consejeria de Agua, Agricultura, Ganaderia y Pesca, Comunidad Autonoma de la Region de Murcia, Spain).

2. Lipid extraction and PL class composition

Total lipid from whole-animal 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, 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 analysed by computer using IQ-Image Quant TL 8.1 software (GE Healthcare Bio-Sciences AB, Sweden).

3. PL fatty acid composition

Individual phospholipid classes from fish 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.

4. Lipid profile and lifespan.

Lipid profiles from *Nothobranchius* species and *Aphyosemion 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-Sanchez et al., 2011), *N. rachovii* (Lucas-Sanchez et al., 2014; Tozzini et al., 2013) and *N. guentheri* (Genade and Lang, 2013; Wang et al., 2017;

Zhou et al., 2019) have been shown. Regarding *Aphyosemion australe*, a maximum lifespan of 156 weeks has been reported (https://en.aqua-fish.net/fish/lyretail-killifish).

5. 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 total PL content (∑PL), individual PL class, single fatty acids, 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 ∑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).

Results and Discussion

Figure 1a) shows total phospholipid (PL) content and percentages of the main PLs that integrate whole fish membranes from *Nothobranchius* species and *Aphyosemion australe*. Total PL content was significantly lower in the two species with the highest maximum lifespan potential (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 the 80.1-83.6% of total PL (Figure 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 compositions 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 *Aphyosemion australe* membranes as compared to the

Nothobranchius species while PS content of whole *Nothobranchius rachovii* membranes was significantly higher than in the other three species. A negative correlation between fish MLSP and PS content was found (r=-0.607*) while maximum lifespan and PE content were positively correlated (r=0.649*). This is interesting since the abundance of PE positively regulates autophagy, regarded as one of the major cytoprotective mechanisms during aging (Feng et al., 2014). PE intracellular levels can rise from PS by the action of phosphatidylserine decarboxylases, and this increase has been associated to a reduction in the aging-associated production of ROS and to 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 *pisd* gene, which is traduced 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 (FA) compositions of whole fish membranes were also performed (Figure 2 and Tables S1-6) and peroxidation indexes (PIn) for every PL class (Figure 2) and for the whole membrane (Figure 1b) were calculated. PE and PS peroxidation indexes negatively correlated with fish maximum lifespan. This is also interesting because, as it has been mentioned above, these are two of the three most abundant PL classes in fish membranes and PE and PS have high contents in docosahexaenoic acid (DHA, 22:6n-3) (22-27% for PS and 37-44% for PE) (Tables S1 and S3). Therefore, PE and PS will greatly contribute to membrane´s susceptibility to suffer oxidative damage, as the longevity-homeoviscous adaptation (LHA) theory of aging 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). Besides, CL contains high levels of linoleic acid (18:2n-6, LA) (16-28% of CL fatty acids) (Table S4), which makes it highly prone to suffer oxidative damage. All these properties make CL a potential regulator of the processes connecting aging and membrane lipid composition (Paradies et al., 2011), Nevertheless, although CL PIn was generally higher in *Nothobranchius* species (higher susceptibility to peroxidation) than in *Aphyosemion australe* (Figure 2), there was no significant correlation between CL PIn and MLSP.

Finally, a negative correlation between membrane total PIn and fish MLSP was found (Figure 1b), this meaning that the most long-lived fish species have lower susceptibilities to oxidative damage, which is in accordance with the LHA theory of aging. Longer-lived

fish have a lower degree of fatty acid unsaturation in cell membranes due to decreases in highly unsaturated fatty acids like DHA (Figure 2, Tables S1-6) as it 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 (Figure 1b). This suggests that the LHA theory of aging alone is not sufficient to explain those differences and other aging effectors, such as mitochondrial ROS production and autophagy, may be operating in an integrated way inside cells to determine longevity.

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Competing interests

The authors report no conflicts of interests.

Author contributions

P.F.A.-P coordinated the different stages of this study and personally participated in fish maintenance, sample collection, biochemical analyses and manuscript writing and editing. GB contributed to the manuscript editing. JdC participated in the experiment design and the manuscript writing and editing.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends

Figure 1. a) Phospholipid (PL) content (percentage of weight of total lipid) and PL class composition (percentage of total PLs) of cell membranes from whole *Aphyosemion australe*, *Nothobranchius korthausae*, *Nothobranchius rachovii* and *Nothobranchius guentheri*. Results are expressed as mean ± SD (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 (species with `b´ have a statically higher value than those with `a´ for the same PL class) (p<0.05). ∑PL, total phospholipids; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; CL, cardiolipin; PE, phosphatidylethanolamine. b) Peroxidation index (PIn) values of total phospholipids (PL) from each of the four fish species. Results are mean ± SD (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: a) PL content and PL class composition, b) whole membrane PIn are presented in the upper boxes (*p< 0.05, **p< 0. 01).

Figure 2. Phospholipid fatty acid composition of whole fish membranes from *Aphyosemion australe*, *Nothobranchius korthausae*, *Nothobranchius rachovii* and *Nothobranchius guentheri*. Each segment of the pie chart represents the following fatty acids (clockwise order): PE, saturated (black: 18:0 and ∑saturated), monounsaturated (dark grey: 18:1n-9 and ∑monounsaturated), n-6 polyunsaturated (light grey: 18:2 n-6, 20:4 n-6 and ∑n-6) and n-3 polyunsaturated (white: 20:5 n-3, 22:6 n-3 and ∑n-3); PC: 16:0, ∑saturated, 18:1 n-9, ∑monounsaturated, 18;2 n-6, 20:4 n-6, ∑n-6, 22:6 n-3 and ∑n-3; PS: 18:0, ∑saturated, 18:1 n-9, ∑monounsaturated, 20:4 n-6, 22:4 n-6, ∑n-6, 22:6 n-3 and ∑n-3; CL: 18:0, ∑saturated, 18:1 n-9, 18:7 n-7, ∑monounsaturated, 18;2 n-6, 20:4 n-6, ∑n-6, 18:3 n-3, 22:6 n-3 and ∑n-3; PI: 18:0, ∑saturated, 18:1 n-9, 18:1 n-7. ∑monounsaturated, 18:2 n-6. 20:4 n-6, ∑n-6, 22:5 n-3, 22:6 n-3 and ∑n-3; SM: 16:0, 18:0, saturated, 18:1 n-9, 24:1 n-9, ∑monounsaturated, 18:2 n-6, 20:4 n-6, ∑n-6 and ∑n-3. Right column graphs present peroxidation index (PIn) values of each PL class for the four fish species. Results are mean ± SD (n= 4). Distinct superscript letters mean statistical differences in PIn values between fish species (compared one to one) for each phospholipid class as determined by a one-way ANOVA and Tukey's post-hoc test (p<

412 0.05). Pearson correlation (r) values between maximum lifespan potential and PIn values
413 for each PL were: PE, -0.743**; PC, -0.030; PS, -0.779**; CL, -0.454; PI, -0.002 and
414 SM, -0.290 (*p< 0.05, **p< 0.01).
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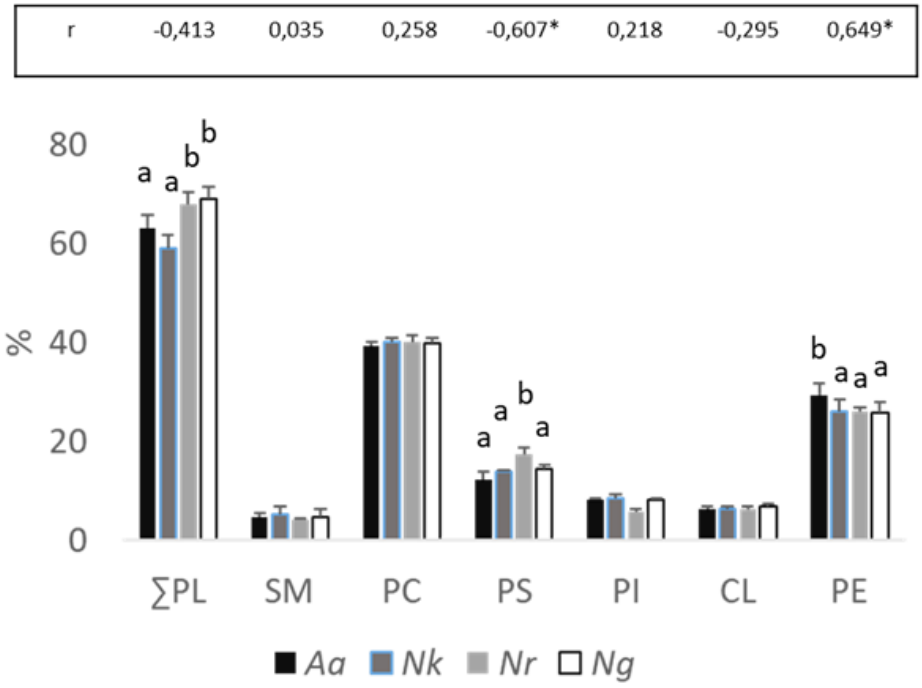
a)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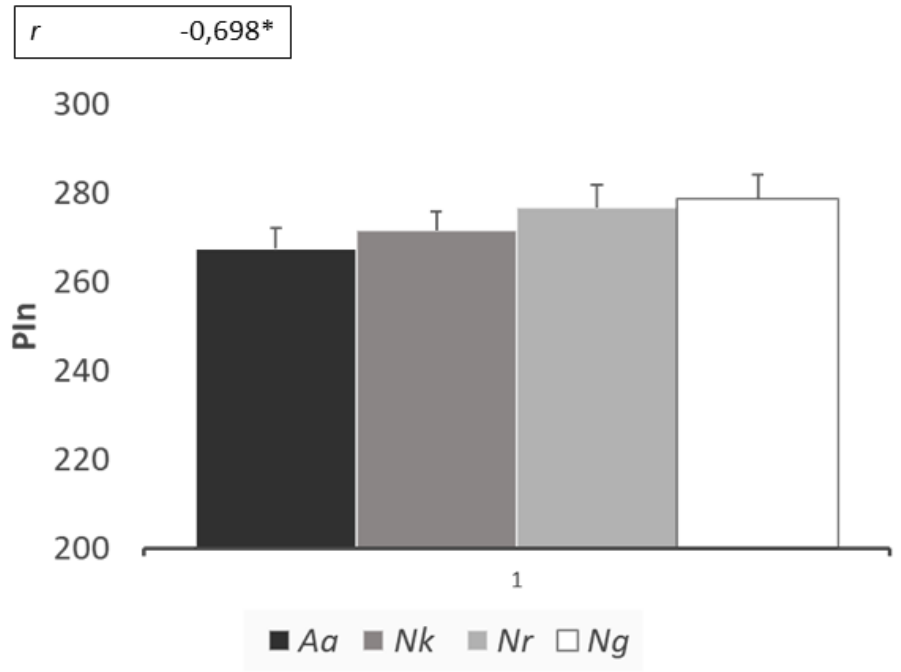
b)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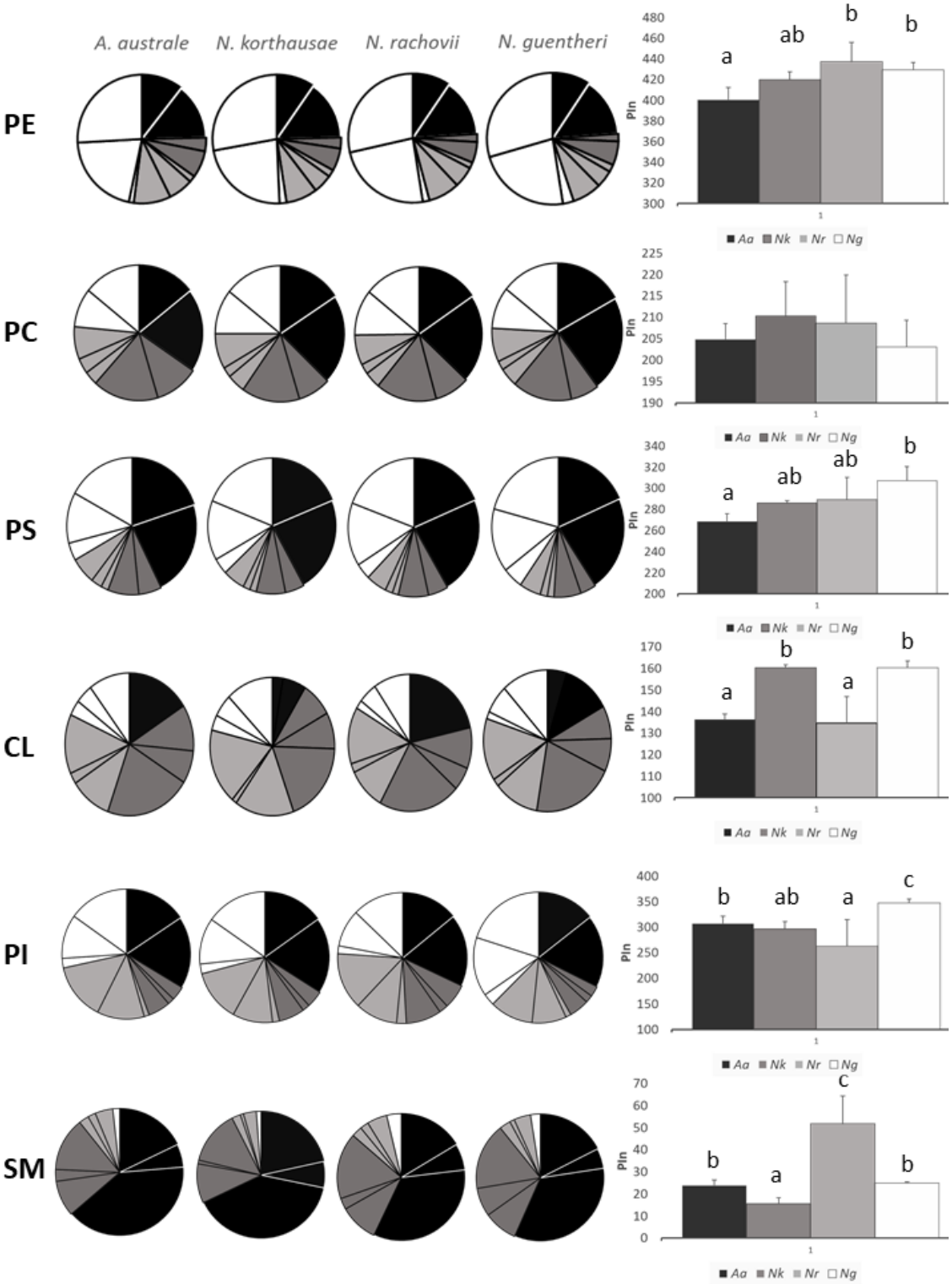
A. australe
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