Experimental Gerontology 47 (2012) 203–210

Contents lists available at SciVerse ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

# Does the oxidative stress theory of aging explain longevity differences in birds? I. Mitochondrial ROS production

Magdalene K. Montgomery [a,b,*], A.J. Hulbert [a], William A. Buttemer [c]

[a] School of Biological Sciences, University of Wollongong, Wollongong, New South Wales 2522, Australia
[b] Diabetes & Obesity Research Program, Garvan Institute of Medical Research, Darlinghurst, New South Wales 2010, Australia
[c] Centre for Integrative Ecology, Deakin University, Waurn Ponds, Victoria 3217, Australia

ARTICLE INFO

*Article history:*
Received 10 June 2011
Received in revised form 8 October 2011
Accepted 14 November 2011
Available online 21 November 2011

Section Editor: Andrzej Bartke

*Keywords:*
Aging
Parrots
Maximum lifespan
Reactive oxygen species (ROS)
Antimycin A
Free radical leak

ABSTRACT

Mitochondrial reactive oxygen species (ROS) production rates are reported to be inversely related to maximum lifespan potential (MLSP) in mammals and also to be higher in short-living mammals compared to short-living birds. The mammal–bird comparison, however, is mainly based on studies of rats and pigeons. To date, there has been no systematic examination of ROS production in birds that differ in MLSP. Here we report a comparison of mitochondrial ROS production in two short-living (quails) and three long-living bird species (parrots) that exhibit, on average, a 5-fold longevity difference. Mitochondrial ROS production was determined both in isolated mitochondria (heart, skeletal muscle and liver) as traditionally done and also in intact erythrocytes. In all four tissues, mitochondrial ROS production was similar in quails and parrots and showed no correspondence with known longevity differences. The lack of a consistent difference between quails and parrots was not due to differences in mitochondrial content as ROS production in relation to oxygen consumption (determined as the free radical leak) showed a similar pattern. These findings cast doubt on the robustness of the oxidative stress theory of aging.

© 2011 Elsevier Inc. All rights reserved.

## 1. Introduction

The oxidative stress theory of aging gained considerable support when Sohal and colleagues discovered that the rates of superoxide and hydrogen peroxide production of liver, heart and kidney mitochondria were inversely related to maximum lifespan potential (MLSP) in mammals (Sohal et al., 1990; Ku et al., 1993). Because the mammal species they studied differed significantly in size, the extent to which interspecific differences in MLSP were attributable to size effects versus differences in rates of mitochondrial formation of reactive oxygen species (ROS) was unclear. This led the group to compare mitochondrial ROS production in short-living rats (MLSP 5y) to that of similar-sized but long-living pigeons (MLSP 35y). They found mitochondrial ROS production to be significantly lower in brain, heart and kidney of pigeons, from which they concluded that pigeon tissues were under less oxidative stress than those of rats (Ku and Sohal, 1993). Subsequent studies have extended this comparison by using different tissues and substrates for the mitochondrial respiration chain, and also conclude that mitochondrial ROS production is higher in rats compared to pigeons (Barja et al., 1994; Herrero and Barja, 1997; Barja, 1998; Lambert et al., 2007, 2010). Although the finding that heart mitochondria of short-living mice (MLSP 4y) have higher ROS production rates than those of long-living budgerigars (MSLP 21y) and canaries (MLSP 24y) (Herrero and Barja, 1998) further supports the oxidative stress theory of aging, Brown et al. (2009) challenge this generality by showing that liver mitochondria from mice have a lower rate of ROS production compared to longer-living house sparrows (MLSP 14y).

While previous comparative studies mainly support the oxidative stress theory of aging, the extent to which variation in mitochondrial ROS production is responsible for differences in MLSP among species is far from certain. Most conclusions are based mainly on studies comparing isolated heart mitochondria using succinate as a respiratory chain substrate. In some cases, however, different conclusions are reached when using other substrates or other tissues. For example, whereas the Barja group demonstrated a higher ROS production in pyruvate-supplemented heart mitochondria of the rat in comparison to the pigeon (Herrero and Barja, 1997), Lambert et al. (2007) did not find a relationship between heart mitochondrial ROS generation and MLSP in a variety of mammals and birds

*Abbreviations:* FRL, free radical leak; HRP, horseradish peroxidase; HVA, homovanillic acid; MLSP, maximum lifespan potential; RCR, respiratory control ratio; ROS, reactive oxygen species; SOD, superoxide dismutase.

* Corresponding author at: Diabetes & Obesity Research Program, Garvan Institute of Medical Research, Darlinghurst, New South Wales 2010, Australia. Tel.: +61 9295 8236, +61 422059907(mobile); fax: +61 9295 8201.
*E-mail addresses:* m.montgomery@garvan.org.au (M.K. Montgomery), hulbert@uow.edu.au (A.J. Hulbert), buttemer@deakin.edu.au (W.A. Buttemer).

0531-5565/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.exger.2011.11.006

using pyruvate, but did so when providing succinate. Similarly, the relationship between MLSP and mitochondrial ROS production originally reported for liver (Barja et al., 1994) was not observed for glutamate/malate- or succinate-supplemented liver mitochondria (Brown et al., 2009).

While mammal–bird longevity differences make them well suited for examining the bases of aging, there has been surprisingly little comparative examination of ROS production rates among bird species with very different MLSP. Here we report a comparison of mitochondrial ROS production of three tissues from two short-living and three long-living bird species that exhibit, on average, 5-fold longevity differences. The two quail species (king quails and Japanese quails) and the three parrot species (budgerigars, lovebirds and cockatiels) overlap in size, but the quails are very short-living and the parrots are very long-living among bird species (Fig.1). Although traits, including MLSP, among closely related species cannot be considered to be statistically independent if phylogenetic signals strongly influence the evolution of those traits (Felsenstein, 1985), the oxidative stress theory of aging predicts convergent evolution of mechanisms responsible for longevity.

Traditionally, mitochondrial ROS generation has been determined using enzymatic detection of hydrogen peroxide production by isolated mitochondria (Barja, 2002). Extrapolation from these in vitro measurements to the in vivo situation is difficult because little is known about changes in mitochondrial superoxide production in response to physiologically relevant changes in substrate supply and energy demand in intact cells. Some of these uncertainties are alleviated by using fluorescent dyes, such as Dihydrorhodamine 123 or MitoSox Red to infer cellular and mitochondrial ROS production in intact cells (Wardman, 2007). Birds, in contrast to mammals, have nucleated erythrocytes that also possess mitochondria and other organelles; thus permitting ROS production to be analyzed in whole cells in their physiological cell environment. We have therefore determined ROS production both in intact red blood cells and in mitochondria isolated from three tissues (heart, skeletal muscle and liver) of the five bird species. ROS production of isolated mitochondria was determined under physiological substrate concentrations of succinate and pyruvate at assay temperatures representing normal bird body temperature. These measurements were undertaken in the absence and presence of the complex III inhibitor antimycin A to assess basal and maximal rates of ROS production, respectively.

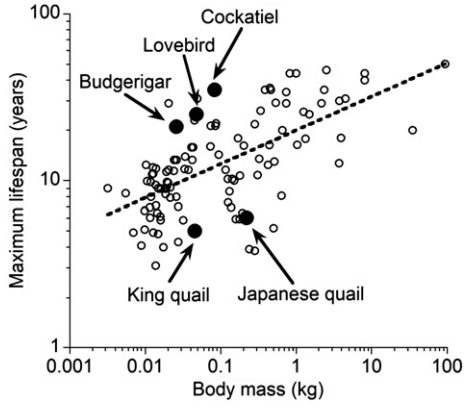


**Fig. 1.** Correlation of body weight and maximum lifespan in birds. The five bird species used in this study are superimposed highlighting that the parrots are long-living and the quails are short-living for birds. Shown is the average body mass, with n = 6 for budgerigars, n = 8 for lovebirds and king quails, n = 9 for cockatiels and n = 10 for Japanese quails.

## 2. Methodology

### 2.1. Holding conditions

All experiments were approved by the University of Wollongong Animal Ethics Committee and were conducted in conformity with the NHMRC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Six male budgerigars (*Melopsittacus undulatus*) (MLSP = 21 y; average mass = 26.0 g) derived from wild-type native budgerigars were purchased from a bird breeder in Queensland, Australia. Ten Japanese quails (*Coturnix japonica*) (MLSP = 6 y; average mass = 221.2 g) were purchased from Kyeema Organics, Windellama, Australia; and were of mixed sex. Eight lovebirds (*Agapornis roseicollis*) (MLSP = 25 y; average mass = 48.9 g), nine cockatiels (*Nymphicus hollandicus*) (MLSP = 35 y; average mass = 83.2 g), and eight king quails (*Coturnix chinensis*) (MLSP = 5 y; average mass = 45.4 g) were from a local breeder (Andrew's Pet Shop, Smithfield, Australia) and were of mixed sex. Data for MLSP were obtained from http://www.eol.org/pages/1177924, for cockatiels, from http://www.cyberquail.com/faq.html for king quails, and from the AnAge database (http://genomics.senescence.info/species) for all other species. Comparison of oxidative stress parameters in species that differ in MLSP can be approached in two ways: (1) animals can be chosen to have the same biological age, meaning that the parrots would be approximately 5-times older than the quails, or (2) animals can be chosen to have the same chronological age. We used the second approach and selected birds that were all approximately 1 year old, with the oxidative stress theory predicting that more rapidly aging species should have higher rates of ROS production.

All birds had free access to water and to the same specially formulated pellet diet for at least 6 weeks. The diet was formulated to ensure adequate n-3 and n-6 polyunsaturated fatty acids and to be palatable to parrots as well as to quails. This common diet was used to ensure that all assessed differences were independent of the birds' diet. Birds were housed in outdoor aviaries a minimum of 2 months prior to sampling. Despite having been purchased from different commercial sources, we believe the extended adjustment period to a common diet under similar holding conditions renders observed differences in mitochondrial ROS production to reflect species-specific characteristics rather than potential effects of previous husbandry conditions.

Two weeks prior to euthanasia, blood was collected and ROS production of erythrocytes was determined immediately afterwards. Following euthanasia, aliquots of liver, pectoral muscle and heart were placed in buffer solutions for mitochondrial extraction (Trzcionka et al., 2008) prior to determining mitochondrial ROS production (see below).

### 2.2. Chemicals

MitoSox Red, Mitotracker Red CMXRos and Dihydrorhodamine 123 were purchased from Invitrogen, Mount Waverley, Australia. All other chemicals were from Sigma-Aldrich, Castle Hill, Australia.

### 2.3. ROS in intact cells

ROS formation in erythrocytes was measured by flow cytometry using dyes, which exhibit fluorescence upon oxidation. We measured the fluorescence output after a 30 min incubation period. The fluorescence output represents the magnitude of ROS (in arbitrary fluorescence units) produced over the 30 min period and not a production rate. Dihydrorhodamine 123 (DHR) was used to detect hydroxyl radicals and peroxynitrate (Guo et al., 2008), whereas superoxide was measured with MitoSox Red (Olsson et al., 2009) and the membrane

potential was determined using Mitotracker Red CMXRos (CMX) (Pendergrass et al., 2004).

Fresh blood was diluted 1:10 in phosphate buffered saline (PBS, 138 mM NaCl, 2.7 mM KCl, pH 7.4 at 25 °C). In a washing step, the blood was further diluted to a final volume of 15 ml and centrifuged (1800 rpm for 5 min) to pellet the cells. The pellet was resuspended in PBS containing one of the following: (1) no additions (unstained control), (2) 100 μM dihydrorhodamine 123 (DHR), (3) 5 μM MitoSOX Red, or (4) 0.1 μM Mitotracker Red CMXRos (CMX). DHR, MitoSOX Red and CMX were added from stock solutions in dimethyl sulfoxide (DMSO), with the final concentration of DMSO being less than 0.2% (v/v). The cells were incubated at 41 °C for 30 min in the dark, PBS added to a final volume of 15 ml and the samples then centrifuged (1800 rpm for 5 min). The pellets were resuspended in PBS and transferred into flow cytometer tubes. The emitted fluorescence of the flow cytometer (Becton Dickinson LSR II, excitation at 488 nm) was collected using bandpass filters of 515 ± 10 nm (DHR), 575 ± 13 nm (MitoSox Red and Mitotracker Red CMXRos) and 695 ± 20 nm (Propidium iodide) acquired 30,000 events for all samples. Propidium iodide, which is excluded by viable cells, but penetrates the cell membrane of dying or dead cells, was added to each sample before the reading (1 mg/ml) to permit exclusion of dead cells from the analysis. Data were analyzed using FlowJo Software (Tree Star, Ashland, USA). The arithmetic mean of the fluorescence values of all 30,000 counted events was determined and used to compare the ROS production between species. The results for ROS production in erythrocytes are shown in arbitrary fluorescent units.

### 2.4. Isolation of mitochondria

We extracted tissues for mitochondrial extraction from an equal number of parrots and quails on the same day to minimize potential differences due to daily variation in the quality of mitochondrial preparation. Mitochondria were isolated by differential centrifugation as described previously (Trzcionka et al., 2008). The protein concentration of mitochondrial suspensions was determined by the Biuret method using BSA as standard (Gornall et al., 1949).

### 2.5. Mitochondrial respiration (respiratory control ratio)

Mitochondrial oxygen consumption was determined as described by Trzcionka et al. (2008). Oxygen consumption was measured using a Clarke-type electrode (Rank Brothers Ltd, Cambridge, UK) maintained at 41 °C and calibrated with air-saturated medium, which was assumed to contain 381 nmol oxygen/ml (Reynafarje et al., 1985). Mitochondrial respiration was initiated by adding 5 mM succinate (state 2 respiration). The respiratory control ratio (RCR) was determined by dividing state 3 respiration (addition of 800 μM ADP to achieve maximum oxygen consumption) by state 4 respiration (5 μg/ml oligomycin, which inhibits the F1Fo-ATP synthase and prevents ATP synthesis).

### 2.6. Mitochondrial $H_2O_2$ production

ROS production in isolated mitochondria was determined by monitoring the oxidation of homovanillic acid (HVA) as described elsewhere (St-Pierre et al., 2002). $H_2O_2$ was detected using a BMG Labtech FLUOstar OPTIMA fluorescence plate reader. Mitochondria were incubated at 0.5 mg/ml at 41 °C in assay medium (120 mM KCl, 3 mM Hepes, 1 mM EGTA, 0.3% BSA, pH 7.2 at 41 °C) containing horseradish peroxidase (HRP, 16 u/ml, One pyrogallol unit will form 1.0 mg purpurogallin from pyrogallol in 20 s at pH 6.0 at 20 °C), superoxide dismutase (SOD, 60 u/ml, 1 u will inhibit reduction of cytochrome c by 50% in a coupled system with xanthine oxidase at pH 7.8 at 25 °C in a 3.0 ml reaction volume), and HVA (0.2 mM).

Mitochondrial ROS production was determined under 2 different conditions: (1) at state 2 (no inhibitors), and (2) at state 2 with the addition of antimycin A (10 μM, inhibits center i of complex III of the respiration chain). After a 5-min incubation period at 41 °C inside the plate reader, ROS production was initiated by injecting (using onboard injectors) a substrate for the mitochondrial respiration chain: either pyruvate and malate (both 2.5 mM) or succinate (5 mM). ROS production, observable as a linear increase in fluorescence, was monitored for 20 min.

### 2.7. Calibrations and corrections

Calibrations and corrections for mitochondrial quenching and background fluorescence were carried out as described by St-Pierre et al. (2002). Calibrations were performed by adding known amounts of $H_2O_2$ (up to 3 μM). The presence of mitochondria quenched the fluorescence; the slopes with liver, heart and pectoral muscle mitochondria were on average 73, 95 and 91% of the corresponding slopes of control curves without mitochondria. Horseradish peroxidase and superoxide dismutase were used in excess, as indicated by further addition of either enzyme having no further effect on the results. Control measurements were carried out in the absence of mitochondria or HVA. With HVA, dependent on the absence or presence of inhibitors, control measurements led to a slight linear increase or decrease in fluorescence, respectively. The final rates of $H_2O_2$ production, as shown in the results section, have been fully corrected by subtracting background rates associated with control measurements.

### 2.8. Free radical leak

The rates of basal ROS production and state 2 respiration of succinate-supplemented mitochondria were used to calculate the percentage of electrons which leak out of sequence and produce superoxide and subsequently hydrogen peroxide in succinate-supplemented mitochondria (Brown et al., 2009). Whereas two electrons are needed for the reduction of 1 mol of $O_2$ to $H_2O_2$, four electrons are transferred in the reduction of 1 mol of $O_2$ to water. Therefore, the percent free radical leak was calculated as the rate of $H_2O_2$ production divided by twice the rate of oxygen consumption, and the result then multiplied by 100 (Gredilla et al., 2001).

### 2.9. Statistical analysis

Data analyses were performed using JMP 5.1 (Statistical Analysis System Institute Inc., Cary, NC, USA). All results are expressed as means with their standard errors, with $p = 0.05$ set as the level of significance. Data were tested for normality using the Shapiro–Wilk W test and homogeneity of variance using the O'Brien and Brown–Forsythe tests. Data not normally distributed were compared using non-parametric Wilcoxon/Kruskal–Wallis tests and any data showing unequal variance were compared using a Welch ANOVA. A one-way ANOVA was undertaken with bird species as the independent variable and ROS production/free radical leak as the dependent variable. Means were then compared using the Tukey–Kramer honestly significant difference test. There was no effect of gender on any of the measurements.

## 3. Results

### 3.1. ROS production in erythrocytes

The production of hydroxyl radicals and peroxynitrate (determined over a 30 min period using Dihydrorhodamine 123) was highly variable, with king quail erythrocytes exhibiting the lowest and the budgerigar erythrocytes showing the highest fluorescence, but with no significant difference between the other species (Fig. 2A).

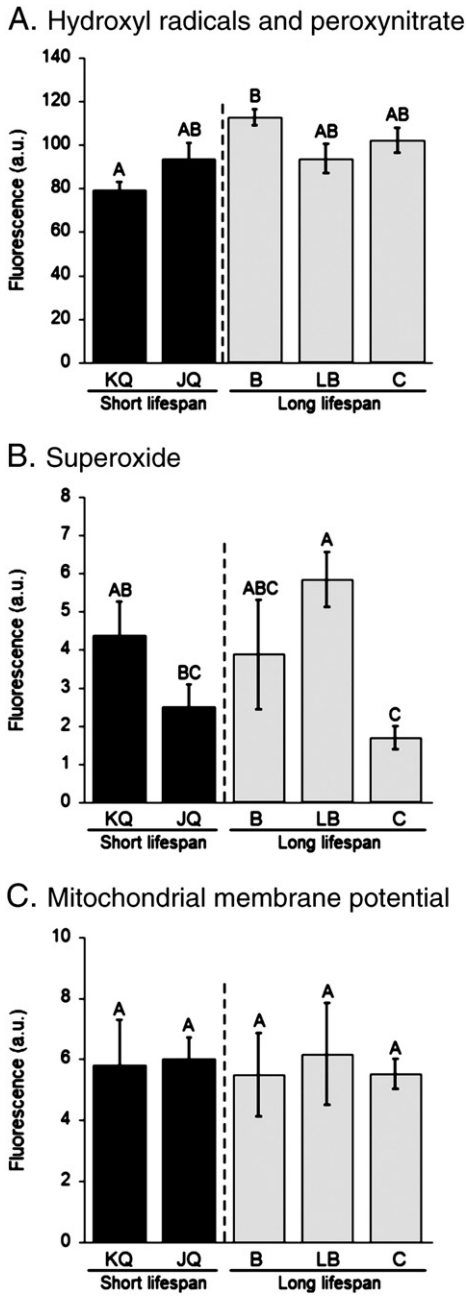


**Fig. 2.** Reactive oxygen species (ROS) production and mitochondrial membrane potential of quail and parrot erythrocytes. (A) Detection of hydroxyl radicals and peroxynitrate using Dihydrorhodamine123 (DHR); (B) Detection of superoxide using MitoSox Red; (C) Determination of mitochondrial membrane potential using Mitotracker CMX ROS. Shown are means ± SEM, n = 6 for budgerigars, n = 8 for lovebirds and king quails, n = 9 for cockatiels and n = 10 for Japanese quails. Bars that don't share a common letter are significantly different ($p<0.05$). KQ = king quails, JQ = Japanese quails, B = budgerigars, LB = lovebirds, and C = cockatiels.

Although there are significant differences in erythrocyte superoxide production between individual species, there is no general pattern between short-living quails and long-living parrots, nor among the parrots with respect to MLSP (Fig. 2B). Superoxide production is higher in lovebirds in comparison to Japanese quails and cockatiels, and also higher in king quails in comparison to cockatiels (Fig. 2B). Because mitochondrial uptake of the fluorescent dye used for superoxide detection, MitoSox Red, is dependent upon mitochondrial membrane potential, we also evaluated mitochondrial membrane potential using Mitotracker Red CMXRos, a fluorescent dye that accumulates inside mitochondria dependent upon the membrane potential. The erythrocyte mitochondrial membrane potential did not statistically differ among any bird species (Fig. 2C), giving us confidence that our MitoSox Red fluorescence measurements were valid indicators of superoxide production.

Based on our superoxide and hydroxyl radical measurements, we conclude that there is no relationship between erythrocyte ROS production and MLSP among these five bird species.

### 3.2. ROS production by isolated mitochondria

To verify that the mitochondria isolated from heart, skeletal muscle and liver were functionally intact, we measured mitochondrial respiration polarographically in the presence and absence of ADP while using succinate as substrate for the respiratory chain. From Table 1, it can be seen that all mitochondrial preparations were coupled and thus functionally intact. There was no significant difference in respiratory control ratios for heart or skeletal muscle mitochondria between the 5 bird species, but budgerigar liver mitochondria were significantly more coupled than those from the other bird species.

Superoxide production was determined in these isolated mitochondria, using both pyruvate (+malate) and succinate as substrates, and in the absence/presence of antimycin A (complex III inhibitor), using homovanillic acid (HVA) as fluorescent dye as described by Barja (2002). The use of HVA requires careful calibration and control for background fluorescence and fluorescent quenching, and these corrections can sometimes lead to negative values when ROS production rates are very low (St-Pierre et al., 2002). We have verified this methodological approach in a rat–pigeon comparison, which showed: (1) ROS production rates in agreement to those reported previously by other groups (e.g. Lambert et al., 2007) and (2) almost identical results when using HVA or Amplex Ultra Red as fluorescent dyes (Montgomery et al., 2011). With pyruvate (+malate) as a substrate, superoxide is generated at both complexes I and III via forward electron transport. In contrast, the addition of succinate leads to superoxide production at complex I mainly through reverse electron flow and at complex III through forward electron transport. The presence of excess antimycin A (with both substrates) yields complex I superoxide plus a maximal production of superoxide from complex III because of a complete reduction of respiratory chain components prior to center i of complex III (Lambert and Brand, 2004). To facilitate the discussion of the results, mitochondrial superoxide production in the absence of antimycin A will be called 'basal ROS production', whereas the rates of superoxide generation in the presence of antimycin A will be referred to as 'maximal ROS production' (see Figs. 3 and 4) as reported in other studies (e.g. Brown et al., 2009). All values shown were obtained from mitochondria under state 2 (non-phosphorylating) conditions.

Using pyruvate (+malate) as a substrate, the basal rates of mitochondrial ROS production are very low in all three tissues and in all species, except in the budgerigar (Fig. 3). Budgerigar mitochondria have significantly higher rates of hydrogen peroxide production in heart and skeletal muscle compared to the other species, but not in the liver. In general, parrot mitochondria produced similar amounts of hydrogen peroxide as quail mitochondria. The maximal rates of ROS production are greater than the basal rates, but not significantly greater in all cases (asterisks inside the bars indicate significant increases in maximal rates compared to basal rates). Budgerigar mitochondria have a higher maximal hydrogen peroxide production in skeletal muscle and liver compared to all other species, but not in heart. However, as with basal ROS production, the maximal rates show no longevity-related pattern (Fig. 3).

In general, mitochondrial ROS production rates in succinate-supplemented mitochondria show a similar interspecific pattern as when given pyruvate (+malate), but the actual rates of hydrogen peroxide production are higher with succinate. The rates of basal ROS production reported here are similar to values reported

**Table 1**
Respiration rates of quail and parrot heart, skeletal muscle and liver mitochondria.

| Parameter | King quail | Japanese quail | Budgerigar | Lovebird | Cockatiel |
|---|---|---|---|---|---|
| *State 2 respiration* (nmol $O_2$/min/mg protein) | | | | | |
| Heart | $13.5 \pm 3.8^{A}$ | $30.5 \pm 4.4^{B}$ | $31.2 \pm 4.1^{B}$ | $16.9 \pm 5.7^{A}$ | $7.4 \pm 1.7^{A}$ |
| Pectoral muscle | $26.4 \pm 5.7^{AB}$ | $44.4 \pm 6.2^{A}$ | $26.3 \pm 8.0^{AB}$ | $8.2 \pm 2.5^{B}$ | $18.0 \pm 3.0^{AB}$ |
| Liver | $22.5 \pm 5.5^{AB}$ | $27.4 \pm 3.0^{AB}$ | $35.8 \pm 11.5^{A}$ | $43.4 \pm 7.8^{A}$ | $16.1 \pm 2.5^{AB}$ |
| *State 3 respiration* (nmol $O_2$/min/mg protein) | | | | | |
| Heart | $43.3 \pm 12.7^{A}$ | $115.2 \pm 19.8^{B}$ | $193.1 \pm 30.1^{B}$ | $106.0 \pm 42.6^{B}$ | $20.7 \pm 6.5^{A}$ |
| Pectoral muscle | $106.3 \pm 20.0^{A}$ | $197.1 \pm 21.3^{B}$ | $73.1 \pm 23.4^{A}$ | $12.0 \pm 3.8^{C}$ | $82.6 \pm 28.3^{A}$ |
| Liver | $66.2 \pm 11.5^{A}$ | $98.6 \pm 9.8^{AB}$ | $234.7 \pm 46.8^{C}$ | $182.7 \pm 30.4^{BC}$ | $69.7 \pm 8.5^{A}$ |
| *State 4 respiration* (nmol $O_2$/min/mg protein) | | | | | |
| Heart | $13.7 \pm 3.1^{A}$ | $35.0 \pm 5.2^{B}$ | $30.3 \pm 4.6^{AB}$ | $26.5 \pm 7.8^{AB}$ | $6.0 \pm 1.6^{A}$ |
| Pectoral muscle | $28.5 \pm 7.3^{AB}$ | $39.6 \pm 6.7^{A}$ | $27.4 \pm 5.9^{A}$ | $5.0 \pm 1.9^{B}$ | $25.2 \pm 8.4^{AB}$ |
| Liver | $12.6 \pm 3.2^{A}$ | $24.1 \pm 2.2^{AB}$ | $19.2 \pm 5.6^{AB}$ | $31.0 \pm 4.9^{B}$ | $21.1 \pm 3.8^{AB}$ |
| *Respiratory control ratio* | | | | | |
| Heart | $4.2 \pm 1.2^{A}$ | $3.5 \pm 0.5^{A}$ | $6.4 \pm 1.2^{A}$ | $3.6 \pm 0.9^{A}$ | $3.7 \pm 0.5^{A}$ |
| Pectoral muscle | $5.1 \pm 1.3^{A}$ | $7.3 \pm 2.5^{A}$ | $2.4 \pm 0.3^{A}$ | $2.8 \pm 0.6^{A}$ | $4.0 \pm 1.6^{A}$ |
| Liver | $5.8 \pm 0.8^{A}$ | $4.2 \pm 0.4^{A}$ | $19.0 \pm 6.3^{B}$ | $6.2 \pm 0.8^{A}$ | $3.9 \pm 0.6^{A}$ |

Mitochondrial respiration was determined in the presence of 5 mM succinate and 7 uM rotenone. State 3 respiration was initiated through the addition of 800 uM ADP and state 4 respiration rates were obtained through the addition of 5ug/ml oligomycin. Shown are means ± SEM, n = 6 for budgerigars, n = 8 for king quails and lovebirds, n = 9 for cockatiels and n = 10 for Japanese quails. Mean values without a common superscript in each row are significantly different ($p < 0.05$).

previously for other Galliformes and non-Galliformes (St-Pierre et al., 2002; Mujahid et al., 2009; Azad et al., 2010). Regardless of the substrate provided, there was no longevity-related pattern in basal ROS production.

### 3.3. ROS production relative to oxygen consumption

Because all of our analyses of mitochondrial ROS production have been determined relative to mitochondrial protein content, it is possible that longevity-related patterns might be masked by species differences in mitochondrial protein content. Another way of comparing mitochondrial ROS production is to express it relative to the rate of mitochondrial oxygen consumption. Using reasonable assumptions (see methods), it is possible to convert both the measured hydrogen peroxide production and oxygen consumption (for each individual mitochondrial preparation) to the same units (electrons) and thus calculate the percent of electrons that leak out of the respiratory chain and produce superoxides, rather than being transported to molecular oxygen as an eventual electron acceptor. This has been called the free radical leak (FRL). We were able to calculate the FRL when succinate was a substrate (from data in Table 1 and Fig. 4) for all measured rates greater than zero. Our FRL is based on electron leakage at complex I through reverse electron flow and on electron leakage at complex III through forward electron transport. Oxygen consumption measurements were carried out in the presence of the complex I inhibitor rotenone. Importantly, rotenone does not affect mitochondrial respiration when using succinate as a substrate (Dargel, 1974; Hinkle and Yu, 1979).

All FRL averages showed high intra-specific variability. The free radical leak ranges from 0.02% in Japanese quail liver to 1.25% in budgerigar skeletal muscle, but shows no longevity-specific pattern, as found with mitochondrial ROS production relative to mitochondrial protein (Fig. 5).

## 4. Discussion

The common view that mitochondrial ROS production is higher in short-living species compared to long-living species has been historically based on either bird–mammal (e.g. Ku and Sohal, 1993; Barja et al., 1994; Herrero and Barja, 1997; Lambert et al., 2007) or within-mammal comparisons (e.g. Sohal et al., 1990). To our knowledge, ours is the first study to compare mitochondrial ROS production among birds with substantial variation in MLSP. The approximately 5-fold difference in MLSP between quails and parrots is similar to the longevity difference in the commonly used rat–pigeon model. Although there are published ROS production values for five bird species, these are restricted to isolated heart and liver mitochondria (Barja and Herrero, 1998; Lambert et al., 2007; Brown et al., 2009) and have added variance due to methodological differences between them.

There is a considerable variation in the magnitude of mitochondrial in vitro ROS production reported in the literature, and our values are lower than most of these findings, but very similar to those reported by St-Pierre et al. (2002). Based on our measurements, we found no pattern between mitochondrial ROS production and longevity among the 5 species, nor did we detect a longevity-related pattern in ROS production between the organs we examined. Given the 5-fold average difference in MLSP between the quail and parrots we studied, we predicted quail mitochondria would show consistently higher rates of ROS production. Instead, of our 12 examinations of mitochondrial ROS production, there was only one instance of quail ROS production being higher than parrots, and this was for Japanese quail muscle mitochondria during maximum ROS production (Fig. 4B). By contrast, 9 of the 12 comparisons showed at least one of the parrot species to have higher rates of ROS production than one of the quail (Figs. 3 and 4). Even among the parrots, there was no consistent pattern between MLSP and mitochondrial ROS production, despite their 2-fold range of MLSP. While part of the lack of significant differences in measured ROS production among the species we studied reflects the large variance in these measurements, the absence of consistently higher mean values in most of these measurements among the two species with the lowest MLSP suggests this pattern would not be changed by having a much larger sample size.

Another indicator of mitochondrial pro-oxidative potential is the relative amount of ROS formed per unit of oxygen consumed. Early studies suggested that up to 4.4% of mitochondrial electron transport is diverted to produce superoxides (Herrero and Barja, 1997). These early estimates have been proven to be excessive, with more recent studies reporting values in the range of 0.15 to 1.0% (Hansford et al., 1997; Lopez-Torres et al., 2002; St-Pierre et al., 2002; Gomez et al.,

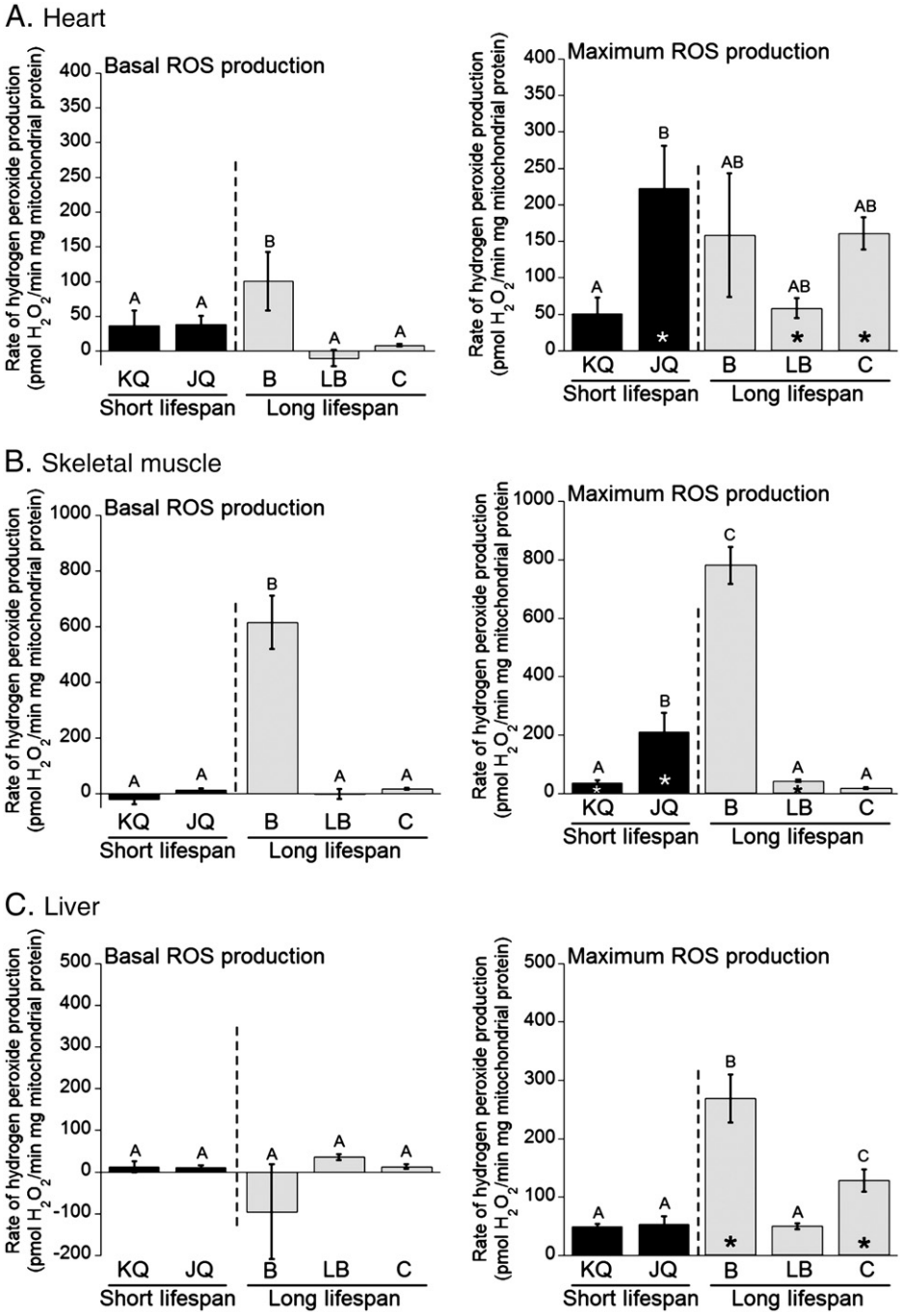


**Fig. 3.** Hydrogen peroxide production in isolated and pyruvate (+malate)-supplemented heart, skeletal muscle and liver mitochondria of quails and parrots. Rates of both basal hydrogen peroxide production (left-hand graphs) and maximal hydrogen peroxide production in the presence of excess antimycin A (right-hand graphs) are shown. Shown are means ± SEM, n = 6 for budgerigars, n = 8 for king quails and lovebirds, n = 9 for cockatiels, n = 10 for Japanese quails. Bars without a common letter are significantly different ($p<0.05$). An asterisk inside the bars indicates statistically significant intraspecific increases in maximal rates compared to basal rates ($p<0.05$).

2009), which agree with values we obtained for parrots and quails. The similar FRL and mitochondrial ROS production rates in the parrots and quails raise doubts about the importance of ROS generation rate in lifespan differences among these bird species.

An advantage in studying birds and reptiles is that their erythrocytes are nucleated and possess mitochondria (Alberio et al., 2005). This makes them amenable for determining erythrocyte mitochondrial ROS production under conditions closer to the in vivo situation, as has been determined previously in reptiles (Olsson et al., 2008, 2009). In the bird erythrocytes we studied, fluorescent dyes were used that readily enter live cells and target the mitochondria (Koppers et al., 2008; Olsson et al., 2009). Thus it is possible to measure mitochondrial ROS production in response to physiologically relevant changes in substrate supply and energy demand in intact cells. We used a similar methodological approach as in the reptile studies and determined hydroxyl radical and peroxynitrate as well as superoxide production in parrots and quails. Superoxide is produced at approximately equal rates in erythrocytes of parrots and quails. The small differences in superoxide generation between bird species occur through mechanisms that are independent of mitochondrial membrane potential. Despite their long lifespan, parrot erythrocyte mitochondria did not produce lower amounts of reactive oxygen intermediates (hydroxyl radicals and peroxynitrate) in comparison to quail erythrocytes. These results substantiate our conclusion that

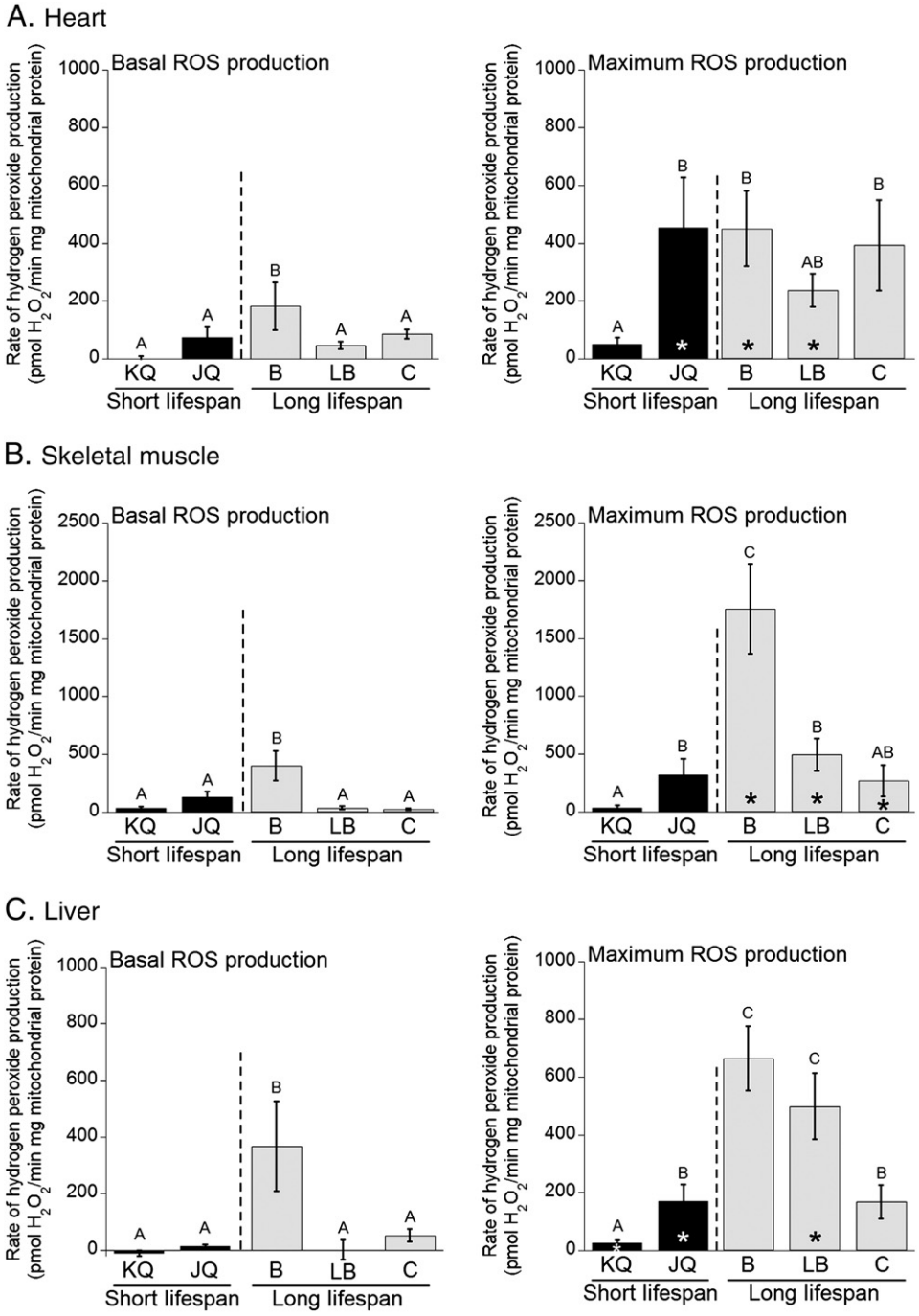


**Fig. 4.** Hydrogen peroxide production in isolated and succinate-supplemented heart, skeletal muscle and liver mitochondria of short-living quails and long-living parrots. Rates of both basal hydrogen peroxide production (left-hand graphs) and maximal hydrogen peroxide production in the presence of excess antimycin A (right-hand graphs) are shown. Shown are means ± SEM, n = 6 for budgerigars, n = 8 for king quails and lovebirds, n = 9 for cockatiels, n = 10 for Japanese quails. Bars without a common letter are significantly different ($p<0.05$). An asterisk inside the bars indicates statistically significant intraspecific increases in maximal rates compared to basal rates ($p<0.05$).

differences in ROS production are not responsible for lifespan variation in these bird species. However, it should be noted that avian erythrocytes have lifespans of approximately 25–48 days (Rodnan et al., 1957; Kalomenopoulou and Koliakos, 1989) and, thus, may have different evolutionary constraints in their rate of ROS production compared to longer-living cells.

Given the lack of a relation between ROS production rate and MLSP regardless of tissue type examined or mitochondrial substrate used, we unequivocally conclude that the 5-fold difference in MSLP between quail and parrots is not explained by differences in mitochondrial ROS production. While this refutes expectations from the oxidative stress theory of aging, this does not justify dismissing the theory altogether. It is possible, for example, that interspecific differences in MLSP reflect differences in oxidative stress and not rates of pro-oxidation per se. We have determined antioxidant protection and oxidative damage (Montgomery et al., in press) and the susceptibility of tissue membranes to peroxidation (Montgomery et al., 2012), and can conclude that none of those parameters is able to explain the lifespan differences in the parrots and quails that we studied. Importantly, all of our studies have examined birds of the same chronological, but not the same biological age. While this is a valid experimental design for testing an underlying assumption of the oxidative stress theory, namely that MLSP is inversely related to rate of accumulated oxidative damage, it presumes that cumulative damage is a linear

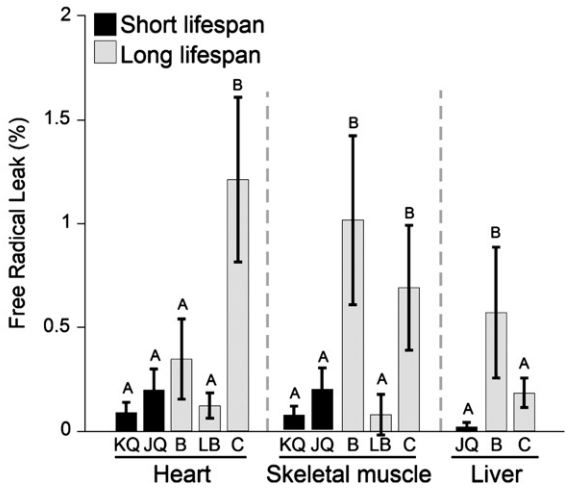


**Fig. 5.** Free radical leak (FRL) of succinate-supplemented heart, skeletal muscle and liver mitochondria of short-living quails and long-living parrots. The FRL is the percentage of electrons that leak out of sequence during mitochondrial respiration towards the production of superoxide. The FRL was calculated only when hydrogen peroxide production was greater than zero. Shown are means ± SEM, n = 6 for budgerigars, n = 8 for king quails and lovebirds, n = 9 for cockatiels and n = 10 for Japanese quails (exceptions: lovebird heart n = 3 and skeletal muscle n = 5, budgerigar heart n = 2 and skeletal muscle n = 3). Bars without a common letter are significantly different ($p < 0.05$).

process. It is very possible that oxidative stress importantly contributes to aging processes, but that the extent of its effects is dependent on age-related declines in other homeostatic control mechanisms. We therefore suggest that longitudinal studies of species with disparate MLSP are needed to resolve the functional importance of oxidative stress to a species MLSP.

## Acknowledgments

We gratefully acknowledge the assistance on diet formulation provided by Kirk C. Klasing and Ron Newman. This research was supported by funding from the Australian Research Council. The study was conceived and planned by all three authors; the experiments were carried out by M.K. Montgomery and the manuscript was written by all three authors.

## References

Alberio, S.O., Diniz, J.A., Silva, E.O., de Souza, W., DaMatta, R.A., 2005. Cytochemical and functional characterization of blood and inflammatory cells from the lizard *Ameiva ameiva*. Tissue Cell 37, 193–202.

Azad, M.A., Kikusato, M., Sudo, S., Amo, T., Toyomizu, M., 2010. Time course of ROS production in skeletal muscle mitochondria from chronic heat-exposed broiler chicken. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 157, 266–271.

Barja, G., 1998. Mitochondrial free radical production and aging in mammals and birds. Ann. N. Y. Acad. Sci. 854, 224–238.

Barja, G., 2002. The quantitative measurement of H2O2 generation in isolated mitochondria. J. Bioenerg. Biomembr. 34, 227–233.

Barja, G., Herrero, A., 1998. Localization at complex I and mechanism of the higher free radical production of brain nonsynaptic mitochondria in the short-lived rat than in the longevous pigeon. J. Bioenerg. Biomembr. 30, 235–243.

Barja, G., Cadenas, S., Rojas, C., Perez-Campo, R., Lopez-Torres, M., 1994. Low mitochondrial free radical production per unit O2 consumption can explain the simultaneous presence of high longevity and high aerobic metabolic rate in birds. Free. Radic. Res. 21, 317–327.

Brown, J.C.L., McClelland, G.B., Faure, P.A., Klaiman, J.M., Staples, J.F., 2009. Examining the mechanisms responsible for lower ROS release rates in liver mitochondria from the long-lived house sparrow (*Passer domesticus*) and big brown bat (*Eptesicus fuscus*) compared to the short-lived mouse (*Mus musculus*). Mech. Ageing Dev. 130, 467–476.

Dargel, R., 1974. Effect of Ca2 + on coupling of rat liver mitochondria. FEBS Lett. 42, 57–60.

Felsenstein, J., 1985. Phylogenies and the comparative method. Am. Nat. 125, 1–15.

Gomez, J., Caro, P., Sanchez, I., Naudi, A., Jove, M., Portero-Otin, M., Lopez-Torres, M., Pamplona, R., Barja, G., 2009. Effect of methionine dietary supplementation on mitochondrial oxygen radical generation and oxidative DNA damage in rat liver and heart. J. Bioenerg. Biomembr. 41, 309–321.

Gornall, A.G., Bardawill, C.J., David, M.M., 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177, 751–766.

Gredilla, R., Sanz, A., Lopez-Torres, M., Barja, G., 2001. Caloric restriction decreases mitochondrial free radical generation at complex I and lowers oxidative damage to mitochondrial DNA in the rat heart. FASEB J. 15, 1589–1591.

Guo, Z., Jiang, H., Xu, X., Duan, W., Mattson, M.P., 2008. Leptin-mediated cell survival signaling in hippocampal neurons mediated by JAK STAT3 and mitochondrial stabilization. J. Biol. Chem. 283, 1754–1763.

Hansford, R.G., Hogue, B.A., Mildaziene, V., 1997. Dependence of $H_2O_2$ formation by rat heart mitochondria on substrate availability and donor age. J. Bioenerg. Biomembr. 29, 89–95.

Herrero, A., Barja, G., 1997. Sites and mechanisms responsible for the low rate of free radical production of heart mitochondria in the long-lived pigeon. Mech. Ageing Dev. 98, 95–111.

Herrero, A., Barja, G., 1998. $H_2O_2$ production of heart mitochondria and aging rate are slower in canaries and parakeets than in mice: sites of free radical generation and mechanisms involved. Mech. Ageing Dev. 103, 133–146.

Hinkle, P.C., Yu, M.L., 1979. The phosphorus/oxygen ratio of mitochondrial oxidative phosphorylation. J. Biol. Chem. 254, 2450–2455.

Kalomenopoulou, M., Koliakos, G., 1989. Total-body hematocrit iron kinetics and erythrocyte life-span in pigeons (*Columba livia*). Comp. Biochem. Physiol. A Physiol. 92, 215–218.

Koppers, A.J., De Iuliis, G.N., Finnie, J.M., McLaughlin, E.A., Aitken, R.J., 2008. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. J. Clin. Endocrinol. Metab. 93, 3199–3207.

Ku, H., Sohal, R., 1993. Comparison of mitochondrial proxidant generation and antioxidant defenses between rat and pigeon — possible basis of variation in longevity and metabolic potential. Mech. Ageing Dev. 72, 67–76.

Ku, H.H., Brunk, U.T., Sohal, R.S., 1993. Relationship between mitochondrial superoxide and hydrogen peroxide production and longevity of mammalian species. Free Radic. Biol. Med. 15, 621–627.

Lambert, A.J., Brand, M.D., 2004. Inhibitors of the quinone-binding site allow rapid superoxide production from mitochondrial NADH:ubiquinone oxidoreductase (complex I). J. Biol. Chem. 279, 39414–39420.

Lambert, A.J., Boysen, H.M., Buckingham, J.A., Yang, T., Podlutsky, A., Austad, S.N., Kunz, T.H., Buffenstein, R., Brand, M.D., 2007. Low rates of hydrogen peroxide production by isolated heart mitochondria associate with long maximum lifespan in vertebrate homeotherms. Aging Cell 6, 607–618.

Lambert, A.J., Buckingham, J.A., Boysen, H.M., Brand, M.D., 2010. Low complex I content explains the low hydrogen peroxide production rate of heart mitochondria from the long-lived pigeon, *Columba livia*. Aging Cell 9, 78–91.

Lopez-Torres, M., Gredilla, R., Sanz, A., Barja, G., 2002. Influence of aging and long-term caloric restriction on oxygen radical generation and oxidative DNA damage in rat liver mitochondria. Free Radic. Biol. Med. 32, 882–889.

Montgomery, M.K., Hulbert, A.J., Buttemer, W.A., 2011. The long life of birds: the rat–pigeon comparison revisited. PLoS One 6 (8), e24138.

Montgomery, M.K., Hulbert, A.J., Buttemer, W.A., in press. Does the oxidative stress theory of aging explain longevity differences in birds? II. Antioxidant systems and oxidative damage. Exp. Gerontol.

Montgomery, M.K., Hulbert, A.J., Buttemer, W.A., 2012. Metabolic rate and membrane fatty acid composition in birds: a comparison between long-living parrots and short-living fowl. J. Comp. Physiol. B 182, 127–137.

Mujahid, A., Akiba, Y., Toyomizu, M., 2009. Olive oil-supplemented diet alleviates acute heat stress-induced mitochondrial ROS production in chicken skeletal muscle. Am. J. Physiol. Regul. Integr. Comp. Physiol. 297, R690–R698.

Olsson, M., Wilson, M., Isaksson, C., Uller, T., Mott, B., 2008. Carotenoid intake does not mediate a relationship between reactive oxygen species and bright colouration: experimental test in a lizard. J. Exp. Biol. 211, 1257–1261.

Olsson, M., Wilson, M., Isaksson, C., Uller, T., 2009. Polymorphic ROS scavenging revealed by CCCP in a lizard. Naturwissenschaften 96, 845–849.

Pendergrass, W., Wolf, N., Poot, M., 2004. Efficacy of MitoTracker Green and CMXrosamine to measure changes in mitochondrial membrane potentials in living cells and tissues. Cytometry A 61, 162–169.

Reynafarje, B., Costa, L.E., Lehninger, A.L., 1985. $O_2$ solubility in aqueous media determined by a kinetic method. Anal. Biochem. 145, 406–418.

Rodnan, G.P., Ebaugh, F.G., Spivey Fox, M.R., Chambers, D.M., 1957. The life span of the red blood cell and the red blood cell volume in the chicken, pigeon, duck as estimated by the use of $Na_2Cr^{51}O_4$: with observations on red cell turnover rate in the mammal, bird and reptile. Blood 12, 355–365.

Sohal, R.S., Svensson, I., Brunk, U.T., 1990. Hydrogen peroxide production by liver mitochondria in different species. Mech. Ageing Dev. 53, 209–215.

St-Pierre, J., Buckingham, J., Roebuck, S., Brand, M., 2002. Topology of superoxide production from different sites in the mitochondrial electron transport chain. J. Biol. Chem. 277, 44784–44790.

Trzcionka, M., Withers, K.W., Klingenspor, M., Jastroch, M., 2008. The effects of fasting and cold exposure on metabolic rate and mitochondrial proton leak in liver and skeletal muscle of an amphibian, the cane toad *Bufo marinus*. J. Exp. Biol. 211, 1911–1918.

Wardman, P., 2007. Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects. Free Radic. Biol. Med. 43, 995–1022.