Low rates of hydrogen peroxide production by isolated heart mitochondria associate with long maximum lifespan in vertebrate homeotherms

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H₂O₂ production that was significant by sign test, but fell short of significance by regression analysis. These findings indicate that enhanced longevity may be causally associated with low free radical production by mitochondria across species over two classes of vertebrate homeotherms. Key words: free radical hypothesis of aging; mitochondria; reactive oxygen species; superoxide.

Introduction

Mechanisms of biological aging, defined as the molecular processes causing the gradual decline in function over time, are poorly understood. One way to address this problem is to compare possible mechanisms across different species exhibiting variation in aging rate or lifespan. Indeed, some of the earliest work on aging (Weissman, 1889; Rubner, 1908; Pearl, 1928) through to modern times is based on the comparative approach (Cutler, 1985; Ku et al., 1993; Austad, 1997; Barja, 2002; Buffenstein, 2005; Hulbert, 2005). Of particular interest are species with exceptional longevities relative to their body mass, such as bats and many birds (Holmes et al., 2001; Brunet-Rossini & Austad, 2004). One such species that has come under scrutiny recently is the naked mole-rat, which is extraordinarily long-lived for an animal with a body mass of approximately 30 g. Its maximum observed lifespan is more than 28 years and it is the longest-lived rodent known (Sherman & Jarvis, 2002; Buffenstein, 2005).

The free radical hypothesis of aging posits that free radicals and/or reactive oxygen species (ROS), produced mostly by mitochondria, react with cell components such as proteins, lipid and DNA to produce a variety of damaged products. Over time these products accumulate because the protection and repair systems that prevent or remove the damage are imperfect. The long-term effect of this accumulation of damage is the decline in cell function, that is characteristic of aging (Harman, 1956; Beckman & Ames, 1998; Balaban et al., 2005).

In support of this hypothesis are comparative data showing that the production of ROS is low in mitochondria isolated from large long-lived mammals compared to small short-lived ones, with mitochondria from mammals of intermediate lifespan and body mass having intermediate rates of ROS production (Sohal et al., 1990b; Ku et al., 1993). In addition, ROS production rates of mitochondria from long-lived birds (pigeons, canaries and parakeets) are lower than those from similar-sized short-lived mammals (rats and mice) (Ku & Sohal, 1993; Herrero & Barja, 1997a, 1998). ROS production rate is low in mitochondria from the white-footed mouse (maximum observed lifespan: 8 years).
compared to the house mouse and short-tailed shrew (2 years) (Sohal et al., 1993; Brunet-Rossini, 2004). ROS production rate is also relatively low in the little brown bat (34 years) (Brunet-Rossini, 2004). Further support for the hypothesis comes from observations that the steady-state levels of oxidative damage in mammals and birds also correlate with lifespan (Barja, 2002).

As explained by Speakman (2005), most comparative studies on lifespan and physiology have not taken into account the possible confounding effects of body mass and phylogeny. For example, the amount of mitochondrial DNA damage (as assessed by the amount of 8-hydroxy-2-deoxyguanosine) correlates with maximum lifespan in mammals (Barja, 2004). However, the short-lived species tended to be relatively small and have high levels of damage, while the long-lived species were large and had low levels of damage. When the effects of body mass were removed mathematically by analysis of residuals, the resulting correlation between damage and lifespan was nonsignificant (Speakman, 2005). The effects of body mass can be circumvented by comparing animals of similar body mass but differing lifespans. In the aging field, this has been achieved by comparing birds such as pigeons (maximum observed lifespan: 35 years) with mammals with similar body masses such as rats. However, in these cases there are issues associated with phylogeny: it may be that certain traits (such as DNA damage) are low in all species for some reason unrelated to their longevity. As yet there are no reports detailing aspects of oxidative damage in short-lived birds, which would help resolve this

potential issue of phylogeny. Finally, some studies have focused on just two species. For statistical and logical reasons, it is questionable to draw inferences about lifespan and physiology solely on the basis of data from two species (Garland & Adolph, 1994). For example, a two-species comparison is effectively an n of 1 with zero degrees of freedom (rendering statistical analysis impossible).

We have revisited the important work on lifespan and ROS production described above experimentally to see if the results still hold when body mass and phylogeny are taken into account. We did this by comparing mitochondrial ROS production in groups or pairs of species with large differences in maximum observed lifespan but only small differences in body mass. We compared the relatively short-lived house mouse with four other species including two species of relatively long-lived bats and the long-lived naked mole-rat. We compared the relatively short-lived guinea pig to the relatively long-lived Damara mole-rat, and we compared the short-lived Japanese quail to the long-lived pigeon. We also obtained data from the rat, baboon and ox to obtain a final set of 12 species. We then subjected the data to residual analysis and phylogenetic independent contrasts to obtain a correlation between maximum lifespan and mitochondrial ROS production that is independent of body mass and phylogeny. A model of mitochondrial hydrogen peroxide (H$_2$O$_2$) generation, showing the sites of superoxide production and inhibitor action at the electron transport chain, is depicted in Fig. 1.

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**Fig. 1** Hydrogen peroxide (H$_2$O$_2$) production by isolated mitochondria. The primary free radical species produced is superoxide (O$_2^-$), most of which is converted to H$_2$O$_2$ by superoxide dismutase (SOD). Endogenous SOD is present in the matrix; H$_2$O$_2$ produced in the matrix can diffuse out to be detected by a coupled fluorometric assay in the bulk medium. The addition of SOD to the assay medium ensures that all superoxide produced to the intermembrane space is also converted to H$_2$O$_2$ and detected in the assay (St-Pierre et al., 2002). (a) During succinate oxidation by succinate dehydrogenase (SDH) the high proton motive force and highly reduced ubiquinol (QH$_2$) pool result in electrons being pushed thermodynamically uphill into complex I (reverse electron transport) reducing NAD$^+$ to NADH. Under these conditions, complex I superoxide generation rates are relatively high and directed into the matrix. Rotenone prevents electrons entering complex I, resulting in a diminished rate of superoxide production (Lambert & Brand, 2004a). The residual rotenone-insensitive rate reflects superoxide production by complex III during cytochrome c reduction. (b) During forward electron transport from pyruvate and malate, superoxide production rates are relatively low (Lambert & Brand, 2004b). The addition of piericidin or rotenone blocks the transfer of electrons from complex I to ubiquinone and causes complex I to become fully reduced, resulting in an increased rate of superoxide production from complex I.
Fig. 2 Hydrogen peroxide (H₂O₂) production by heart mitochondria from mammals with diverse maximum lifespans. Substrate: (a, c) succinate; (b, d) succinate + rotenone. Standard incubation conditions were KHB buffer at 34 °C with oligomycin, H₂O₂ detection system and superoxide dismutase (SOD) as described in Experimental procedures. Values are means ± standard error. In (a) n = 7 and in (b) n = 5 for both species. In (c) and (d) n = 3 for both species. Statistics were calculated by the two-tailed t-test.

Table 1 Lifespan and body mass data for the animals used in the study

<table>
<thead>
<tr>
<th>Label</th>
<th>Scientific name</th>
<th>Common name</th>
<th>Maximum lifespan (years)</th>
<th>Body mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><em>Mus musculus</em></td>
<td>C57BL/6 mouse</td>
<td>3.5</td>
<td>22 ± 2*</td>
</tr>
<tr>
<td>B</td>
<td><em>M. musculus</em></td>
<td>BALB/c mouse</td>
<td>3.5</td>
<td>21†</td>
</tr>
<tr>
<td>C</td>
<td><em>M. musculus</em></td>
<td>Wild (Idaho) mouse</td>
<td>4</td>
<td>31 ± 0.7*</td>
</tr>
<tr>
<td>B</td>
<td><em>Rattus norvegicus</em></td>
<td>Rat</td>
<td>5</td>
<td>216 ± 8*</td>
</tr>
<tr>
<td>C</td>
<td><em>Pomeroyus leucopus</em></td>
<td>White-footed mouse</td>
<td>8</td>
<td>21 ± 0.5*</td>
</tr>
<tr>
<td>D</td>
<td><em>Heterocephalus glaber</em></td>
<td>Naked mole-rat</td>
<td>28</td>
<td>30 ± 7*</td>
</tr>
<tr>
<td>E</td>
<td><em>Cryptomys damaras</em></td>
<td>Damara mole-rat</td>
<td>15†</td>
<td>127 ± 32*</td>
</tr>
<tr>
<td>F</td>
<td><em>Cavia porcellus</em></td>
<td>Guinea pig</td>
<td>8</td>
<td>222 ± 10*</td>
</tr>
<tr>
<td>G</td>
<td><em>Papio cynocephalus</em></td>
<td>Baboon</td>
<td>37.5</td>
<td>20 250 ± 8584*</td>
</tr>
<tr>
<td>H</td>
<td><em>Tadarida brasiliensis</em></td>
<td>Brazilian free-tailed bat</td>
<td>12</td>
<td>8.9 ± 0.3*</td>
</tr>
<tr>
<td>I</td>
<td><em>Myotis lucifugus</em></td>
<td>Little brown bat</td>
<td>34</td>
<td>8.0 ± 0.4*</td>
</tr>
<tr>
<td>J</td>
<td><em>Bos taurus</em></td>
<td>Ox (domestic cow)</td>
<td>30</td>
<td>750 000†</td>
</tr>
<tr>
<td>K</td>
<td><em>Coturnix japonica</em></td>
<td>Japanese quail</td>
<td>6</td>
<td>115†</td>
</tr>
<tr>
<td>L</td>
<td><em>Columbia livia</em></td>
<td>Domestic pigeon</td>
<td>35</td>
<td>300§</td>
</tr>
</tbody>
</table>

*Measured (mean ± standard deviation).  
†Values taken from the AnAge online database (http://genomics.senescence.info/species), Ku et al. (1993) and Miller et al. (2002), except †R. Buffenstein (unpublished observation).  
§Estimated. Maximum lifespan values are from captive individuals except *Myotis lucifugus* and *T. brasiliensis*.

Results

Hydrogen peroxide production in mitochondria from small mammals

Differences in mitochondrial H₂O₂ production rate between species have been observed during succinate oxidation (Ku et al., 1993; Ku & Sohal, 1993; Herrero & Barja, 1997a). In addition, we previously demonstrated that when mitochondria oxidize succinate under conditions of a high pH gradient across the mitochondrial inner membrane, the H₂O₂ production rate is very high (Lambert & Brand, 2004a). Thus, the succinate/high pH condition was initially chosen to test whether heart mitochondrial H₂O₂ production rates were different between the short-lived house mouse (*C57BL/6 strain*) and longer-lived mammals (species used are listed in Table 1). Figures 2a and 3a show the
results: while there was an apparent trend towards low H$_2$O$_2$ production in longer-lived species, the differences between house mouse, naked mole-rat, white-footed mouse and the two species of bats were not significant.

Rotenone caused a substantial drop in the rate of H$_2$O$_2$ production by mitochondria oxidizing succinate, indicating that most of the H$_2$O$_2$ in the absence of rotenone came from complex I during reverse electron transport (Lambert & Brand, 2004a). As shown in Figs 2b and 3b, there were no significant differences in H$_2$O$_2$ production rate with succinate + rotenone, indicating that there are no differences in superoxide production at complex II, the ubiquinone pool or complex III.

Hydrogen peroxide production in wild-derived vs. laboratory mice

Most comparative studies on ROS production and lifespan have compared laboratory rodents with domesticated animals or birds. Laboratory mice are relatively inbred and have been selected for fast maturation, high fecundity and docility. These factors may have resulted in high ROS production by their mitochondria compared to their wild ancestors. To investigate this hypothesis, we compared rates of H$_2$O$_2$ production in mitochondria from wild-derived (Idaho) mice and two strains of laboratory mice. When respiring on succinate at 37 °C, the rates in nmol H$_2$O$_2$ min$^{-1}$ mg mitochondrial protein$^{-1}$ were: Idaho, 2.49 ± 0.32 (n = 5); C57BL/6, 2.51 ± 0.30 (n = 8); BALB/c, 2.50 ± 0.17 (n = 3). There were no significant differences between any of the strains as judged by ANOVA.

Hydrogen peroxide production in Damara mole-rat and guinea pig mitochondria

We chose a pair of slightly larger mammals (Damara mole-rats and guinea pigs) with different longevities to test the generality of our findings on heart mitochondria with mice and bats. Figure 2c shows that during succinate oxidation, the rate of H$_2$O$_2$ production was significantly lower in heart mitochondria from Damara mole-rats compared to guinea pigs. No differences were seen at sites other than complex I (succinate + rotenone; Fig. 2d). Thus, the difference between Damara mole-rats and guinea pigs was at complex I during reverse electron transport.

Hydrogen peroxide production in rat, quail and pigeon mitochondria

To investigate further any possible relationships between lifespan and H$_2$O$_2$ production rate by heart mitochondria, we extended our observations to a long-lived and a short-lived bird. We first tested whether the reported differences between rats and pigeons (Ku & Sohal, 1993; Barja et al., 1994) could be repeated. Indeed, during succinate oxidation, the rate of H$_2$O$_2$ production was over twofold higher in the rat compared to the pigeon and this difference was significant (Fig. 4a). There was no difference in the presence of succinate + rotenone (Fig. 4b), showing that the differences in Fig. 4a originate at complex I during reverse electron transport. The rate during succinate oxidation in quail mitochondria was not different from that in the rat (Fig. 4c). Figure 4d shows that rotenone caused a large drop in the rates of H$_2$O$_2$ production, which again indicates that most of the signal originates at complex I during reverse electron transport. Figure 4d also shows a significant difference (P = 0.03) between rats and quails at complex II and/or the ubiquinone pool and/or complex III. Post-hoc examination showed that when oxidizing succinate, the rate in quail mitochondria was 2.1-fold higher than the rate in pigeon mitochondria. This suggests that H$_2$O$_2$ production rate is not low in all birds regardless of their lifespan.

Correlations between lifespan and hydrogen peroxide production

The original design of the experiment was to compare pairs and groups of species of similar body mass but with different longevities. However, by considering the dataset as a whole, we were able to test more generally for effects of body mass and phylogeny. We also obtained unpaired data from mitochondria of two relatively large species, baboon and ox, and included
these in our analysis. We pooled data from all species, assuming that the 3°C lower assay temperature in Fig. 2 does not alter the outcome of the analysis significantly. This was tested in mouse mitochondria, where the difference in H₂O₂ production rate caused by a 3°C increase in temperature was a negligible 10%.

Figure 5a shows that the relationship between mitochondrial H₂O₂ production with succinate and maximum lifespan was similar to the one previously shown for a more limited dataset of domesticated mammals not selected for unusually long or short lifespans (Sohal et al., 1990b; Ku et al., 1993). There was no relationship between mitochondrial H₂O₂ production with succinate + rotenone and maximum lifespan (Fig. 5b). We also measured H₂O₂ production using pyruvate and malate as substrates (these substrates provide electrons that enter complex I via the forward direction as opposed to the reverse). As expected, the rates with pyruvate and malate were generally low compared to the rates with succinate (Lambert & Brand, 2004b). To fully reduce complex I during forward electron transport we added inhibitors (either piericidin or rotenone). As expected, the rates of H₂O₂ production were increased under the inhibited condition (Lambert & Brand, 2004b). However, under both conditions with forward electron transport there were no apparent relationships between H₂O₂ production rate and maximum lifespan (Fig. 5c,d).

Focusing on the condition with succinate as substrate, we statistically removed any effects of body mass by analysis of residual H₂O₂ production and residual maximum lifespan (Speakman, 2005). Figure 6a shows that a linear fit of the relationship between maximum lifespan and ln rate of H₂O₂ production (prior to residual analysis) was significant. Figure 6b shows the relationship between lifespan and body mass. This relationship was weak because of our selection of several species (mole-rats, bats, pigeons) with unusually long lifespans for their body mass. Figure 6c shows the relationship between H₂O₂ production and body mass. The plot of the residuals (the vertical distances from each of the points to the regression lines in Fig. 6b,c) showed a highly significant correlation between maximum lifespan and H₂O₂ production when any underlying effects of body mass, already reduced by our selection of species, were removed completely (Fig. 6d).

To account for the fact that species do not represent statistically independent data, we generated phylogenetically independent contrasts (from the residuals in Fig. 6d) using standard methods (Felsenstein, 1985; Garland et al., 2005). A phylogenetic tree was derived from molecular sequence data from various sources (van Tuinen et al., 2000; Murphy et al., 2001; Huclon et al., 2002; Faulkes et al., 2004) and is shown in Fig. 7a. Independent contrasts were calculated using a spreadsheet, and confirmed using published phenotypic diversity analysis programs (Garland et al., 1999; Garland & Ives, 2000). Figure 7b shows the relationship between residual maximum lifespan and residual H₂O₂ production for the phylogenetically independent contrasts in our dataset (the nodes in Fig. 7a). The null hypothesis states that H₂O₂ production associates equally with either increases or decreases in lifespan, that
Fig. 5 Correlations between $\text{H}_2\text{O}_2$ production and maximum lifespan across vertebrate homeotherms. Substrate: (a) succinate; (b) succinate + rotenone; (c) pyruvate + malate; (d) pyruvate + malate + rotenone or piericidin. For (a) and (b) $n=12$ species, that is the ten species examined in Figs 2–4 plus the bat and ox. Forward electron transport was not determined for white-footed mouse, thus $n=11$ for (c) and (d). Letters 'A–L' denote the species listed in Table 1.

Fig. 6 Analysis of residuals for mitochondria respiring on succinate. (a) Ln maximum lifespan vs. Ln $\text{H}_2\text{O}_2$ production rate; (b) Ln maximum lifespan vs. Ln body mass; (c) Ln $\text{H}_2\text{O}_2$ production rate vs. Ln body mass; (d) residual Ln maximum lifespan vs. residual Ln $\text{H}_2\text{O}_2$ production rate. The correlation in (a) is significant: $r^2=0.54, F_{1,10}=11.7, P=0.007$. The correlation in (d) is significant: $r^2=0.69, F_{1,10}=22.5, P=0.0008$. Letters 'A–L' denote the species listed in Table 1, see Experimental procedures for more details.
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Fig. 7 Phylogenetically independent contrast (PIC) analysis for mitochondria respiring on succinate. (a) Phylogenetic tree used in the analysis. A tree with 12 species results in 11 contrasts at nodes numbered as 1–11. (b) PIC of residual Ln maximum lifespan vs. PIC of residual ln H$_2$O$_2$ production rate with the x-axis ‘positiveized’ (Garland et al., 1992). Ten of the 11 points show that H$_2$O$_2$ production associates negatively with maximum lifespan, the dashed line at y = 0 is to emphasize this fact. The relationship is significant (P = 0.011) by the sign test. The presence of the outlier at node 11 renders the relationship nonsignificant by regression analysis, see Experimental procedures for more details.

is, the points in Fig. 7b should be distributed equally above and below the dashed line. This was clearly not the case; ten of the 11 contrasts showed a negative association between maximum lifespan and H$_2$O$_2$ production that was significant by the sign test (P = 0.011). A linear regression through the points in Fig. 7b did not give a significant correlation due to the presence of an outlier, which was the naked mole-rat–Damaraland mole-rat contrast. However, this is not necessarily grounds for accepting the null hypothesis (Harvey & Pagel, 1991).

Analysis of residuals and phylogenetically independent contrasts for each of the other three conditions (succinate + rotenone, pyruvate + malate, pyruvate + malate + rotenone or piericidin) did not reveal any significant relationships between H$_2$O$_2$ production rate and maximum lifespan (data not shown).

Discussion

In this study, we exploited differences in longevity among homeothermic vertebrates to critically test the hypothesis that longevity correlates with radical production by isolated heart mitochondria. Our design overcomes some of the most serious criticisms of previous studies by correcting for effects of body mass and phylogeny that may have confounded earlier work.

Indeed, as shown in Fig. 8, the significant relationship between lifespan and ROS production by heart mitochondria reported by Ku et al. (1993) became nonsignificant (P = 0.62) when the published data were subjected to residual analysis. In other words, in that particular dataset, the changes in ROS production rates and longevity can be explained by their dependence on body mass, confounding any interpretation of the effects of ROS production on lifespan. Similarly, published correlations between lifespan and ROS production in mitochondria from kidney (Ku et al., 1993) and liver (Sohal et al., 1990b) became nonsignificant when reanalysed in the same way (P values were 0.28 and 0.15 for kidney and liver, respectively). Nonetheless, the results we report here suggest that our hypothesis is supported: in general, maximum lifespan associates negatively with H$_2$O$_2$ production by isolated mitochondria, independently of body mass or phylogeny. However, further studies with greater numbers of species are still required to test the generality of the hypothesis, particularly since one exception was found: the naked mole-rat (see below).

We worked with isolated mitochondria, and therefore had to make choices about incubation conditions. The reduction of oxygen to superoxide is second order and depends on the concentrations of the two reactants, oxygen and X*, where X* is the free radical reductant of oxygen (Turner, 1997). In the mitochondrial electron transport chain there are two main sites of superoxide production. In complex III, X* is thought to be the semiquinone at centre ‘o’, in complex I it is thought to be either a semiquinone, an iron-sulphur centre or the flavin group (Brand et al., 2004). When measuring mitochondrial H$_2$O$_2$ production in vitro, at high, essentially constant oxygen concentration, any change in superoxide production rates reflects changes in the concentration of X*. The concentration of X* depends on the respiratory substrate, the presence or absence of inhibitors of electron transport, and on energization, that is, the magnitude of the protonmotive force components ΔΨ and Δφ (Lambert & Brand, 2004a). Therefore, to ensure that all major sites of production were included (Fig. 1), we measured H$_2$O$_2$ generation by mitochondria with different relevant combinations of substrates and inhibitors.

Differences in mitochondrial ROS generation between species are reported mostly during reverse electron transport into complex I, that is, when intact mitochondria are respiring on succinate in the absence of rotenone (Sohal et al., 1990b; Ku & Sohal, 1993; Ku et al., 1993; Herrero & Barja, 1997a; Brunet-Rossini, 2004). In general, our own findings agree that differences between species only occur with succinate in the absence of rotenone. In vivo, mitochondria do not oxidize succinate exclusively, so it is not known to what extent, if any, reverse electron transport occurs. Although forward electron transport is more physiological, mitochondria in vivo do not oxidize pyruvate exclusively either, because other substrates, such as fatty acids and glycerol-3-phosphate, are also oxidized (feeding electrons into the Q pool in the process). When the Q
pool is relatively reduced, forward electron transport in complex I may stall, resulting in high rates of superoxide production. We suggest that succinate-supported superoxide production during reverse electron transport may mimic this condition. The precise details of which sites, and under what conditions mitochondria produce ROS in vivo are still unclear, and further work is required in this area.

What causes the differences in \( \text{H}_2\text{O}_2 \) production rates between species that give rise to the correlation with lifespan? There are no consistent major differences in known processes that remove radicals and repair the damage they cause in long-lived mammals (Cutler, 1985; Sohal et al., 1990a; Lopez-Torres et al., 1993; Perez-Campo et al., 1993; Brunet-Rossini, 2004), particularly naked mole-rats (Andziak et al., 2005). It is unlikely that superoxide production rates are the same between species but superoxide dismutase (SOD) activity is different, giving rise to different rates of \( \text{H}_2\text{O}_2 \) production, as we measured SOD activity in most of our samples and found no correlations with lifespan (data not shown). It may be that both superoxide and \( \text{H}_2\text{O}_2 \) production rates are the same between species, but the differences are at the levels of processes that remove \( \text{H}_2\text{O}_2 \) before it diffuses out of the mitochondria matrix and is consumed by the detection system. These removal processes could include glutathione peroxidase, catalase or peroxiredoxin activities. Again, this is probably not true, as we measured glutathione peroxidase activity and the ability of mitochondria to remove an external spike of \( \text{H}_2\text{O}_2 \) and found no correlations with maximum lifespan (data not shown). In addition, if the apparent differences between rates of \( \text{H}_2\text{O}_2 \) production were due to differences in rates of superoxide dismutation or \( \text{H}_2\text{O}_2 \) removal, then a correlation between \( \text{H}_2\text{O}_2 \) production rate and lifespan should be seen under all conditions. This was not the case, for example, in the presence of succinate + rotenone, or pyruvate + malate, the rates of \( \text{H}_2\text{O}_2 \) production did not generally correlate with lifespan. Thus, the causes of the differences in \( \text{H}_2\text{O}_2 \) production are likely to be at the level of the superoxide production by the electron transport chain. As the experiments with succinate and succinate + rotenone show, the differences are most likely at complex I during reverse electron transport.

There was one exception to the general result that lifespan was negatively associated with \( \text{H}_2\text{O}_2 \) production. Heart mitochondria from long-lived naked mole-rats produced more ROS than expected (or the naked mole-rats lived longer than expected) compared to the other species that were examined. However, these effects were not strong enough to efface the general trend seen after correction for body mass in Fig. 6d. Although the inverse relationship between maximum lifespan and mitochondrial \( \text{H}_2\text{O}_2 \) production after correction for phylogeny in Fig. 7b was significant by sign test, there was no significance by regression analysis due to the presence of an outlier generated from the naked mole-rat–Damara mole-rat contrast. Naked mole-rats live longer than Damara mole-rats, but heart
mitochondria from naked mole-rats produced $H_2O_2$ at higher rates than mitochondria from Damara mole-rats. This pair of related species did not follow the general rule, generating the outlier in Fig. 7b. It is noteworthy that the value of 15 years for Damara mole-rat maximum lifespan is based on a relatively small sample size compared to the sample size of naked mole-rats from which the value of 28 years is drawn. If Damara mole-rat maximum lifespan is greater than 15 years then the effect of the outlier will be diminished.

It may be that low free radical production generally results in long lifespan, but naked mole-rats are specific exceptions. The possible reasons for this exception are not known, but naked mole-rats are "unusual" in several other ways, being highly eusocial, having poor endothermy and exhibiting nonavoidance of inbreeding depression (Buffenstein, 2005). They have virtually undetectable cellular glutathione peroxidase activity (Andziak et al., 2005) and very high levels of oxidative damage in certain tissues (Andziak et al., 2006). Alternatively, it may be that some of the underlying assumptions involved in the analysis are invalid. The method of phylogenetically independent contrasts assumes equal rates of evolution along all branches of the tree for both ROS production and lifespan. If this assumption is invalid for either trait in naked mole-rats, then the calculated branch length will be incorrect, resulting in a spurious contrast value.

By what mechanism could low mitochondrial ROS production cause extended lifespan? The traditional view according to the free radical hypothesis of aging would be that the low ROS production rates in long-lived species lead to low accumulation rates of molecular damage, and thus a slow decline in cellular function over time. There are reports that whole cells from long-lived mammals and birds are more resistant to external stresses than cells from short-lived species (Ogburn et al., 1998; Kapahi et al., 1999). It may be that low ROS production contributes to this resistance, for example, when compared to cells producing high amounts of ROS, cells that produce low amounts of ROS fare better when exposed to more ROS or other stresses. An intriguing alternative (but not necessarily mutually exclusive) possibility may be that ROS influence lifespan via signalling mechanisms (Linnane & Eastwood, 2006).

Free radicals and ROS do serve in signal transduction pathways (Droge, 2002), but basic information on signalling and ROS in different species is lacking.

In conclusion, our results show that the correlations between mitochondrial ROS production and lifespan as originally proposed by the laboratories of Sohal and Barja are correct. For the first time we subjected ROS production rates and lifespan data to residual analysis and phylogenetic independent contrasts. We show that after these corrections (with the exception of naked mole-rats) lifespan still associates negatively with $H_2O_2$ production by heart mitochondria in vertebrate homeotherms, strengthening this strand of evidence for the mitochondrial free radical hypothesis of aging. It should be stressed, however, that if there is a causal relationship between the rate of ROS production as measured in isolated mitochondria and lifespan, then the site of superoxide production during reverse electron transport in complex I has to be active in vivo. No associations between ROS production during forward electron transport and lifespan were found.

**Experimental procedures**

**Materials**

Amplex red (10-acetyl-3,7-dihydroxyphenoxazine) was purchased from Invitrogen, Paisley, UK. Potassium chloride was from BDH, Poole, UK. HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) was from Fisher, Loughborough, UK. All other chemicals were from Sigma, Poole, UK.

**Animals**

Female naked mole-rats and male Damara mole-rats were obtained from colonies maintained at the City College of New York as described previously (Andziak et al., 2005). Female C57BL/6 mice and female Hartley guinea pigs were from Charles River, USA (Wilmington, MA, USA). Female Wistar rats and female BALB/c mice were from Charles River, UK (Margate, UK). Male White-footed mice were from the Peromyscus Genetic Stock Center, University of South Carolina, SC, USA. Male wild-derived mice from the Idaho line were generated as described (Miller et al., 2002). Little brown bats and Brazilian free-tailed bats were wild captured from a barn in Framingham, MA, USA and from beneath a highway bridge near D'Hanis, TX, USA, respectively, and of mixed sex. Pigeons were of unknown sex and obtained from Cambridge University Veterinary School, UK. Japanese quails were from a commercial supplier in Cambridge, UK, and of unknown sex. Ox hearts were obtained from an abattoir near Cambridge, UK, from animals of unknown sex. Yellow baboons were from the South-West Foundation for Biomedical Research, San Antonio, TX, USA, and of mixed sex. In most cases, the ages of the animals used were approximately 5–7% of the maximum recorded lifespan for the species. The exceptions were the two bat species and pigeon, for which it was not possible to determine the ages of the animals used, and the baboons, which were approximately 60% of the maximum recorded lifespan. Values for the maximum observed lifespan and body mass are given in Table 1. We compared $H_2O_2$ production rate by heart mitochondria from male mice to female mice (C57BL/6 strain) and did not find any significant differences (data not shown). We assume that the effect of sex is negligible in the other species. As the ages of some of the animals were unknown, we cannot rule this out as a possible confounding factor in our analysis.

**Isolation of mitochondria**

Standardized isolation methodologies were employed with heart tissue as previously described (Tyler & Gonce, 1967), with modifications. Briefly, heart tissue was chopped with scissors...
and minced with a scalpel blade prior to incubation with protease in 250 mM sucrose, 5 mM Tris and 2 mM EGTA, pH 7.4 at 4 °C. The tissue was homogenized and the mitochondria were isolated by differential centrifugation. For mice, naked mole-rats and bats, the whole hearts from four to ten individuals were pooled and treated as one preparation. For rats and quails, two whole hearts were pooled and treated as a single preparation. The whole heart from individual guinea pigs, Damara mole-rats and pigeons was used for a single preparation. For ox and baboon, approximately 10 g of right ventricular tissue was used for the preparation. Mitochondrial protein content was determined by the biuret assay using bovine serum albumin (BSA) as standard.

Measurement of mitochondrial hydrogen peroxide production

Hydrogen peroxide generation rate was determined by monitoring the oxidation of either p-hydroxyphenylacetic acid (PHPA) or amplex red with the reaction being coupled to the enzymatic reduction of H₂O₂ by horseradish peroxidase. Control experiments with mitochondria from rat skeletal muscle indicated that the two probes were equivalent (data not shown). Mitochondria (0.35 mg mitochondrial protein per mL) were incubated in 'standard KHEB buffer' containing 120 mM KCl, 3 mM HEPES, 1 mM EGTA, 0.3% BSA (w/v) (pH 7.2 at either 34 °C or 37 °C). Incubations also contained 6 U mL⁻¹ horseradish peroxidase, 30 U mL⁻¹ SOD, 1 μg mL⁻¹ oligomycin and either 330 μM PHPA or 50 μM amplex red. Appropriate corrections for background signals were applied (St-Pierre et al., 2002) and standard curves, generated using known amounts of H₂O₂, were used to calibrate the raw signals. Rates of H₂O₂ production in nmol min⁻¹ mg mitochondrial protein⁻¹ were calculated by linear regression from the essentially linear rate of fluorescence increase. Different respiratory substrates (5 mM succinate or 2.5 mM pyruvate + 2.5 mM malate) and inhibitors (1 mM rotenone or 1 μM piericidin) were employed to assess superoxide production by different parts of the electron transport chain under different conditions.

Analysis of residuals and phylogenetic independent contrasts

The method of analysis of residuals and phylogenetic independent contrasts (as they relate to aging in particular) is described by Speakman (2005). The methods are described in greater depth in Harvey and Pagel (1991), Felsenstein (1985) and Garland et al. (1992, 2005). The rationale for residual analysis is that body mass may influence both lifespan and ROS production, and body mass varies between species. If the possible effects of body mass on the two traits are not removed, then any description of a relationship between lifespan and ROS production will be confounded. A plot of ln maximum lifespan vs. ln body mass was constructed from the data in Table 1 and a line was fitted to the data by least squares regression (Fig. 6b). The residuals given by vertical distances of each data point to the regression line were then calculated from the regression equation. Species that are long-lived for their body mass have positive residuals (i.e. they lie above the regression line as in naked mole-rats); species that are short-lived for their body mass have negative residuals (i.e. they lie below the regression line as in mice). The procedure was repeated for ln ROS production vs. ln body mass (Fig. 6c). The two sets of residuals are independent of body mass and the relationship between them was plotted in Fig. 6d.

The residuals were then subjected to analysis of phylogenetically independent contrasts. The rationale behind this approach is that because species share common ancestors by the process of evolution, they do not represent statistically independent data. For example naked mole-rats are not independent of Damara mole-rats (they are both in the family Bathyergidae), house mice and rats are not independent of each other (they are both in the family Muridae). These four species in turn are not independent of each other as they are all rodents. The analysis corrects for this nonindependence mathematically. This is achieved by construction of a phylogenetic tree using known molecular sequence data; the important aspects of the tree are the branch lengths (representing evolutionary time) and the hierarchy of the tree (the branching structure). In our case, we have a tree with 12 'tips' as we have 12 species, and 11 'nodes' (numbered as 1–11 in Fig. 7a). The tip values for residual lifespan are subtracted from each other, giving a contrast for this trait (e.g. naked-mole rat – Damara mole-rat = node 11). The process is repeated for the internal nodes. The internal nodal values are estimates of the ancestral state, these are the calculated averages of the daughter nodal values, weighted according to the daughter branch lengths. The same procedure is applied to the values of residual ROS production, giving a set of contrasts for each trait that can be used in conventional statistical analysis.

Statistics

In Figs 2–4, values are given as means ± standard error (or ± range, if n = 2). In all cases n is the number of separate mitochondrial preparations. The significance of differences between means was assessed by ANOVA or Student’s t-test. In Figs 6 and 7, significance of correlation coefficients and the sign test were calculated as described (Sokal & Rohlf, 1981). P values < 0.05 were taken to be significant.

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