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Mitochondrial ageing of a polar and a temperate mud clam

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Abstract

We investigated mitochondrial ageing in a temperate (*Mya arenaria*) and an Antarctic (*Laternula elliptica*) mud clam, with similar lifestyle (benthic filter feeders) but different maximum life spans (MLSP), 13 and 36 years, respectively. The short-lived temperate *M. arenaria* showed a more pronounced decrease in mitochondrial function (respiration, respiratory control ratio, proton leak, membrane potential) with age than the long-lived Antarctic *L. elliptica*. H_2O_2 generation rates at habitat temperature were far higher in the short-lived *M. arenaria* compared to *L. elliptica*. Reactive oxygen species (ROS) production as proportion of the mitochondrial oxygen consumption rate ($\%H_2O_2/O_2$) increased significantly with age in *M. arenaria*, whereas in *L. elliptica* the proportion remained unchanged. Lower rates of mitochondrial H_2O_2 generation were presumably due to mild uncoupling as *L. elliptica* mitochondria showed higher proton leak compared to *M. arenaria* mitochondria. The results are discussed in to the light of the "Free Radical-Rate of Living theory", (Pearl, R., 1928. The Rate of Living. Alfred Knopf, New York; Harman, D., 1956. Aging: a theory based on free radical and radiation biology. J. Gerontol. 11, 298–300) and the "Uncoupling to Survive" hypothesis (Brand, M.D., 2000. Uncoupling to survive? The role of mitochondrial inefficiency in ageing. Exp. Gerontol. 35, 811–820).

Keywords: Mitochondrial ageing; Marine bivalves; Polar ecology

1. Introduction

With the exception of some simple forms like hydra, planarians and turbellarians (Child, 1915; Balazs and Burg, 1962; Haranghy and Balazs, 1964; Martinez, 1998), all multicellular organisms age. Two theories link the process of ageing and the maximum life span (MLSP) of a species to mitochondrial oxygen free radical (ROS) formation:

(i) The "Free Radical-Rate of Living theory", (Pearl, 1928; Harman, 1956) predicts a negative correlation between SMR and MLSP due to increased mitochondrial production of oxygen free radicals at higher standard metabolic rate (SMR) (Ku et al., 1993).
(ii) The "uncoupling to survive" hypothesis (Brand, 2000) is based on the same assumption of a negative correlation between ROS production and MLSP, but further predicts that mitochondrial uncoupling mechanisms may modulate reactive oxygen species (ROS) production, altering the strict dependency of ROS formation on SMR.

When comparing marine and freshwater ectotherms of similar lifestyle, several groups discovered higher MLSPs in species from permanently cold compared to temperate environments (Brey, 1991; Brey et al., 1995; Ziuganov et al., 2000; Cailliet et al., 2001; La Mesa and Vacchi, 2001). In Antarctica, marine ectotherms experience year round permanent cold temperatures between −1.9 and +2.0 °C and most species have lower SMRs than related species from temperate environments, where temperatures fluctuate from 0 to 18 °C (Clarke, 1983; Heilmayer et al., 2004). According to the "Free Radical-Rate of Living theory", these lower SMRs and the correspondingly low ROS generation by aerobic mitochondrial activity might result in a slow down of physiological ageing and may explain the higher MLSP of polar ectotherms compared to their tem-

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perate relatives. However, aside from higher mitochondrial densities in several polar (Johnston et al., 1998) and subpolar species (Sommer and Pörtner, 2002), mitochondrial adaptations in the cold involve higher cristae densities and altered membrane fatty acid composition, that may modulate free radical leakage from mitochondria and compromise the simple relationship between SMR and MLSP (Archer and Johnston, 1991; Johnston et al., 1994; St.-Pierre et al., 1998; Sommer and Pörtner, 2002).

For an analysis of whether MLSP is set by the level of SMR and by associated differences in mitochondrial functioning in marine ectotherms, we investigated age dependent changes of mitochondrial energy coupling and ROS formation in isolated mitochondria from mantle tissue of the Antarctic mud clam *Laternula elliptica* (Pholadomyoida) and the North Sea mud clam *Mya arenaria* (Myoida). Both clams are representatives of the same ecotype (benthic filter feeders and burrowing clams) and important key species in their respective habitat, but have adapted to different temperature regimes over long evolutionary time scales (Soot-Ryen, 1952; Petersen et al., 1992; Jonkers, 1999). With a maximum age of approximately 36 years, *L. elliptica* has a three-fold longer MLSP than *M. arenaria* with ~13 years MLSP. Although the animals belong to different bivalve subclasses their similarity in size, morphology and lifestyle should justify a comparison of physiological ageing parameters between both species.

2. Materials and methods

2.1. Sampling and maintenance

2.1.1. Laternula elliptica

Antarctic *L. elliptica* were collected by divers in the Potter Cove, King George Island, South Shetland Island (62°14′S, 58°40′W) in November–February 2002/2003 at 5–10 m depths, 34 PSU and temperatures between −1 and +2 °C. Animals were maintained in aquaria with seawater from the cove at 0 °C for several days until they were used for experimentation. Water was exchanged once a week to ensure a good water quality and food supply. All measurements were carried out at the Dallmann-Laboratory, King George Island, Antarctica.

2.1.2. Mya arenaria

North Sea *M. arenaria* were sampled with a box corer at a shallow subtidal muddy site close to Harlingen (53°09′N, 05°19′E), The Netherlands, in summer 2002 and 2003. At the Alfred-Wegener-Institute, animals were kept in aquaria with sediment at 10 °C seawater and 28 PSU salinity. Animals were fed plankton tablets (REBIE, Germany) twice a week. All measurements were undertaken at the Alfred-Wegener Institute for Polar- and Marine Research in Bremerhaven, Germany.

2.2. Age determination

Age determination for *M. arenaria* was done using a Von Bertalanffy (1934) growth model (VBGF) based on length-at-age data of the same subtidal population:

$$St = 117.17 \times (1 - e^{-0.12\times(t+0.054)})$$

Due to the high variability in the relation between shell length and age, *L. elliptica* were individually aged by individual ring counts in polished cuts through the shell umbo (Brey and Mackensen, 1997). For a more detailed description see Philipp et al. (2005 in press).

2.3. Mitochondrial measurements

2.3.1. Isolation of mitochondria

Mitochondria were isolated from the mantle tissue of freshly sacrificed bivalves. Depending on size, tissues of 1–9 individuals of *L. elliptica* and up to 22 *M. arenaria* specimens were pooled for one experiment. About 4 g of mantle tissue was finely chopped in 10 ml ice-cold homogenisation buffer (400 mM sucrose, 70 mM Hepes, 100 mM KCl, 3 mM EDTA, 6 mM EGTA, 1% bovine serum albumine, 1 μl/ml aprotinine, pH 7.3) modified after Moyes et al. (1985) and Heise et al. (2003).

Briefly, the tissue was homogenised in a pre-cooled glass/teflon-homogeniser, the homogenate centrifuged at 1300 × *g* for 15 min at 2 °C and the supernatant collected. The pellet was resuspended, homogenised, and again, mitochondria extracted at the same speed. The two supernatants were combined and centrifuged at 10,500 × g for 15 min to sediment the mitochondria. The resulting mitochondrial pellet was resuspended in 1.5–2 ml assay medium (560 mM sucrose, 100 mM KCl, 10 mM KH_2PO_4, 70 mM Hepes, 5 mM glutamate, 1 μg/ml aprotinine and 1% bovine serum albumine at pH 7.3).

2.3.2. Respiration and membrane potential of isolated mitochondria

Measurements were carried out using a water-jacketed respiration chamber under gentle stirring. Mitochondrial respiration was recorded by oxygen microoptodes (TX PreSens GmbH, Neuweiler, Germany) and membrane potential determined using TPMP-sensitive electrodes according to Brand (1995). Measurements were carried out at 0 °C for *L. elliptica* and 10 °C for *M. arenaria* mitochondria. Oxygen concentrations were calculated using the oxygen solubility (βO_2) according to Johnston et al. (1994) and the atmospheric pressure of the day. For mitochondrial respiration and membrane potential measurements 5 mM succinate was added as substrate and 5 μM rotenone to prevent respiration of endogenous NAD-linked substrates (Brand, 1995). State 3 respiration was induced by addition of 0.075–0.15 mM (*M. arenaria*) and 0.06–0.09 mM (*L. elliptica*) ADP. Non-phosphorylating respiration, comprising oxygen consumption by proton leak and

ROS formation (state 4+), was recorded after adding 2 μg/ml, of the F_0F_1-ATPase inhibitor oligomycin. The respiratory control ratio (RCR), which describes how effectively the respiratory chain is coupled to the ATPase, was calculated according to Estabrook (1967), using state 4+ respiration. The percentage of proton leak of state 3 oxygen consumption was calculated as (state 4+/state 3) × 100. Membrane potential measurements are described in detail in Keller et al. (2004).

2.3.2.1. Production of hydrogen peroxide (H_2O_2) by isolated mitochondria. Mitochondrial hydrogen peroxide production was measured fluorimetrically ($\lambda_{excitation}$ = 312 nm and $\lambda_{emission}$ = 420 nm) recording the reaction of H_2O_2 with homovanilic acid (HVA) in the presence of horse radish peroxidase (HRP) modified after Miwa et al. (2003). Measurements of the H_2O_2 generation rate of *L. elliptica* mitochondria were performed with a Shimadzu (RF-1501) fluorometer at the Dallmann Laboratory, whereas *M. arenaria* mitochondria were measured with a Perkin Elmer (LS 50B) fluorometer in Bremerhaven. Incubation of 275 μl *L. elliptica* mitochondrial suspension in 775 μl assay medium, containing 10 μM rotenone, 0.3 mM HVA, 6.5 U/ml HRP was performed in a water-jacketed respiration chamber under gentle stirring outside the fluorometer at 0 °C. Initial fluorescence recordings on a two channel chart recorder (Kipp & Zonen, Netherlands) were run after adding 200 μl of the incubated mitochondrial mixture to 800 μl phosphate buffer (10 mM KH_2PO_4, 100 mM Na_2HPO_4, pH 7.1). After 45 min of state 2 respiration with succinate, fluorescence values were recorded again (200 μl incubated mitochondrial suspension plus 800 μl phosphate buffer) and the difference to baseline fluorescence was calculated. H_2O_2 generation in state 3 was measured after adding ADP to the incubated mitochondria. In each experiment, fluorescence was calibrated with an H_2O_2 standard (0.2 nM; Merck, Germany). Each value was determined with 2–3 replicate measurements and the whole experiment was repeated at least twice with every mitochondrial isolate. Controls run without mitochondria, showed no fluorescence produced under any assay condition.

Using the LS 50B Perkin Elmer fluorometer with a cooled sample compartment and magnetic stirring, the H_2O_2 generation in states 2 to 4+ were recorded for one and the same mitochondrial aliquot directly in the fluorometer. *M. arenaria* mitochondrial solution (150 μl) was incubated with 850 μl assay medium, with the following chemicals added in the order: 5 μM rotenone, 5 μM of the myokinase inhibitor Ap5A (P^1,P^5-adenosine-5′-pentaphosphate), 0.1 mM HVA, 2.5 U/ml HRP at 10 °C. When a steady fluorescence signal was reached, succinate (state 2), ADP (state 3) and oligomycin (state 4+) were added following the same protocol as in the respiration measurements. Again each measurement was calibrated with an H_2O_2 standard.

A comparison with both fluorometers was run on *M. arenaria* mitochondria to assure that methodological effects did not compromise the results. Both, H_2O_2 generation rates and oxygen consumption rates were measured in parallel and related to mitochondrial protein (Keller et al., 2004).

2.4. Enzyme assays

For enzyme assays animals were rapidly dissected and mantle tissue freeze clamped and stored in liquid nitrogen until processing. Assay temperature for photometric measurements was set to in situ temperature for both animals, 0 °C for *L. elliptica* and 10 °C for *M. arenaria*, and 20 °C for both species as a reference temperature. Assays were run at least in duplicate for each sample and results expressed as international units (μmol of substrate converted to product min^{-1}) mg^{-1} protein. Protein content was determined with the Biuret method modified after Kresze (1988).

2.4.1. Cytochrome c oxidase (COX) and citrate synthase (CS) measurements

For COX (EC 1.9.3.1) and CS (EC 4.1.3.7) measurements, frozen mantle tissue was ground in liquid nitrogen and homogenised with a glass homogeniser (Nalgene, USA) in Tris–HCl buffer (20 mM Tris–HCL, 1 mM EDTA, 0.1% (v/v) Tween® 20, pH 7.4) 1:3 (w/v) for COX, and 1:4 (w/v) for CS.

For COX measurements, homogenates were centrifuged for 10 min at 1000 × *g* and 2 °C. COX activity was determined after Moyes et al. (1997) by measuring the oxidation rate of cytochrome *c* at 550 nm in 20 mM Tris–HCL buffer with 0.5% Tween 20, pH 8.0 Activity was calculated using the mmolar extinction coefficient ε_{550} mM 19.1 mM^{-1} cm^{-1} after Hardewig et al. (1999).

Homogenates for CS activity were sonicated for 15 min in Branson Sonifier 450 (output control 8, duty cycle 50%) cooled to 0 °C and centrifuged at 7400 × *g* for 5 min at 2 °C. CS activity was measured after Sidell et al. (1987) recording the absorbance increase of 5 mM DTNB (5,5′dithiobis(2-nitrobenzoic acid)) in 100 mM Tris–HCL (pH 8.0), 20 mM acetyl-CoA and 20 mM oxaloacetat at 412 nm. Activity was calculated using the mmolar extinction coefficient ε_{412} of 13.61 mM^{-1} cm^{-1}.

The temperature coefficient Q_{10} was calculated as:

$$Q_{10} = e^{10(d\ln EA/dT)}.$$

With dlnEA being the difference in ln enzyme activity at the higher and lower temperature and d*T* being the difference between assay temperatures.

2.5. Calculations and statistics

Analyses of variance (ANOVA) and covariance (ANCOVA) were used to analyse the relationship between parameters versus age and to identify differences between species after testing the data for normality. Data of mitochondrial respiration were $\log_{10}$ transformed for

linearization. Lines shown in graphs are regression lines and the upper and lower 95% confidence bands.

3. Results

3.1. Age dependent changes in the function of isolated mitochondria

Oxygen consumption of mitochondria isolated from mantle tissue of the temperate *M. arenaria* and the polar mud clam *L. elliptica* at in situ temperatures are presented in Fig. 1.

A significant decline of respiratory capacity with chronological age was recorded in both species. The slope of the decrement was, however, more than two times steeper in *M. arenaria* than in *L. elliptica* mitochondria. This resulted in lower respiration rates of mitochondria isolated from aged *M. arenaria* compared to aged *L. elliptica* mitochondria. Coupling of respiration to mitochondrial phosphorylation was best in isolated mitochondria of young *M. arenaria* and decreased significantly with age (Fig. 2). Mitochondria isolated from *L. elliptica* had generally lower RCRs than *M. arenaria* mitochondria. RCRs decreased significantly with age in both species, however; again the change was more pronounced in *M. arenaria*. The non-phosphorylating leak of protons into the mitochondrial matrix increased significantly with age in both species (Fig. 3). In the temperate species the age related increase in proton leakage was significantly more rapid compared to the polar species, when plotted against chronological age. Generally, isolated mitochondria from the polar mud clam had a higher proton leak than *M. arenaria* mitochondria (Fig. 3).

Mitochondrial membrane potential ($\Delta\Psi$) of state 4+ respiration with nigericin declined significantly with age in *M. arenaria*, whereas in *L. elliptica* $\Delta\Psi$ remained stable around 134 mV (S.D. 8.202 mV) with no age dependent variations (Fig. 4).

The generation of hydrogen peroxide (H_2O_2) in state 2 with succinate was measured in isolated mitochondria of both species and is presented in Fig. 5 on milligram protein basis. Isolated mitochondria from young *M. arenaria* produced more than three times the amount of H_2O_2 per milligram protein (0.1 nmol H_2O_2 mg^{-1} protein) than *L. elliptica* mantle mitochondria (0.03 nmol H_2O_2 mg^{-1} protein). H_2O_2 generation per milligram mitochondrial protein with age was constant in *M. arenaria*, whereas in *L. elliptica* a significant decrease with age was observed.

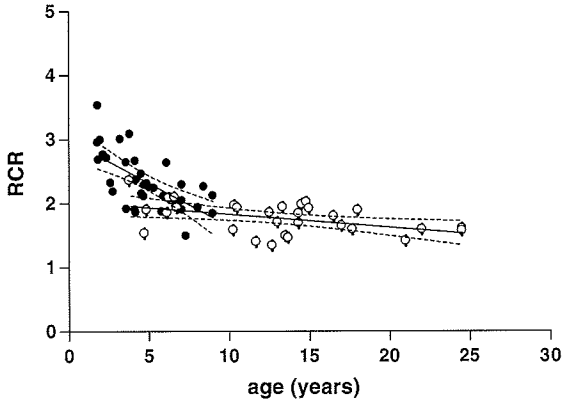

Fig. 2. Respiratory coupling ratio (RCR) of mitochondria isolated from mantle tissue of *M. arenaria* (filled circles, $N = 35$, age range = 2–9 years) and of *L. elliptica* (open circles, $N = 30$, age range = 4–24) vs. chronological age (years). Each circle represents between one and three replicate measurements per mitochondrial isolation. Slopes differed significantly between species ($p < 0.001$, ANCOVA). *M. arenaria*: RCR = −0.136 × age (years) + 2.997, $r^2 = 0.428$; *L. elliptica*: RCR = −0.021 × age + 2.04, $r^2 = 0.222$. Values are $p < 0.001$ for *M. arenaria* and $p = 0.009$ for *L. elliptica*.

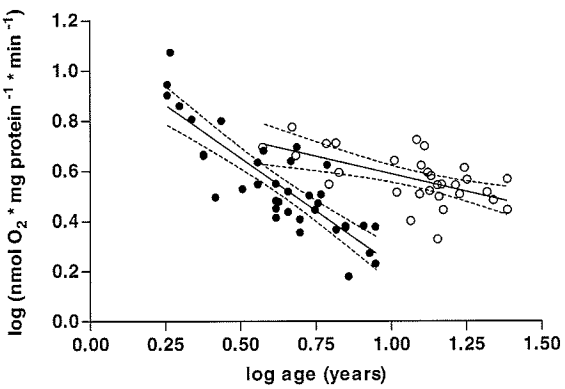

Fig. 1. State 3 respiration of mitochondria isolated from *M. arenaria* (filled circle, $N = 37$, age range = 2–9 years) and *L. elliptica* (open circles, $N = 30$, age range = 4–24 years) mantle tissue vs. chronological age. Measurements were carried out at mean in situ temperatures (10 °C *M. arenaria* and 0 °C *L. elliptica*). Each circle represents between one and three replicate measurements per mitochondrial isolation. Slopes differed significantly between species ($p < 0.001$, ANCOVA). *M. arenaria*: log MO_2 = −0.8470 × log age + 1.076, $r^2 = 0.730$; *L. elliptica*: log MO_2 = −0.28 × log age + 0.87, $r^2 = 0.344$. For all slopes $p < 0.001$.

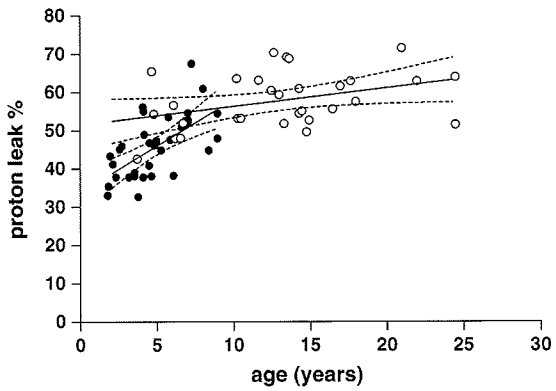

Fig. 3. Percentage of state 4+ in state 3 respiration of mitochondria isolated from mantle tissue of *M. arenaria* (filled circles, $N = 33$, range = 2–9 years) and *L. elliptica* (open circles, $N = 30$, age range = 4–24 years) vs. chronological age (years). Each circle represents between one and three replicate measurements per mitochondrial isolation. Slopes differed significantly between species ($p < 0.005$, ANCOVA). *M. arenaria*: leak = 2.4 × age + 33.7, $r^2 = 0.394$, *L. elliptica*: leak = 0.48 × age + 51.5, $r^2 = 0.137$. $p < 0.001$ for *M. arenaria* and $p = 0.044$ for *L. elliptica* (ANOVA).

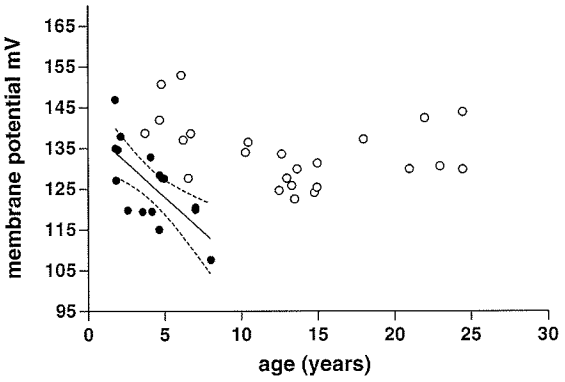

Fig. 4. Membrane potential of mitochondria isolated from mantle tissue of *M. arenaria* (filled circles, $N = 16$, range = 2–8 years) and *L. elliptica* (open circles, $N = 24$, age range = 4–25 years) vs. chronological age (years) measured at in situ temperature. Each circle represents between one and three replicate measurements per mitochondrial isolation. Slopes differed significantly between species ($p = 0.0051$, ANCOVA). *M. arenaria*: membrane potential = $-5.404 \times$ age $- 131.2$, $r^2 = 0.495$, $p = 0.0023$; *L. elliptica*, no significant relationship.

The proportion of ROS production of the mitochondrial oxygen consumption rate $\%H_2O_2/O_2$ [((nmol H_2O_2 mg protein^{-1} min^{-1}/nmol O_2 mg protein^{-1} min^{-1})/100) $\times$ 2, for a detailed description see Keller et al. (2004)] could be calculated for states 3 and 2. State 2 equals state 4 in the sense that ADP is absent and the F_0F_1 ATPase is not inhibited by oligomycin. Under non-phosphorylating conditions (state 2), a far higher fraction of up to six times more oxygen was converted to ROS in mitochondria isolated from *M. arenaria* than from *L. elliptica* (Fig. 6A). The $\%H_2O_2/O_2$ increased significantly with age in mitochondrial isolates from the temperate species, whereas in the polar species the percent ROS production was generally low, and moreover decreased mildly with age. Percentage of H_2O_2/O_2 during maximal energetic coupling in state 3 was extremely low in both species (Fig. 6B).

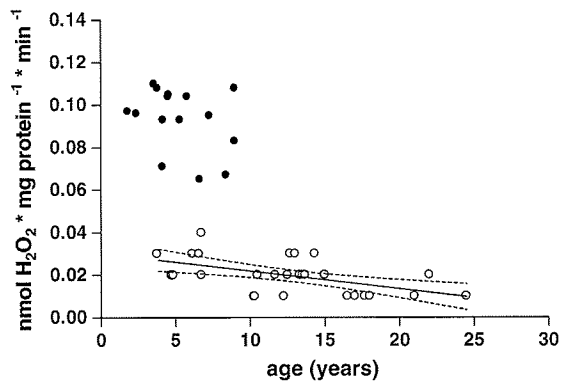

Fig. 5. Generation of hydrogen peroxide (H_2O_2) of isolated mitochondria from mantle tissue of *M. arenaria* (filled circles, $N = 15$, age range = 3–9 years) and *L. elliptica* (open circle, $N = 29$, age range = 4–24 years) over chronological age in state 2. Measurements were carried out at mean in situ temperatures (10 °C *M. arenaria*, 0 °C *L. elliptica*). Each circle represents between one and three replicate measurements per mitochondrial isolation. Slopes of both species were not, but intercepts were significantly different with $p < 0.001$ (ANCOVA). *L. elliptica*: $H_2O_2 = -0.0008 \times$ age $+ 0.030$, $r^2 = 0.320$, $p = 0.0014$; *M. arenaria*, no significant relationship.

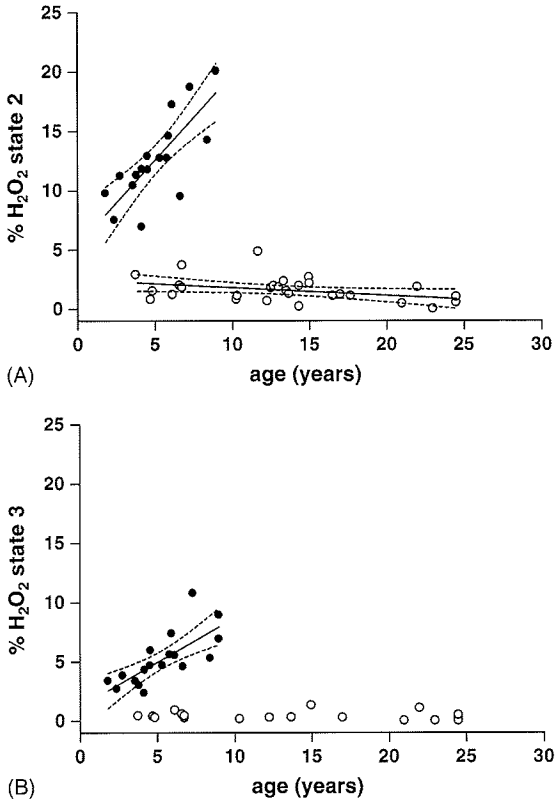

Fig. 6. Percentage of H_2O_2 production in oxygen consumption in state 2 (A) and state 3 (B) of mitochondria isolated from mantle tissue of *M. arenaria* (filled circles) and *L. elliptica* (open circles) depending on chronological age. Measurements were carried out at mean in situ temperatures (10 °C *M. arenaria* and 0 °C *L. elliptica*). Each circle represents between one and three replicate measurements per mitochondrial isolation. Slopes differed significantly between species ($p < 0.001$, ANCOVA). (A) *M. arenaria*: $\%H_2O_2 = 1.44 \times$ age $+ 5.33$, $r^2 = 0.637$, $n = 18$, age range = 3–9 years, $p < 0.001$; *L. elliptica*: $\%H_2O_2 = -0.067 \times$ age $+ 2.5$, $r^2 = 0.145$, $n = 29$, age range = 4–24 years, $p = 0.041$; (B) *M. arenaria*: $\%H_2O_2 = 0.755 \times$ age $+ 1.171$, $r^2 = 0.547$, $n = 18$, age range = 3–9 years, $p < 0.001$; *L. elliptica*, no significant relationship ($n = 18$, age range = 4–24 years).

3.2. Activities of mitochondrial enzymes

Table 1 lists the activities of the mitochondrial enzymes citrate synthase (CS) and cytochrome *c* oxidase (COX) in the mantle tissue of *M. arenaria* and *L. elliptica*, at reference and at in situ temperature. At reference temperature, *L. elliptica* displayed higher CS and COX activities per milligram protein than *M. arenaria*. Even at in situ temperature COX activities were higher in *L. elliptica* compared to *M. arenaria*, whereas CS activities were higher in *M. arenaria* (Table 1). No significant change in enzyme activity with age was found in either species. COX Q_{10} values were the same in both species 2.92 ± 0.57 (*L. elliptica*) and 2.53 ± 0.87 (*M. arenaria*), but for CS *L. elliptica* displayed a higher Q_{10} (2.8 ± 0.42) than *M. arenaria* (1.83 ± 0.15).

Table 1
Citrate synthase (CS) and cytochrome *c* oxidase (COX) activity in the mantle tissue of *M. arenaria* and *L. elliptica* over all ages

Species	Citrate synthase mean ± S.D. ($\times 10^{-2}$)	Cytochrome *c* oxidase mean ± S.D. ($\times 10^{-4}$)
M.arenaria (20 °C)	1.8 (0.6)	6.5 (2.4)
L.elliptica (20 °C)	4.8 (1.3)*	71.5 (19.0)*
M.arenaria in situ (10 °C)	1.0 (0.4)*	2.6 (1.8)
L.elliptica in situ (0 °C)	0.6 (0.3)	8.5 (3.7)*

Enzyme activities were assayed at in situ temperature and 20 °C reference temperature. Data are expressed as international units per milligram protein. Shown are means and S.D. over all ages. Chronological age of *M. arenaria* ranged from 2 to 8 years and from 2 to 30 years for *L. elliptica*. *N* (*N* in situ) = 17 (17) (M. a) – 35 (34) (L. e) for CS measurements and 16 (16) (M. a) – 37 (37) (L. e) for COX measurements.
* Significantly different between species (student's *t*-test, $p < 0.05$).

4. Discussion

The present study clearly demonstrates age related changes of mitochondrial functions in both investigated bivalves. This is in line with other studies which found important changes in isolated mitochondria from aging humans (Trounce et al., 1989; Cooper et al., 1992; Boffoli et al., 1994), other vertebrates like rats (Nohl and Hegner, 1978; Ventura et al., 2002) and invertebrates (Sohal et al., 1995), see Shigenaga et al. (1994) for review. Moreover, Hagen et al. (1997) documented that mitochondrial functions not only decline when investigated in isolated mitochondria, but also in isolated cells of aging rats.

In both investigated mud clams respiratory capacities and RCRs of isolated mitochondria decreased, whereas proton leak increased with age. Both, magnitude and timescale of these changes, however, differ strikingly between both species. In the shorter-lived temperate mud clam age-related changes proceed more rapidly than in the longer-lived polar species.

The results can also be plotted against relative age, i.e. the percentage of an age t at the maximal age of the species. Relative age was calculated by using the maximum age found within the species population and assuming this to be the maximum age the species can attain. This assumption is supported by the literature for the different species (Ralph and Maxwell, 1977; Winther and Gray, 1985; Urban and Mercuri, 1998; Strasser, 1999) and additionally for *L. elliptica* by over 650 individual age determinations of animals sampled around King George Island (this study, Voigt, 2004, Urban and Mercuri, 1998 and T. Brey unpublished data). When plotted against relative age (Fig. 7), species-specific slopes of respiratory capacity and RCR were still significantly higher in *M. arenaria* than in *L. elliptica*. This indicates that mitochondrial ageing is not only faster but also more pronounced in the temperate than the polar species.

We hypothesized that an explanation for accelerated ageing in *M. arenaria* could be the higher rates of mitochondrial ROS formation. H_2O_2 generation per milligram mitochondrial protein was far lower in the polar mud clam, although protein specific respiration rates at in situ temperature were similar or even higher in *L. elliptica* mitochondria. Along with decreasing rates of mitochondrial respiration, H_2O_2 generation declined significantly in aged *L. elliptica* individuals. The clear-cut relationship between mitochondrial ROS generation and respiratory rate is still subject to controversy (Barja, 1999). In our study, *M. arenaria* mitochondrial respiration rates declined dramatically with age, whereas H_2O_2 generation per milligram mitochondrial protein remained constant, because a higher percentage of consumed oxygen was converted to H_2O_2 ($\%H_2O_2/O_2$) in mitochondria of aged compared to younger *M. arenaria*. In the longer-lived *L. elliptica* no significant change in $\%H_2O_2/O_2$ with age was found and the overall $\%H_2O_2/O_2$ ratio was far lower than in the shorter-living *M. arenaria*. Our findings agree with a study comparing $\%H_2O_2/O_2$ of isolated mitochondria from pigeon and rats with different MLSPs, in which isolated mitochondria of long-lived pigeons showed significantly lower $\%H_2O_2/O_2$ than mitochondria from the shorter-lived rats (Barja et al., 1994).

It remains open, which mitochondrial mechanism causes the higher H_2O_2 generation rates in *M. arenaria* and what causes the increase in $\%H_2O_2/O_2$ with age. A basic difference is the comparably higher proton leak in isolated mitochondria of the polar *L. elliptica* compared to the temperate *M. arenaria* which, according to Brand (2000), could account for the lower ROS generation in *L. elliptica*. According to Porter et al. (1996), proton leak increases as a function of inner mitochondrial membrane surface area (cristae density) and can be modulated by the degree of unsaturated fatty acids in the membrane (Porter et al., 1996). Increased cristae density has already been detected in active rainbow trout following cold acclimation (St.-Pierre et al., 1998). An elevated content of unsaturated membrane fatty acids is common in marine fish from low latitudes (Cossins et al., 1978; White and Somero, 1982) and held responsible for the higher proton leak in some subpolar species (Guderley, 2004).

Whether either mechanism accounts for the higher proton leak rate in the polar mud clam remains speculation. However, Ahn et al. (2000) reported similar levels of total unsaturated fatty acids in the soft tissue of *L. elliptica* and several marine bivalves from warmer waters, raising some doubt as to the involvement of membrane unsaturation in elevated proton leak rates in the polar clam. An indication for potentially higher cristae density in *L. elliptica* than in *M. arenaria* mitochondria may be seen in the three-fold higher activity (U/mg protein at in situ temperature) of cytochrome

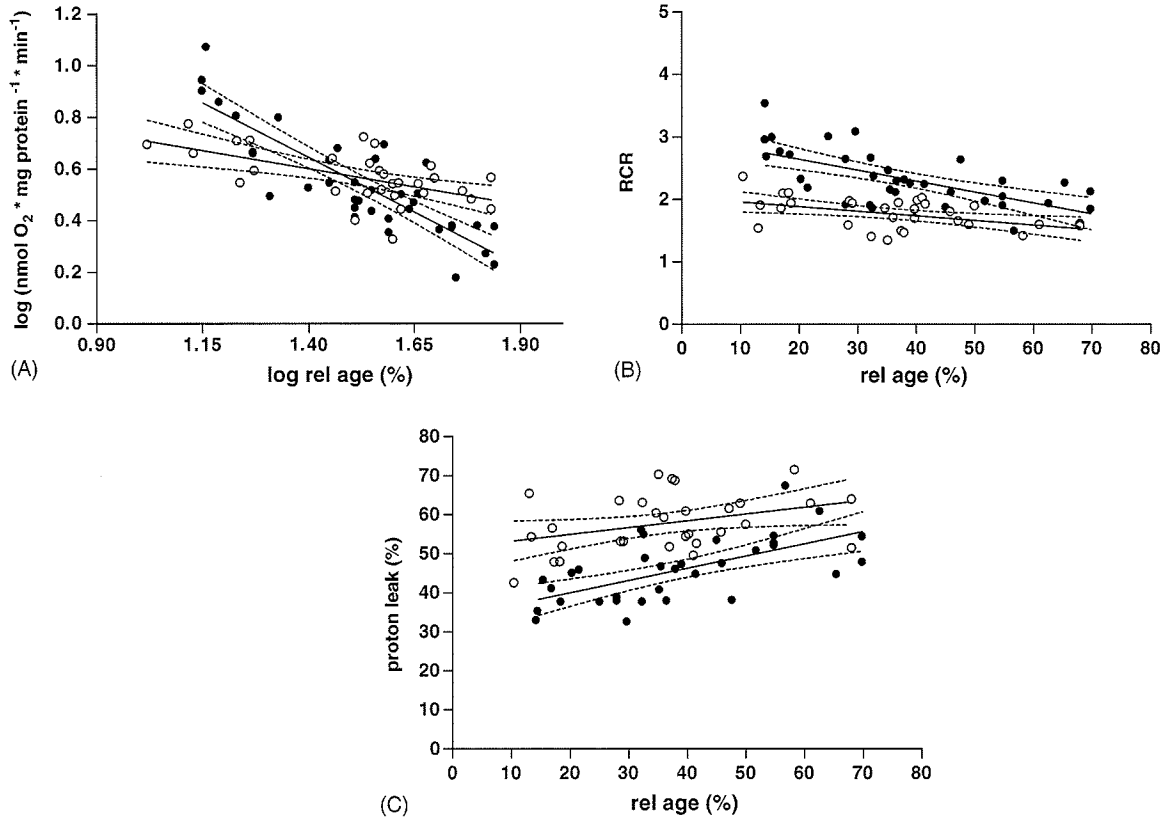

Fig. 7. Functions of isolated mitochondria (A) state 3 respiration (B) RCR and (C) proton leak vs. relative (rel.) age. Legend details and p values for the individual slopes see Figs. 1–3, respectively. Slopes differed significantly between species in (A) $p < 0.001$, ANCOVA and (B) $p = 0.031$, ANCOVA, and were not significantly different in (C). (A) *M. arenaria*: log $MO_2 = -0.847 \times$ log rel.age + 1.83, $r^2 = 0.730$; *L. elliptica*: $MO_2 = -0.28 \times$ log rel.age + 0.99, $r^2 = 0.344$. (B) *M. arenaria*: RCR = $-0.0175 \times$ rel.age + 2.996, $r^2 = 0.428$; *L. elliptica*: RCR = $-0.007 \times$ rel.age + 2.04, $r^2 = 0.221$. (C) *M. arenaria*: leak = $0.314 \times$ rel.age + 33.74, $r^2 = 0.393$; *L. elliptica*: leak = $0.174 \times$ rel.age + 51.49, $r^2 = 0.137$.

c oxidase (COX), the enzyme being located in the mitochondrial inner membrane. In contrast, the activity of matrix located citrate synthase (CS) was slightly higher in *M. arenaria* (Table 1). At reference temperature CS values were two-fold higher in *L. elliptica* than *M. arenaria*, whereas the difference in COX activities was even more pronounced and 11-fold higher in *L. elliptica*. COX-Q_{10} values were similar in both animals and the higher activities in *L. elliptica* are therefore attributed to higher COX quantities possibly paralleled by higher cristae density, rather than a qualitative difference in enzyme activities between the two species. In line with this, St.-Pierre et al. (1998) found significantly higher COX activities (U mg^{-1} mitochondrial protein) in winter compared to summer acclimatised rainbow trout while CS activities (U mg^{-1} mitochondrial protein) were only mildly increased in winter animals. This went along with higher mitochondrial cristae density in winter compared to summer animals. We conjecture that ROS formation in *L. elliptica* is minimized by mild uncoupling (Brand, 2000) due to proton leakage, presumably because of higher cristae density, and that this may be a strategy employed by the polar clams, to prolong mitochondrial and animal lifetime in the cold.

The higher proton leakage (Porter et al., 1996) could explain the higher mitochondrial respiration rates at in situ temperature in *L. elliptica* compared to *M. arenaria* in individuals older than 35% of maximum age. In contrast, comparing whole animal respiration, the polar *L. elliptica* displayed more than two times lower standard metabolic rates (SMR) than the temperate clam at in situ temperature and at all ages (Philipp et al., 2005). This discrepancy between lower whole animal SMR and higher mitochondrial respiratory capacity in the polar clam could be explainable when assuming lower numbers of highly active mitochondria per gram wet mass in *L. elliptica*. These animals may employ two defence strategies to suppress the production of hazardous ROS: a low SMR at in situ temperatures ("Free Radical-Rate of Living" theory) and mild uncoupling by an elevated mitochondrial proton leak ("Uncoupling to survive" theory). However, ultrastructural analyses are necessary to confirm our assumptions concerning lower mitochondrial volume and higher cristae density in the polar species.

A correction of the oxygen consumption of *M. arenaria* mitochondria ascribed to proton leak for the amount of oxygen that goes into H_2O_2 formation (Heise et al., 2003) resulted in a lower calculated proton leak (Fig. 8). Net proton leak was calculated after correcting states 3 and 4+ respiration for the percentage of H_2O_2 formed in each state. A similar correction cannot be done for our *L. elliptica*

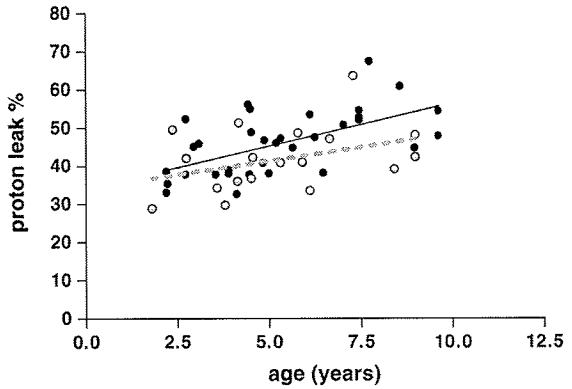

Fig. 8. Proton leak of *M. arenaria* uncorrected (black dots, $N = 33$) and corrected (grey dots, $N = 18$) for ROS generation. Regression details and p value for uncorrected values see legend of Fig. 3. For corrected values no significant relationship between age and proton leak was found.

data, as no H_2O_2 measurements of state 4+ mitochondria were performed. However, because of the low overall H_2O_2 generation in state 2 and stable or even decreasing H_2O_2 generation rates with age, a correction of the proton leak in *L. elliptica* mitochondria can result in only minor changes of the calculated data. The difference in proton leak between *L. elliptica* and *M. arenaria* mitochondria would then be even more apparent.

The significant increase of proton leak with age found prior H_2O_2-correction in *M. arenaria* is abolished when corrected for H_2O_2 production (Fig. 8), so, we conclude that the significant decrease in membrane potential with age in the temperate clam must be attributed to a slow down of mitochondrial respiration and not to a higher proton leak in older animals.

In the present study, H_2O_2 generation at complex III was measured in the presence of succinate and rotenone (St-Pierre et al., 2002; Keller et al., 2004). Addition of SOD (50U/ml) doubled H_2O_2 generation in *M. arenaria*. Due to insufficient sample size the SOD effect could not be measured for *L. elliptica*. However, this indicates that at least in *M. arenaria* mitochondria, ROS are generated not only on the matrix, but also on the cytoplasmic side of the mitochondrial inner membrane (St-Pierre et al., 2002). According to Barja et al. (1994), species with high mitochondrial ROS production are more likely to suffer damage of the inner mitochondrial membrane and of the mitochondrial DNA (mt-DNA), located adjacent to the membrane. Assuming that our in vitro measurements reflect in vivo conditions, continued oxygen radical formation on both sides of the membrane in *M. arenaria* mitochondria and consistently higher than in *L. elliptica,* might give rise to elevated oxidative stress and exacerbate membrane and mt-DNA damage in *M. arenaria*. Like in a "vicious cycle" (Lenaz, 1998), this ensues higher rates of incomplete oxygen reduction with age and may eventually accelerate and exacerbate the aging process in the North Sea mud clam.

We have previously reported changes of antioxidant capacities and tissue redox state (ratio of oxidised to reduced glutathione) in mantle tissue of both species with age (Philipp et al., 2005). Tissue redox state was more oxidised in the temperate clam and increased with age, whereas in the polar species a less oxidized tissue redox state was maintained stable throughout all ages. Moreover, *L. elliptica* displayed higher H_2O_2 scavenging capacities (catalase, glutathione) than *M. arenaria*. Taken together, higher H_2O_2 generation rates, the increase in $\%H_2O_2/O_2$ with age in *M. arenaria* mitochondria as well as lower H_2O_2 scavenging ability may explain the higher GSSG:GSH ratio and lower MLSP in the temperate compared to the polar species.

According to Vladimir Skulachev's (2001), "Samurai law of biology" one would expect destruction of the progressively damaged and ROS generating mitochondria in *M. arenaria* in the sense that "it is better to die than to be wrong". According to the Samurai law, mitochondria and cells, but also organs or individuals commit suicide (apoptosis) before they turn into "unhopeful monsters" causing detrimental damage in the organism or the population (Skulachev, 2001). In our study, already young *M. arenaria* show higher lipofuscin accumulation than *L. elliptica* and older *M. arenaria* (Philipp et al., 2005), which may be indicative of a higher mitoptotic potential (mitochondrial apoptosis, Skulachev, 2001) in *M. arenaria*, as damaged mitochondria contribute to lipofuscin formation (Miquel, 1998; Brunk and Terman, 2002b). Further degradation of ROS producing mitochondria may be impaired due to the already lipofuscin loaded lysosomes (Terman, 2001; Brunk and Terman, 2002a,b), leaving wasted and ROS generating mitochondria to accumulate in the tissue.

5. Conclusions

The results from this and a previous study (Philipp et al., 2005) explain a three-fold higher MLSP found in a polar compared to a temperate mud clam along the lines of two different ageing theories: the "Free Radical-Rate of Living theory "(Pearl, 1928; Harman, 1956) and the "Uncoupling to Survive" hypothesis (Brand, 2000). In line with the first theory, the longer-lived polar *L. elliptica* shows lower SMRs than the shorter-lived temperate *M. arenaria* (Philipp et al., 2005). On the other hand, isolated mitochondria from *L. elliptica* display similar capacities but far lower H_2O_2 generation rates than those of *M. arenaria*, possibly due to the higher proton leak in the polar mitochondria. The higher H_2O_2 generation in *M. arenaria* mitochondria may account for the more pronounced decrease in mitochondrial function (respiration rate, RCR) with age compared to the polar species. Lower SMR in combination with low mitochondrial H_2O_2 generation and higher antioxidant defence capacities throughout lifetime could explain the longer MLSP in the polar compared to the temperate species.

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