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RESEARCH ARTICLE

Lack of age-related respiratory changes in *Daphnia*

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Abstract Aging is a multifaceted process of accumulation of damage and waste in cells and tissues; age-related changes in mitochondria and in respiratory metabolism have the focus of aging research for decades. Studies of aging in nematodes, flies and mammals all revealed age-related decline in respiratory functions, with somewhat controversial causative role. Here we investigated age-related changes in respiration rates, lactate/pyruvate ratio, a commonly used proxy for NADH/NAD+ balance, and mitochondrial membrane potential in 4 genotypes of an emerging model organism for aging research, a cyclic parthenogen *Daphnia magna*. We show that total body weight-adjusted respiration rate decreased with age, although this decrease was small in magnitude and could be fully accounted for by the decrease in locomotion and feeding activity. Neither total respiration normalized by protein content, nor basal respiration rate measured in anaesthetized animals decreased with age. Lactate/pyruvate ratio and mitochondrial membrane potential ($\Delta\Psi_{mt}$) showed no age-related changes, with possible exceptions of $\Delta\Psi_{mt}$ in epipodites (excretory and gas exchange organs) in which $\Delta\Psi_{mt}$ decreased with age and in the optical lobe of the brain, in which $\Delta\Psi_{mt}$ showed a maximum at middle age. We conclude that actuarial senescence in *Daphnia* is not caused by a decline in respiratory metabolism and discuss possible mechanisms of maintaining mitochondrial healthspan throughout the lifespan.

Keywords Aging · Daphnia · Respiration · Mitochondrial membrane potential

Introduction

Ever since—and even somewhat before—the role of mitochondrial membranes in oxidative phosphorylation has been elucidated, researchers asked the question of whether the efficiency of this crucial step of energy metabolism declines with age (Weinbach and Garbus 1956; Gold 1968). Nearly 60 years of research resulted in a substantial body of evidence showing that respiratory metabolism, oxygen consumption and mitochondrial properties are engaged in a complex relationship with age and aging in almost all organisms studied. Membrane phosphorylation is the chief

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generator of reactive oxygen species, a central factor of damage to membranes, proteins and DNA that accumulate with age. Such damages in turn reduce the intensity of membrane phosphorylation, resulting in reduced oxygen consumption and shifts in the balance of red-ox and phosphorylation reactions, most importantly in NAD + /NADH and ATP/ADP ratios. The decline of NAD + /NADH and ATP/ADP ratios, in turn, affects a variety of pathways that play either protective or destructive roles in cell and tissue maintenance. Reduced NAD + levels limit the activity of sirtuins (NAD-dependent protein deacetylases) and the PARP family of DNA repair enzymes (Griffiths et al. 2020), resulting in imbalance of post-translational regulation of protein activity, changes chromatin remodeling, decline in autophagy, and impaired DNA repair, thus further extending damage. On the other hand, lower level of ATP and reduced respiration can induce several interconnected protective pathways, including insulin/IGF-1 signaling, AMP-activated protein kinase (AMPK), target of rapamycin (mTOR) and HIF-1a pathways. These pathways are known to affect longevity via enhanced autophagy, mitochondrial biogenesis, antioxidant protection, stress tolerance, and immunity (Guarente and Kenyon 2000; Hekimi and Guarente 2003; Lee et al. 2010; Hekimi 2013; Burkewitz et al. 2016). In other words, the relationships between aging and respiration are complex and rich in positive and negative feedback effects which make it difficult to determine whether respiratory decline constitutes the cause (or a cause), or a consequence of aging. Majority of data on age-related respiratory function decline come from studies on nematodes, flies, and mice (Braeckman et al. 2002; Ferguson et al. 2005; Brys et al. 2007) and within the context of these model organisms it has been repeatedly suggested that gradual decline in mitochondrial function is, in fact, the underlying proximal cause of aging (Ames et al. 1995; Feng et al. 2001; Shoyama et al. 2009; Wang & Hekimi 2015; Gaffney et al. 2018). How universal is this relationship is unclear at the moment.

Gradual decline of mitochondrial function appears inevitable. Mitochondrial DNA is not protected by histones and many DNA repair mechanisms are less efficient or absent in mitochondria, while mtDNA's proximity to the site of ROS production makes it particularly vulnerable to mutations (Ames et al. 1995; Eichenlaub-Ritter et al. 2011; Palozzi et al. 2018). Other age-related changes in mitochondria include fragmentation (Jiang et al. 2015; Brandt et al. 2017; Chaudhari and Kipreos 2018; Gaffney et al. 2018) and inner membrane architecture changes (Brandt et al. 2017). It is particularly difficult to unequivocally establish whether these changes are the cause or a consequence of aging (Regmi et al. 2014).

Although repair, or elimination, or asymmetric inheritance of defective or genetically impaired mitochondria has been well described in nematodes and mammals (Palozzi et al. 2018; Lyamzaev et al. 2020; Liu et al. 2021). It is not clear how effective this process might be in an aging multicellular organism (Palozzi et al. 2018; Monaghan and Metcalfe 2019). In particular, one central mechanism of maintaining mitochondrial health is the fission-and-fusion cycle (Chan 2012; Horbay and Bilyy 2016), is associated with mitosis and therefore may not be functioning at all in post-mitotic organisms like adult *C.elegans* or imago *Drosophila.* Data from animals that are not post-mitotic are scarce and contradictory. For example, in mice (Brandt et al. 2017) there are less age-related structural damage to mitochondria in a post-mitotic tissue (heart), than in mitotically more active tissue (liver), contrary to the prediction based on mitosis-related mitochondria cleansing.

Besides age-related decline in mitochondrial function, the studies of aging and longevity phenotypes of mutants with impaired mitochondria have been extremely insightful. In *C.elegans, Drosophila,* and mice spontaneous mutants and knockouts for genes essential for electron-transport chain function or regulated by such genes typically have superficially normal phenotype, reduced membrane phosphorylation and respiration, and extended lifespan (Gems et al. 1998; Dell'agnello et al. 2007; Copeland et al. 2009; Kenyon 2010; Hekimi 2013; Maglioni et al. 2019). For some of these mutants the extended lifespan has been proven to be associated with up-regulation of HIF-1a pathway (Lee et al. 2010; Hekimi 2013) and/or enhanced immune response (Hekimi 2013); others are implicated in lifespan-regulating sirtuin, insulin/IGF-1-like, or autophagy-related pathways (Guarente and Kenyon 2000; Hekimi and Guarente 2003). Nearly all data on such mutants and knockouts emanate from the study of two or three model organisms, so would be interesting to compare age-specific respiration parameters in naturally

occurring short- and long-lived genotypes in an organism beyond the worm and the fly.

Zooplankton crustacean *Daphnia* is becoming a model organism of choice for aging research due to its ability to reproduce by cyclic parthenogenesis, thus providing an opportunity to test genetically identical individuals in different environments and to generate genetically uniform cohorts (Dudycha 2003; Yampolsky and Galimov 2005; Dudycha and Hassel 2013; Schwartz et al. 2016; Constantinou et al. 2019). Unlike nematodes and insects, cladoceran crustaceans, including *Daphnia,* retain mitosis-based cell replenishment in many tissues throughout the lifespan (Beaton and Hebert 1994; Beaton and Peters 2017), which creates an opportunity to study aging processes in a model more similar, in that respect, to mammals. Additionally, high permeability of *Daphnia* gut epithelium to a variety of hydrophilic and hydrophobic moieties and intensive water turnover through the gut results in a relative ease of drug delivery and therefore of pharmacological perturbations in the studies of lifespan and healthspan. However, at the moment, age-related changes in key phenotypes with potential for use as a model for biomedical research have not been characterized well (Cho et al. 2021). Some notable exceptions are heart rate and locomotion activity shown to markedly decline with age (Constantinou et al. 2019; Cho et al. 2021), but data on whether these changes are accompanied by decline in respiration rate and redox metabolism are scarce. The fraction of *Daphnia* energy budget allocated to respiration has been shown to remain remarkably stable over the lifespan, with differences observed between genotypes attributable to differences in locomotory activity (Glazier and Calow 1992). Here we report age-related changes (or lack thereof) in active and basal respiration rate, feeding rate, mitochondrial membrane potential and lactate-pyruvate ratio (a proxy for NADH/NAD+ ratio) in four clones of *Daphnia magna* in a latitudinal common garden study of individuals of different age from different, uniformly maintained cohorts. We find little evidence of decline of mitochondrial function, both on whole-organism and tissue-specific level, even after onset of actuarial senescence, and discuss possible reasons for that.

Materials and methods

Daphnia clones, maintenance and cohorts set-up

Four geographically distinct *Daphnia magna* clones previously characterized for life-history and longevity (Coggins et al. 2021; Anderson et al. 2021) were used in these experiments. Details of geographic origin of these 4 clones and previously measured median lifespans are given in Supplementary Table 1. The four clones also very reliably differ in adult body size (the two longer-lived clones being smaller, meaning that any clonal effect measured is normalized to individual wet weight). Stocks and experimental cohorts were maintained at 20 °C in groups of 5 adult females in 100 mL jars with COMBO water (Kilham et al. 1998) under 12:12 photoperiod and fed with *Scenedesmus* culture to the concentration of 100,000 cells/ml daily. Water was replaced and neonates removed every 3 days. This regimen was maintained in the stocks for 2 generations prior to the start of the experiment. Neonates less than 24 h of age were collected from the stock jars and used to establish cohort 1, each next cohort was established from neonates born to the previous cohort 25–35 days apart. Each cohort consisted of 25 individuals per clone, 100 total, maintained in groups of 10–12 in 100 mL of COMBO water until the age of 6 days when they were sexed transferred into new jars in groups of 5 females per 100 mL. The number of surviving individuals was recorded at the time of each water change and individuals from the same clone were combined to maintain the density of 5 individuals per jar. When such pooling was not possible, the volume of water and the amount of food in jars containing less than 5 individuals were adjusted accordingly. Individuals used for experiments were recorded as censored and not returned to the cohorts. Survival data were analyzed using Kaplan–Meier and Proportional Hazards models using JMP Reliability and Survival platform (Ver. 10; SAS Institute 2012). Only the first 3 cohorts were followed to maximal lifespan, so only these cohorts are used in formal survival analysis. Efforts have been made to ensure equal representation of all 4 clones in all ages in all experiments, despite lower portion of cohorts in two out of four clones surviving to the most advanced age; this was not, however, always possible. Specifically, in the

rhodamine assays (see below) the two short-lived clones are not represented in the most advanced age classes.

Respiration rates

Respiration rates were measured using Loligo respirometer (Loligo® Systems, Viborg, Denmark) with either 1700 µL well (for active metabolism) or 200 µL well plates. Each plate included 3 blanks. Single *Daphnia* were placed individually into the wells containing either 1700 µL of COMBO water or 200 µL 1% urethane solution in COMBO water (Philippova and Postnov 1988) for active and basal metabolism, respectively, with water, plates, and sensors equilibrated to 20 °C. Oxygen concentrations were measured every 15 s after initial 15-min break-in period for 45 min or until the concentration decreased by 2 mg/L from the blank readings. Oxygen consumption per minute was calculated from a linear regression of oxygen concentration over time; mean blank estimates subtracted. After measurements *Daphnia* were removed from Loligo wells, their body length and wet weight were measured, and individuals were frozen for lactate, pyruvate, and protein content determination. Respiration rates were normalized by individuals' wet weight only for presentation purposes. To detect a significant change across ages, however, respiration rates were calculated per individual and wet weight was used as a co-variable. The same approach was used for the analysis of feeding rate. Additionally, respiration rates were also normalized by individuals' protein content (see below). In both normalizations, when either wet weight or protein content was used as a covariable, none of the interactions between the covariable and main effects were significant, so they were dropped from the models.

Feeding rate

Feeding (filtering) rate was determined by the decrease of chlorophyll fluorescence using Loligo respirometers equipped with regular plastic 24-well plates, each well containing a single *Daphnia* and COMBO water initially containing *Scenedesmus* algae with the concentration of 200,000 cells/mL. Measurements were done in the initial algae suspension and after 18 h of feeding in a dark incubator at 20 °C and the amount consumed was determined from the difference in fluorescence using a calibration with a linear range between 25,000 and 400,000 cells/mL. After measurements *Daphnia* wet weight was determined to be used for normalization purposes and individuals were placed back into their corresponding cohort jars.

Lactate/pyruvate ratio

To quantify red-ox equilibrium whole body extract lactate to pyruvate ratio was measured in *Daphnia* of different age. Lactate to pyruvate ratio is commonly used as a proxy to cytosolic NADH to NAD balance, as unambiguous determination of NAD + and NADH is difficult due to larger portion of these coenzymes being bound to proteins and both substrates of lactate dehydrogenase are at equilibrium with the reduced and oxidized forms of the coenzyme (Williamson et al. 1967; Mintun et al. 2004). The estimated NAD + / NADH ratio is inversely proportional to the lactate/pyruvate ratio, with the coefficient of proportionality being the inverse of the equilibrium constant of the lactate dehydrogenase reaction. This approach is not problem-free and deviations from the equilibrium may result in biased estimates of NAD + /NADH balance (Sun et al. 2012), but it is conventional as a measure of red-ox balance changes over age or in diagnostics of various clinical conditions related to mitochondrial health and hypoxia (Debray et al. 2007; Rimachi et al. 2012).

Lactate and pyruvate concentrations in whole-body extracts were measured using CellBiolab lactate (MET-5013) and pyruvate (MET-5029) fluorometric assay kits. The same individuals used in respiration measurements were weighed to determine wet weight and frozen at − 80 °C. Each *Daphnia* was homogenized in 100 µL ice-cold PBS with a pestle and the homogenates were centrifuged at 4 °C for 4 min. 25 µL of supernatant were pipetted into each of the 96-well lactate and pyruvate assay plates with well contents according to manufacturer's protocols. Additionally, 10 µL of the supernatant was used, in duplicate to quantify soluble proteins by Bradford assay with BSA in concentrations between 0.007 and 2 mg/mL used for plate-specific calibration; regression on log-transformed BSA concentration and 595 nm absorption values linear within the range between 0.03 and 1 mg/mL.

Mitochondrial membrane potential

Individual *Daphnia* were placed in 0.5 mL of COMBO water containing 4 µM solution of rhodamine123 (made from 1000X stock in DMSO) in the dark for 24 h. After threefold rinsing with COMBO water individuals were photographed using a Leica DM3000 fluorescent microscope with a 10 × objective (0.22 aperture) equipped with Leica DFc450C camera using the 488 nm excitation / broadband (> 515 nm) emission filter. The following ROI were selected peduncle of antenna-2 (striated muscle), heart, brain, optical lobe, 2nd epipodite or "gills" (thoracic appendage branch with osmoregulatory functions), and non-neural (parenhymal) head tissue. Median fluorescence (background subtracted) was recorded with exposure of 100 ms with gain 1. The concentration of 4 µM rhodamine123 was chosen as being close to Michaelis–Menten constant of the process of accumulation of the dye in mitochondria (Hasan 2019; Anderson et al. in preparation). Average values of 2–3 replicate individuals from the same clone and the same age measured on the same date are reported.

Results

Cohorts

As expected, the two short-lived and the two long-lived clones differed from each other in lifespan, although the very old individuals from the short-lived FI tended to survive the longest (Supplementary Figure S1). By the highest age studied *Daphnia* in all clones experience actuarial aging with mortality increasing approximately sevenfold relative to initial mortality (Supplementary Figure S1B).

Respiration rates

Wet weight-normalized respiration rate decreased linearly with age, both in active and in anesthetized *Daphnia* (Fig. 1). However, the decline of respiration per individual was statistically significant, with wet weight as a co-variable, only for active metabolism measurements (Table 1). Respiration rates normalized by protein content showed no decline with age, either in active or in anesthetized animals (Supplementary

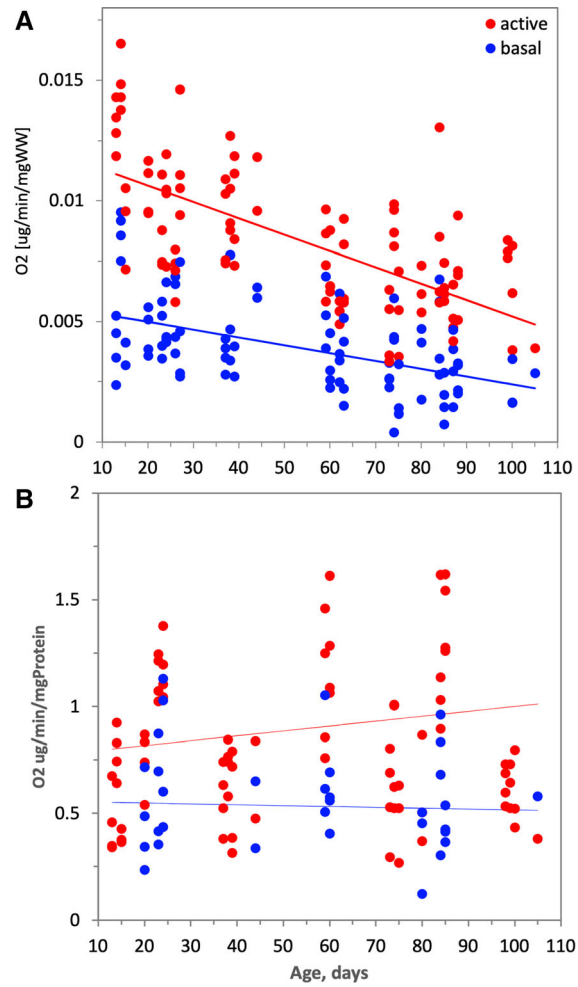

Fig. 1 Active (red) and basal (blue) respiration rate normalized as either wet weight (**A**) or protein content per individual (**B**), as a function of age. Each dot represents an average of 2–4 replicate individuals measured on a given day. Regression lines drawn through all replicates; thick lines $P < 0.05$, thin lines $P > 0.05$. See Table 1 and Supplementary Table S2 for statistical tests

Fig. S2); statistical analysis with the use of protein content as a covariable actually showed a miniscule, but statistically significant ($P < 0.0021$) increase in protein content-specific respiration rate with age (Supplementary Table S3). Regression coefficient (SE) for the age variable was estimated at R = 6.5E-5 (2.1E-5), which translates in an increase of oxygen consumption, protein content corrected, from 0.025 to 0.03 µg/min/individual over the median lifespan of 60 days. The reason for this discrepancy was a significant decline of protein content per wet weight with age (Supplementary Fig. S3). This decline was

Table 1 Analysis of variance of the effect of age on active and basal respiration rate (μg O_2 /min/individual) and feeding rate in 4 clones of *D.magna*

Source	DF	Sum of Squares	F Ratio	Prob > F
Active respiration				
Clone	3	0.00022	0.627	0.6
Age	1	0.00088	7.52	**0.0065**
Clone*age	3	0.00013	0.381	0.77
WWmg	1	0.00347	29.775	**< .0001**
Error	305	0.03551		
Basal respiration				
Clone	3	0.00009	0.293	0.83
Age	1	0.00012	1.225	0.27
Clone*age	3	0.00043	1.476	0.22
WWmg	1	0.00037	3.747	0.054
Error	261	0.02559		
Feeding rate				
Clone	3	3998.88	5.51	**0.004**
Age	1	1390.44	5.75	*0.023*
Clone*age	3	1100.78	1.52	0.23
WWmg	1	469.59	1.94	0.17
Error	31	7501.17		
Lactate:pyruvate ratio				
Clone	3	14.68	0.95	0.42
Age	1	14.02	2.73	0.10
Clone*age	3	1.49	0.1	0.96
Error	80	411.44		

Wet weight included in the models as a covariable, except for lactate-pyruvate ratio. P-values < 0.01 shown in **bold**, < 0.05 in *italics*

not an artifact of overestimation of protein content in the oldest (also largest) or underestimation of protein content in the youngest (smallest) individuals due to extrapolation outside of Bradford calibration curves, as only 14 observations feel outside of the log-linear range of the calibration and their removal does not change this result (Supplementary Fig. S3). There was no evidence of clone-by-age interaction for either wet weight or protein content normalization. There was also no correlation between respiration rate and either the number of eggs in the clutch carried at the time of measurement or with the development stage of the clutch (data not shown). There was, however, a slight difference in active respiration rate (Supplementary Fig. S2) between individuals carrying a clutch as compared to those carrying no clutch and without visible ovaries or those carrying no clutch but with visible ovaries (i.e., within 24 h of producing a new clutch). This difference remained significant when both wet weight and age were accounted for by including these terms as co-variables. It is unlikely that this difference indicates disproportionally higher respiration rate of embryos (Glazier 1991), as it was only observed in actively moving individuals. Rather, it may indicate higher expenditures on active swimming while carrying a clutch.

Feeding rate

There was a consistent decline in feeding rate normalized by wet weight with individual's age (Fig. 2). This decline is not a normalization artifact, as the age term remains significant with wet weight used as a covariable (Table 1). There was also a significant clone effect with different clones showing different feeding rates with body size accounted for.

Lactate/pyruvate ratio

Overall lactate/pyruvate ratio was found to be low: 3.70 ± (SE) 0.23. Contrary to the predictions, the lactate/pyruvate ratio did not increase with age. If

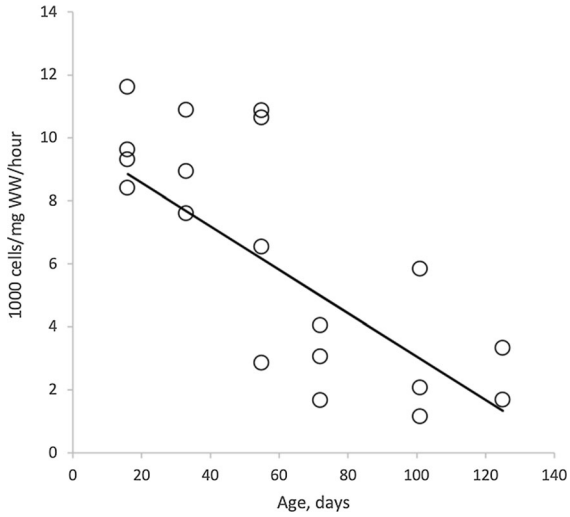

Fig. 2 Feeding rate (10^3 cells/mg wet weight / hour) as a function of age

anything, there was a slight decrease, not reaching a statistical significance and with less than 25% relative change over the entire lifespan in magnitude (Fig. 3, Table 1). The slight decrease was caused by an equally slight increase in pyruvate concentration with completely constant lactate concentration (both normalized by protein content) over age (data not shown).

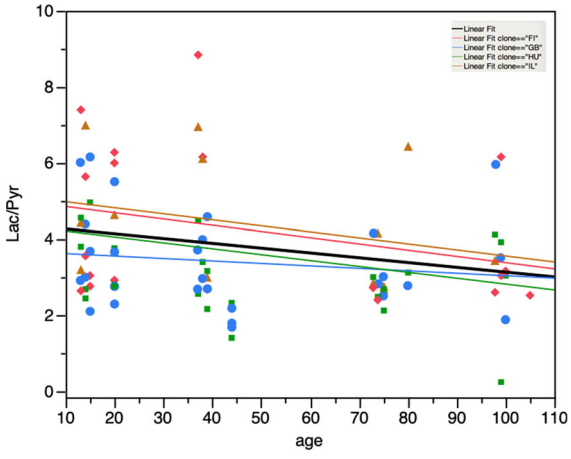

Fig. 3 No significant changes in lactate/pyruvate ratio with age (days) in 4 *Daphnia* clones. Overall regression coefficient $R = -0.0126 \pm 0.0077$. Symbols here and below represent different clones to illustrate no clonal differenced

Mitochondrial membrane potential

Rhodamin-123 assay of mitochondrial membrane potential ($\Delta\Psi_{mt}$) showed little change with individuals' age in most organs and tissues studied (Fig. 4, Table 2, Supplementary Table S3). The only exceptions were the optical lobe (Fig. 4 D), in which there seemed to be a maximum of membrane potential around the age of 75–80 days with a slight decline afterwards (the quadratic term of a second-degree polynomial regression $R = -0.00223 \pm 0.00095$; $P < 0.0254$) and the epipodite (gill) tissue in which $\Delta\Psi_{mt}$ showed a consistent decline with age (Fig. 4E). Such differential effect of age in different tissues (ROIs) resulted in a significant age*ROI interaction term (Table 2).

Discussion

We measured several parameters of *Daphnia* respiratory red-ox metabolism across a wide range of ages, including highly advanced age when actuarial senescence is very apparent and to which only $\sim$ 10% of the initial cohort survives. We did not observe any significant changes in basal respiration rate, lactate and pyruvate concentrations and their ratio, and, with one exception, $\Delta\Psi_{mt}$. The exception is total respiration rate normalized by individuals' wet weight that significantly declined with age, the effect that can be accounted for by age-related decrease of locomotory activity (Constantinou et al. 2019; Cho et al. 2021) and/or by the decrease of filtering activity as described here. Thus, *Daphnia* is capable of maintaining active aerobic respiration even in the part of the lifespan when many other organs fail, and the age is approaching maximal lifespan. Likewise, none of the respiratory parameters measured differed significantly among the four clones used in the experiment, despite a marked difference in lifespan. This indicates that respiratory decline is not a necessary condition for aging or genetic differences in longevity in *Daphnia.* It also suggests that mitochondrial damage remediation mechanisms may be more efficient in *Daphnia* than in similar size and lifespan model organisms such as *Caenorhabditis elegans* or *Drosophila. Daphnia magna* is a long-lived species among cladocerans, with lifespan under laboratory conditions up to three times longer than, for example, in a comparable size

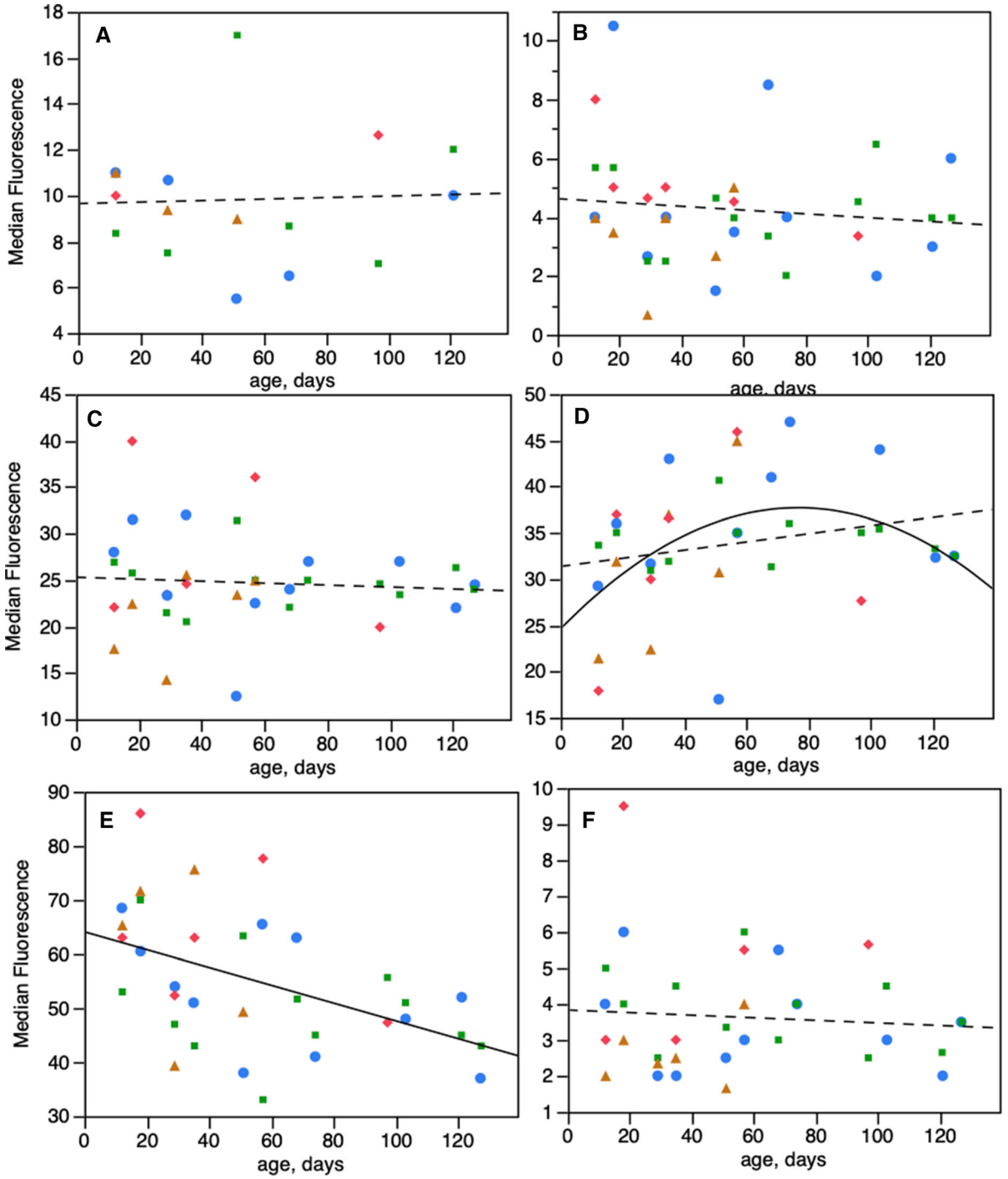

Fig. 4 Rhodamine-123 fluorescence (median, background-subtracted) in various tissue of *Daphnia* as a function of age. High values are indicative of higher mitochondrial membrane potential. Averages of 2–4 replicates per clone per age measured on the same data are shown. **A**: antenna-2 (striated muscle); **B** heart; **C** brain; **D** optical lobe; **E** epipodite; **F** non-neural head tissue. Regressions shown: dashed line where not significant, solid lines where significant ($P < 0.005$; Bonferroni-adjusted $P < 0.03$), quadratic regression shown where the 2nd degree term significant ($P < 0.025$; non-significant with Bonferroni adjustment). Note different scales of fluorescence intensity of different plots

Table 2 Analysis of variance of the effects of age, clone, and tissue (region of interest, ROI) on rhodamine-123 fluorescence

Source	DF	Sum of Squares	F Ratio	Prob > F
Clone	3	206.09	0.94	0.42
age	1	115.47	1.59	0.21
Clone*age	3	51.31	0.23	0.87
ROI	5	77,296.49	212.26	< .0001
Clone*ROI	15	615.98	0.56	0.9
Age*ROI	5	1694.01	4.65	0.0004
Clone*age*ROI	15	706.37	0.65	0.84
Error	387	28,185.84		

ROI's studied include: muscle tissues (antenna-2, heart), neural tissue (brain, optical lobe), non-neural head tissue and epipodite (gill)

See Fig. 4 and Supplementary Table S3 for the analysis for each ROI separately

D. pulex which typically lives for 30–60 days in similar conditions (Latta et al. 2011; Plaistow et al. 2015). It is not known whether *D. magna*'s extended lifespan is achieved by higher investments into maintaining mitochondria.

What this observation means for potential interventions aiming to delay aging in humans is open to interpretation. If it is indeed true that a relatively long lifespan in *D. magna* is achieved by maintaining respiration and red-ox metabolism, this might be an argument in favor of NAD + supplementation to avert aging. On the other hand, the very fact that *Daphnia* succumbs to aging and die despite maintaining specific respiration rate, lactate/pyruvate ratio, and mitochondrial membrane potential comparable to those of young individuals, suggests that there are other, non-respiratory related caused of aging and just intervening to maintain, for example NAD + levels might not achieve the desired goal.

How does maintained respiration balance out with age-related changes in other traits? We observed that while the total body weight-adjusted respiration rate declined with age, the basal rate measured in urethane-anesthetized animals did not once the body weight adjustment is applied (Table 1). Urethane reversibly paralyzes striated muscles, in *Daphnia* resulting in ceased antennal beat and thoracic appendages movement. Both result in decreased oxygen demand, but the immobilization of thoracic appendages also affects the supply of oxygen. Although epipodites are not an exclusive site of dissolved gases exchange with the environment (Pirow et al. 1999; Smirnov 2017), the circulation of water created by the movement of thoracic limbs is critical for oxygen supply. In immobilized animals oxygen enters tissues only through diffusion. We believe that it is primarily the immobilization of these appendages that created a lower, and age-independent level of oxygen consumption in urethane-treated animals. It is unlikely that immobilizing antennae could have had a profound effect on respiratory demand in our experimental set-up because not much swimming activity occurs in the 1.7 mL, 12 mm diameter Loligo wells even in untreated individuals, where their locomotion is likely to be suppressed by frequent encounters with the well walls. Post-measurement observations showed that these *Daphnia* were invariably found at the top of the well, possibly to avoid the fluorescence excitation light coming from the bottom of the well, showing no swimming activity. However, it is impossible, in this experiment, to distinguish between reduced supply and reduced demand effects of immobilization. Because the movement of thoracic appendages is also required for filter-feeding, the observed decline in feeding rate with age should also result in both reduced demand and reduced supply of oxygen. It is probably not by chance that the only tissue in which we observed a decline of $\Delta\Psi_{mt}$ with age was the epipodites, or "gills"—leaf-shaped leaf-shaped branches of thoracic limbs in cladocerans. These mitochondria-rich organs (Kikuchi 1983) have the function of dissolved gasses and ions exchange with the environment and osmoregulation in the adult *Daphnia* (Pirow et al. 1999; Smirnov 2017). It is tempting to assume that the apparent decrease of mitochondrial membrane potential in these organs with age is also related to the decrease in filtering appendages movement rate, resulting in lower energetic demands for their gas exchange, excretory, and osmoregulatory functions.

When measured respiration rates were normalized by protein content rather than wet weight, there was a miniscule increase of respiration rate with age. This was caused by a significant decline of protein content per wet weight in older individuals, obscuring true respiratory changes or lack thereof. Age-related decline of weight-specific protein content has been long known in insects (Utsumi and Natori 1980; Jit

and Sharma 1983), and, in the context of age-related sarcopenia, in humans (Proctor et al. 1998), but to our knowledge has not been previously demonstrated in *Daphnia.* It may be related to reduced muscular activity, reduced yolk proteins production, or increase in body lipid content (Constantinou et al. 2019) or relative thickness and weight of the carapace (Glazier and Calow 1992).

Whether or not the observed constancy of specific respiration rates and $\Delta\Psi_{mt}$ can be explained by continuous mitoses in adult *Daphnia* (as opposed to worms and flies) remains speculative. A suite of mitochondrial health maintaining mechanisms that function during the fission-and-fusion cycle (Chan 2012) include protein content and membrane composition homogenization, complementation (and possibly repair) of mtDNA mutations, elimination of mitochondria that have lost their membrane potential and/or their DNA, and facilitation of apoptosis by mitochondrial fission. All these processes critically depend on events during mitosis or S-phase and apoptosis and additionally require replenishing that eliminates cells from a population of dividing stem cells. None of these events are directly possible in post-mitotic organisms like nematode or nearly post-mitotic ones like insects, but may well be occurring in *Daphnia,* in which mitoses continue throughout adult life in at least some tissues. It is not known whether cell division (and thus possible mitosis-associated mitochondrial quality maintenance through fission–fusion cycles) occurs in most of the tissues studied here in the $\Delta\Psi_{mt}$ assays, except, again, epipodites. In epipodites the number of cells remains constant, and growth occurs via increasing cell volume and endopolyploidy (Beaton and Hebert 1989; Yampolsky, L.Peshkin and M.Kirschner, unpublished data); they are also the only tissue studies in which $\Delta\Psi_{mt}$ shows a decline with age.

Keeping in mind that members of all clonal cohorts are genetic copies of each other (safe for de novo mutations) and shared current, maternal and grandmaternal environments, thus making them likely to be epigenetically uniform, it is impossible to fully exclude cohort heterogeneity. It is entirely possible that even initially identical individuals may diverge in their pathways of accumulating damage throughout the lifespan. Thus, when measuring respiration and $\Delta\Psi_{mt}$ in old *Daphnia* we may be biased by measuring it in unusually healthy individuals that successfully survived to an old age. It would be necessary to conduct a longitudinal experiment with individually maintained *Daphnia,* tracking individual changes in respiration rate, and correlating them with lifespan. Such a study will have a number of limitations: lactate/pyruvate ratio could only be measured in embryos or neonates rather than maternal tissue, while the rhodamine assays may have an effect on survival and/or further assays in the same individual (it does not cause immediate mortality if the specimen is exposed to the excitation light for less than a minute; L.Yampolsky, unpublished). Yet, such an experiment might reveal a decrease in respiration immediately prior to death. If such decreases become more apparent with age, this finding might refute the conclusion of the present study. It would then be interesting to investigate what genetic or epigenetic changes or damages cause such divergence of respiratory health in identical individuals.

However, until such a longitudinal study is conducted, our conclusion stands, and it raises the question about the generality of age-related respiratory decline. Is maintenance of respiratory metabolism and mitochondrial membrane potential throughout the lifespan a *Daphnia*-specific phenomenon? Can it be used to pinpoint potential maintenance investments and/or rejuvenation mechanism that *Daphnia* employs to preserve mitochondrial function and red-ox balance all the way into very advanced age? Is the observed decrease in total oxygen consumption with age fully explainable by the reduced decline in feeding rate and the associated decline in thoracic limb movement and is such decline operating through decreased demand or decreased supply of oxygen? More generally, it is not clear whether the observed decrease in total respiration rate is a result of age-related damages (i.e., reduction of mitochondrial efficiency) or of age-related decrease in demand. The observed decrease in $\Delta\Psi_{mt}$ in the very organ responsible for oxygen delivery to tissues is consistent with both possibilities.

Conclusions

Contrary to expectations, we did not detect any intrinsic, motility-independent age-related changes in respiration rate, lactate/pyruvate ratio and mitochondrial membrane potential ($\Delta\Psi_{mt}$) in *Daphnia magna.* The only possible exception is the decrease of $\Delta\Psi_{mt}$ in

epipodites (gills)—thoracic limbs branch responsible for gas and ion exchange with the environment, indicative of either reduced demand or reduced supply of oxygen in older *Daphnia.* Relatively long lifespan of *D.magna* among daphnids and among invertebrates of similar size may be explained by more efficient mitochondrial maintenance mechanisms due to retained mitoses in adult tissues.

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Data Availability Data are available as on-line Supplementary data, https://doi.org/10.6084/m9.figshare.17303144.

Code availability Not applicable.

Declarations

Conflict of interest All authors declare that they have no conflict of interest.

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