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Does the oxidative stress theory of aging explain longevity differences in birds? II. Antioxidant systems and oxidative damage

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ABSTRACT

The oxidative damage hypothesis of aging posits that the accumulation of oxidative damage is a determinant of an animal species' maximum lifespan potential (MLSP). Recent findings in extremely long-living mammal species such as naked mole-rats challenge this proposition. Among birds, parrots are exceptionally long-living with an average MLSP of 25 years, and with some species living more than 70 years. By contrast, quail are among the shortest living bird species, averaging about 5-fold lower MLSP than parrots. To test if parrots have correspondingly (i) superior antioxidant protection and (ii) lower levels of oxidative damage compared to similar-sized quail, we measured (i) total antioxidant capacity, uric acid and reduced glutathione (GSH) levels, as well as the activities of enzymatic antioxidants (superoxide dismutase, glutathione peroxidase and catalase), and (ii) markers of mitochondrial DNA damage (8-OHdG), protein damage (protein carbonyls) and lipid peroxidation (lipid hydroperoxides and TBARS) in three species of long-living parrots and compared these results to corresponding measures in two species of short-living quails (average MLSP = 5.5 years). All birds were fed the same diet to exclude differences in dietary antioxidant levels. Tissue antioxidants and oxidative damage were determined both 'per mg protein' and 'per g tissue'. Only glutathione peroxidase was consistently higher in tissues of the long-living parrots and suggests higher protection against the harmful effects of hydroperoxides, which might be important for parrot longevity. The levels of oxidative damage were mostly statistically indistinguishable between parrots and quails (67%), occasionally higher (25%), but rarely lower (8%) in the parrots. Despite indications of higher protection against some aspects of oxidative stress in the parrots, the pronounced longevity of parrots appears to be independent of their antioxidant mechanisms and their accumulation of oxidative damage.

1. Introduction

In the 1950's, after the long-known toxicity of oxygen was linked to the production of free radicals by aerobic metabolism (Gerschman et al., 1954), the 'free radical theory of aging' was born (Harman, 1956). Over the years, this theory has evolved into the 'oxidative stress theory of aging', which is currently the most widely accepted explanation of lifespan determination. The main physiological mechanisms of this theory are the mitochondrial production of free radicals (currently called reactive oxygen species; ROS) and the antioxidant defence mechanisms that minimise oxidative damage. In its classic form, the oxidative stress theory of aging purports that an imbalance between ROS production and antioxidant defences leads to cumulative oxidative damage of a variety of biological molecules and eventually leads to the breakdown of homeostatic regulatory systems and, ultimately, to death (Hulbert et al., 2007). This prediction was validated empirically when it was discovered that DNA and protein damage increase with age in vertebrates and invertebrates (Agarwal and Sohal, 1994a, 1996; Sohal et al., 1994). However, it is not only ROS production and antioxidant defences that might be important in lifespan determination, but also the susceptibility of cells and tissues to oxidative damage. A modification of the oxidative stress theory, the "membrane pacemaker theory of aging", suggests that membrane phospholipids vary in their susceptibility to peroxidation, leading to the production of secondary lipid-based ROS (Hulbert et al., 2007). In our view, it is the production of both primary (superoxide and hydrogen peroxide) and secondary ROS (such as lipid

Abbreviations: 8-OHdG, 8-hydroxy-2-deoxy-guanosine; BHT, butylated hydroxytoluene; CAT, catalase; CuZn-SOD, copper–zinc superoxide dismutase; DHA, Docosahexaenoic acid; DNPH, 2,4-dinitrophenylhydrazine; DTT, dithiothreitol; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FRA, ferric reducing ability; GSH, reduced glutathione; GPx, glutathione peroxidase; LQ, longevity quotient; MDA, malondialdehyde; MLSP, maximum lifespan potential; Mn-SOD, manganese superoxide dismutase; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SOD, superoxide dismutase; TAC, total antioxidant capacity; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances.

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hydroperoxide) that might determine a species maximum lifespan (MLSP).

Antioxidant defence mechanisms vary in their importance. The functional significance of superoxide dismutase (SOD), especially the mitochondrial Mn-SOD, for protection against aging-related damage and rates of physiological decline becomes evident in knockout animal models. Mice lacking the Mn-SOD die soon after birth (Lebovitz et al., 1996; Murakami et al., 1998), whereas animals lacking the cytosolic Cu/Zn-SOD have a 'normal' phenotype and live to adulthood, but with a shorter lifespan (Ho et al., 1998; Sentman et al., 2006). These findings demonstrate the importance of a superoxide degradation system, especially at the source of generation in mitochondria. A similar situation has been shown for GPx where knockout-mice lacking the cytosolic GPx lived to adulthood (Ho et al., 1997), whereas other forms of GPx were shown to be essential for all stages of life (Yant et al., 2003). Research taking more moderate approaches of over- and under-expression of both the Cu/Zn-SOD (Huang et al., 2000; Seto et al., 1990; Staveley et al., 1990) and the mitochondrial Mn-SOD (Jang et al., 2009; Mockett et al., 1999; Perez et al., 2009a; Van Remmen et al., 2003) in animal models has shown no effect on MLSP in both vertebrates and invertebrates (for current review see Page et al., 2010). Similarly, dietary SOD supplementation increases the average lifespan (i.e., health), but has no effect on MLSP (Lopez-Torres et al., 1993). The conclusion from such studies is that the various isoforms of antioxidant enzymes vary in their effects on lifespan, and that animals have antioxidant activities that are sufficient for maximum protection from oxidative processes. Although this implies that animals should experience the same levels of oxidative stress, differences in susceptibility of their tissues to oxidative damage along with differences in rates of repair would still support the oxidative stress theory's proposal that variable rates of cumulative damage are responsible for differences in MLSP.

The long lifespan of birds is surprising as they have, on average, (1) a higher metabolic rate, (2) higher blood glucose, (3) a higher body temperature than similar-sized mammals (Holmes and Austad, 1995), and the highest pulmonary oxygen partial pressure of all vertebrate groups (Buttemer et al., 2010). Despite being predisposed to higher oxygen radical generation, their long lifespan suggests that birds are resistant to aging processes. This suggestion goes back to the early 90's when Monnier realized that birds must have special mechanisms to prevent tissue damage, such as those through exposure to advanced Maillard products (Monnier, 1990; Monnier et al., 1991). Early research showed that the susceptibility to oxidative damage (x-ray induced protein damage) in the tissues of mammals and birds was inversely related to their MLSP (Agarwal and Sohal, 1996). Furthermore, studies on avian and mammalian renal tubular epithelial cells (Ogburn et al., 1998) and primary embryonic fibroblast-like cells (Ogburn et al., 2001) showed avian cells to be more resistant to oxidative challenge than murine cells. Recent studies have confirmed this pattern in finding the fibroblasts of shorter-lived laboratory mice to be more susceptible to oxidative damage than fibroblasts of longer-lived wild-caught mice (Miller et al., 2011), as well showing an inverse relation between MLSP and fibroblast sensitivity to oxidative stress in birds (Harper et al., 2011). While variation among species in tissue resistance to oxidative damage is likely to correlate with interspecific differences in longevity, the oxidative stress theory of aging also predicts cumulative oxidative damage among species to be inversely related to their MLSP. Pamplona and colleagues examined lifespan influences on cumulative oxidative damage between size-matched bird and mammalian species that differed in MLSP. They found that the brains of long-living bird species (budgerigars and canaries) had lower levels of protein and lipoxidative damage than the brains of shorter-living mice (Pamplona et al., 2005). In contrast, oxidatively damaged proteins were similar in liver mitochondria of both short-living rats and long-living pigeons (Pamplona et al., 1996), and higher in pigeon skeletal muscle than in rat skeletal muscle (Portero-Otin et al., 2004). These contradictory findings raise doubts about the contribution of cumulative oxidative damage in determining longevity differences between species.

Further uncertainty about the role of oxidative damage and antioxidant protection in the determination of a species' maximum lifespan comes from comparisons of naked mole-rats (the longest living rodent) with similar-sized mice (Buffenstein et al., 2008). Naked mole-rats, with a MLSP of >28 years, have a longevity quotient (LQ = actual MLSP/MLSP predicted from body mass) of 5, whereas the LQ of similar-sized mice is 0.7 (Buffenstein, 2005). Naked mole-rats exhibit low antioxidant protection (Andziak et al., 2005), higher levels of lipid peroxidation, protein and DNA oxidative damage in comparison to mice, and these differences are evident from a very young age (Andziak and Buffenstein, 2006; Andziak et al., 2006). Despite this disparity between the magnitude of oxidative damage and maximum lifespan, naked mole-rats were found to have inherently higher protein stability and increased capacity to degrade oxidised or misfolded proteins through age-related increases in proteasome activity (Pérez et al., 2009). Although naked mole-rats appear to have higher protein integrity, their steady-state levels of protein damage exceed those of mice throughout their whole lifetime. Similarly high levels of oxidative damage are also apparent in other long-living species, such as birds and bats (Portero-Otin et al., 2004; Wilhelm Filho et al., 2007). The inconsistency of these and other studies has provoked some researchers to doubt the importance of antioxidant systems and oxidative damage in the determination of an animal's maximum lifespan, as well as the general validity of the oxidative stress theory (Buffenstein et al., 2008; Salmon et al., 2010).

The uncertainty about the pertinence of the oxidative stress theory in explaining MLSP variation among animals, particularly birds, motivated us to examine the main physiological components of this aging theory in birds. We selected species that overlapped in size, but had 5-fold differences in MLSP. Included in these comparisons were ROS production rates in several tissues (Montgomery et al., in press) and membrane fatty acid susceptibility to oxidative damage (Montgomery et al., 2011), but we report here tissue antioxidant defence mechanisms and oxidative damage levels in three species of long-living parrots (average LQ = 2.4) and two species of size-matched, but shorter-living quails (average LQ = 0.5). If the oxidative stress theory of aging is valid, quail are predicted to have higher rates of tissue oxidative damage leading to a higher accumulation at the same chronological age as comparably sized parrots. We have accordingly selected parrots and quails of the same age (approximately one year old), which, at the time of this study, the quails had reached 16–20% of their MLSP, whereas the parrots had attained only 3–5% of their maximum lifespan.

2. Methodology

2.1. Animals and tissue sampling

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 quail (*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. For cockatiels MLSP was obtained from http://www.eol.org/pages/

1177924, while for king quails MLSP was from http://www.cyberquail.com/faq.html and for all other species from the AnAge database (http://genomics.senescence.info/species). All birds used in this study were young adults and approximately one year old.

All birds had free access to water and to the same specially formulated pellet diet for at least six weeks to ensure that all assessed differences were not attributable to dietary differences. The diets were formulated to provide adequate n−3 and n−6 polyunsaturated fatty acids and to be palatable to parrots as well as fowl. Birds were housed in outdoor aviaries a minimum of 2 months prior to sampling.

Following euthanasia, aliquots of liver, heart and pectoral muscle were transferred to cryovials and stored in liquid nitrogen for subsequent analyses. Blood was collected into heparinised vials following cardiac puncture and centrifuged to extract plasma, which then was stored in liquid nitrogen prior to later analyses. Because this study was part of a detailed investigation of oxidative stress parameters in parrots and quails, there were insufficient amounts of some tissues for all analyses. For budgerigars, reduced glutathione and catalase activities were not determined in liver and heart, nor was catalase activity measured in plasma. Furthermore, lipid hydroperoxide levels were not determined in budgerigar plasma, nor were mitochondrial 8-OHdG levels measured in budgerigar liver. Lipid hydroperoxide levels in liver and 8-OHdG levels in the heart were only measured in Japanese quails and cockatiels.

2.2. Chemicals

Quantichrom Glutathione and Uric Acid Assay Kits were purchased from BioCore, Alexandria, Australia. Catalase, lipid hydroperoxide and 8-hydroxy-2-deoxy-Guanosine EIA kits were purchased from Sapphire Bioscience, Redfern, Australia. The Wizard SV Genomic DNA Purification Kit was purchased from Promega, Alexandria, Australia. All other chemicals were obtained from Sigma, Castle Hill, Australia.

2.3. Total antioxidant capacity

Total antioxidants in plasma were determined using two different assays: the total antioxidant capacity (TAC) assay and the ferric reducing ability (FRA) assay. The TAC assay was carried out as described previously (Erel, 2004) using a spectrophotometer (Varian Cary 300) controlled at 25 °C. Trolox (0.2 mM–1 mM), a vitamin E derivative, was used as an antioxidant standard and results are shown in mM Trolox equivalents. Total antioxidant status in heart, pectoral muscle and liver was determined with the FRA assay. Tissues were homogenised in buffer (100 mM KH_2PO_4, 1 mM EDTA) and a FRA reagent was prepared as described elsewhere (Benzie and Strain, 1996). Blank, standards (ferrous sulphate) or samples were mixed with the reagent and, after a 4-min incubation, the absorbance was determined at 593 nm and 37 °C using a temperature-controlled spectrophotometer (Varian Cary 300).

2.4. Enzymatic antioxidants

Superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) activity were determined in plasma and tissue homogenates. Tissues were homogenised with an Ultra Turrax (11,000/min, 15 s) in buffer (SOD: 210 mM mannitol, 70 mM sucrose, 20 mM HEPES, 1 mM EGTA, pH = 7.5 at 25 °C) (GPx: 50 mM Tris–HCl, 5 mM EDTA, 1 mM dithiothreitol (DTT), pH = 7.5 at 25 °C) (CAT: 50 mM KH_2PO_4, 1 mM EDTA, pH = 6.7 at 25 °C). Homogenates were centrifuged at 4 °C and 1500 g for 5 min (SOD) or at 10,000 g for 15 min (GPx, CAT) and the supernatant used for the assay. SOD activity was measured as the inhibition of the rate of cytochrome c reduction by superoxides which was followed at 550 nm and 25 °C using a temperature-controlled spectrophotometer (Varian Cary 300), adapted from a method described elsewhere (McCord and Fridovich, 1969). The activity of GPx was determined indirectly as the decrease in NADPH absorption at 340 nm and 25 °C, as described elsewhere (Maral et al., 1977). A reaction cocktail was prepared by mixing azide buffer (9.2 ml, 50 mM NaH_2PO_4, 0.4 mM EDTA, pH = 7.0 at 25 °C, addition of 1 mM Na-azide), glutathione reductase (100 μl, 100 μ/ml) and GSH (50 μl, 200 mM) into a 1 mg beta-NADPH vial. The reaction cocktail was mixed with blank, standard or sample and H_2O_2 (5 μl, 0.042% (v/v)) was added to start the reaction. GPx standards were diluted in 10 mM NaH_2PO_4, 1 mM DTT, pH = 7.0 at 25 °C. Catalase was measured in plasma, liver and heart of all animals using the Catalase Assay Kit from Cayman Chemicals (Sapphire Bioscience, Redfern, Australia).

2.5. Non-enzymatic antioxidants

Plasma uric acid levels were determined using a commercially available kit (QuantiChrom™ Uric Acid Assay Kit, Bioassay Systems). GSH levels were determined in blood, heart and liver using a commercially available kit (QuantiChrom™ Glutathione Assay Kit, Bioassay Systems). Tissues were homogenised with an Ultra Turrax (11,000/min, 15 s) in 50 mM KH_2PO_4, 1 mM EDTA, pH = 6.7. Tissue homogenates were centrifuged for 15 min at 10,000 g and 4 °C, and the supernatant used for the assay.

2.6. DNA damage — 8-OHdG

DNA damage was determined in freshly extracted heart and liver mitochondria using the 8-hydroxy-2-deoxy-guanosine (8-OHdG) EIA Kit from Cayman Chemicals (Sapphire Bioscience, Redfern, Australia). Briefly, mitochondria were isolated and mitochondrial DNA was extracted using a commercially available kit (Wizard SV Genomic DNA Purification System, Promega). The DNA was heat denatured at 95 °C for 10 min and digested for 1 h at 50 °C using nuclease P1 (43 ng/μg DNA, Sigma-Aldrich N8630). Nuclease P1 was diluted in acetate buffer (20 mM $C_2H_3O_2Na \cdot 3H_2O$, 5 mM $ZnCl_2$, 50 mM NaCl, pH = 5.3 at 50 °C). The pH of each sample was adjusted to 8.0 with 1 M Tris and alkaline phosphatase was added (1 μ/100 μg DNA). The mixture was incubated at 37 °C for 30 min, boiled for 10 min and then placed on ice. The reported GC content of mitochondrial DNA is similar in all species examined (NCBI genome database: budgerigar 44% (NC009134), rosy-faced lovebird 48% (NC011708), cockatiel 46% (NC015192), king quail 44% (NC004575), Japanese quail 44% (NC003408).

2.7. Protein damage — protein carbonyls

Tissues were homogenised in buffer (50 mM KH_2PO_4, 1 mM EDTA, pH = 6.7) and the homogenate centrifuged for 15 min at 10,000 g and 4 °C. Nucleic acids were removed using 1% streptomycin (incubation at room temperature for 15 min, followed by centrifugation at 6000 g, 10 min, 4 °C) (Pamplona et al., 1996), and the supernatant used for the assay. Protein carbonyls were derivatized to 2,4-dinitrophenylhydrazone by reaction with 2,4-dinitrophenylhydrazine (DNPH) as described previously (Levine et al., 1990). The procedure included incubation with DNPH for 1 h, several washing steps with trichloroacetic acid and an ethanol–ethyl acetate mixture, and a final resuspension of the dinitrophenylhydrazone in guanidine hydrochloride.

2.8. Measures of lipid peroxidation

Lipid hydroperoxides were determined in plasma and liver using a Lipid Hydroperoxide Assay Kit from Cayman Chemicals (Sapphire Bioscience, Redfern, Australia). The TBARS (thiobarbituric acid reactive substances) assay was used to measure malondialdehyde (MDA). Tissues were homogenised in buffer (100 mM KH_2PO_4,

1 mM EDTA). Blank, standard (tetramethoxypropane) or samples were mixed with butylated hydroxytoluene (BHT, 3 mM) and thiobarbituric acid (TBA, 0.4% (w/v) in 10% acetic acid solution (v/v), pH 5.0), and incubated for 1 h at 90 °C. Butanol was added and the samples centrifuged for 10 min at 7500 rpm. The absorbance of the butanol phase was determined at 532 nm. To minimise artificial lipid peroxidation during the TBARS reaction due to high temperatures and low pH values which are necessary to be applied, we used excess BHT which is an antioxidant and which was shown to decrease lipid peroxidation during the measurement (Jentzsch et al., 1996).

2.9. Statistical analysis

Data analyses were performed using JMP 5.1 (Statistical Analysis System Institute Inc., Cary, NC, USA). Results are expressed as means with their standard errors, with P 0.05 set as the significance level. 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 completed with the bird species as the independent variable and each antioxidant/oxidative damage biomarker as the dependent variable. Means were then compared using the Tukey–Kramer honestly significant difference test. When examining the relation between measured variables and MLSP, we used linear regression of species means followed by a one-way ANOVA to test for significance of the slope. We recognise that traits among closely related pecies cannot be considered to be statistically independent if phylogenetic signals strongly influence the evolution of those traits (Felsenstein, 1985), but the oxidative stress theory of aging predicts convergence of longevity traits at mechanistic levels. Furthermore, by choosing species from two groups that overlap in size, we have eliminated the known influence of body mass on MLSP in our comparisons (Ricklefs, 2010; Wasser and Sherman, 2010). All bird species, except the budgerigars, were of mixed sex. Antioxidant levels and oxidative stress biomarkers were statistically compared between genders. There was no effect of gender on any of the measurements so we assume that the effect of gender is negligible for all birds used in this study.

3. Results

3.1. Antioxidant levels in plasma

We measured total antioxidant capacity of plasma using two different methodological approaches: TAC (total antioxidant capacity) and FRA (ferric reducing ability). Whereas in the TAC assay the antioxidant system is challenged via the production of a hydrogen radical and the total antioxidant response against this radical is measured (Erel, 2004), the FRA assay utilizes the ability of reductants, in this case biological antioxidants, to reduce ferric ions (Benzie and Strain, 1996). As each approach measures only certain antioxidant molecules and, therefore, has its limitations (Prior and Cao, 1999), we performed both assays on all plasma samples.

In the TAC assay, proteins and uric acid compose the majority of the measured serum total antioxidant reaction (Erel, 2004). The TAC assay is commonly used by field biologists (e.g., Cohen et al., 2007) to indicate an individual's antioxidant capacity, as it is relatively non-invasive and requires only a small amount of blood. In contrast, the antioxidants measured with the FRA assay comprise mainly uric acid, GSH, alpha-tocopherol, ascorbic acid and bilirubin-related components (Janaszewska and Bartosz, 2002). Because the antioxidant activity of proteins in the FRA assay is low, high plasma protein concentration (especially albumin) won't muffle the antioxidant response and therefore, variations in other, less concentrated antioxidants can be quantified (Benzie and Strain, 1996).

Before summarizing relations between MLSP and measured variables, we call attention to our use of species' averages for all correlation analyses instead of using a multi-level approach that considers individuals as nested components. Although the latter method is more likely to identify significant correlations, it is also biassed in producing these more often when variance is high. Accordingly, we have taken a statistically conservative approach, with the consequence of reducing the likelihood of finding significant relations between measurement variables and MLSP. We have therefore identified both statistically significant and near-significant correlations in the text and figures.

The results for both TAC and FRA are depicted in Fig. 1A. Both assays show that plasma total antioxidant capacity does not correlate with MLSP of birds, although the FRA assay tends towards a negative correlation ($p = 0.06$).

Reduced glutathione (GSH) and uric acid are important non-enzymatic antioxidants and both show a trend towards a negative correlation with MLSP ($p = 0.052$; Fig. 1B and C, respectively). Both antioxidants are the main reactants in the FRA assay and the slightly negative correlation of FRA and maximum lifespan is lost when the uric acid and GSH contributions are subtracted. Although the FRA and TAC assays are used to measure the total antioxidant response of a system, differences in values between species are mainly due to differences in a small number of antioxidants and do not truly reflect a total antioxidant response.

The activities of SOD, GPx and CAT enzymes were measured to obtain insights into the capacity of plasma to degrade superoxide and H_2O_2. Whereas SOD and GPx levels are similar in all bird species we examined, CAT levels show a trend towards a negative correlation with MLSP (Fig. 1). As we were interested in the functional aspects of a tissue's antioxidant mechanisms, we did not differentiate between different enzyme isoforms (e.g., different SOD enzymes).

3.2. Antioxidant levels in tissues

Previous studies of tissue antioxidants often use different denominators to express their results, most commonly either ‘per mg protein’ or ‘per g tissue’ (e.g. Andziak et al., 2005; Cutler, 1984). Species may vary in the protein content of their tissues and consequently the choice of the denominator might affect interpretation of the data. Therefore, we determined the protein content of liver, heart and skeletal muscle (Table 1) and present all antioxidant values on a ‘per mg of protein basis’ in Figs. 2, 3 and 4, and on a ‘per g of tissue’ basis in Table 1. Tissue protein content in the birds examined tends to increase with MLSP in heart and skeletal muscle, but only liver protein content shows a significant, but negative, correlation with lifespan (Table 1).

Total antioxidant capacity of liver, heart and skeletal muscle was only determined using the FRA assay. In the liver, total antioxidant capacity as well as GSH does not correlate with maximum lifespan, either on a ‘per mg protein’ (Fig. 2) or on a ‘per g tissue’ (Table 1) basis. Examining the enzymatic antioxidants in liver, it is only glutathione peroxidase that tends to have a positive correlation with MLSP ($p = 0.08$; Fig. 2). On a “per g of tissue” basis, catalase, SOD and GPx show no tendency to correlate with MLSP (Table 1).

In the heart and in skeletal muscle, the same antioxidants were examined as in the liver, with none of them correlating with MLSP on either ‘per mg of protein’ (Figs. 3 and 4) or ‘per g of tissue’ bases (Table 1). As in liver, however, skeletal muscle glutathione peroxidase levels tend to increase with MLSP ($p = 0.1$; Fig. 4)

3.3. Oxidative damage in plasma and tissues

Protein carbonyl levels were measured as a biomarker for oxidative protein damage, whereas the extent of lipid peroxidation was

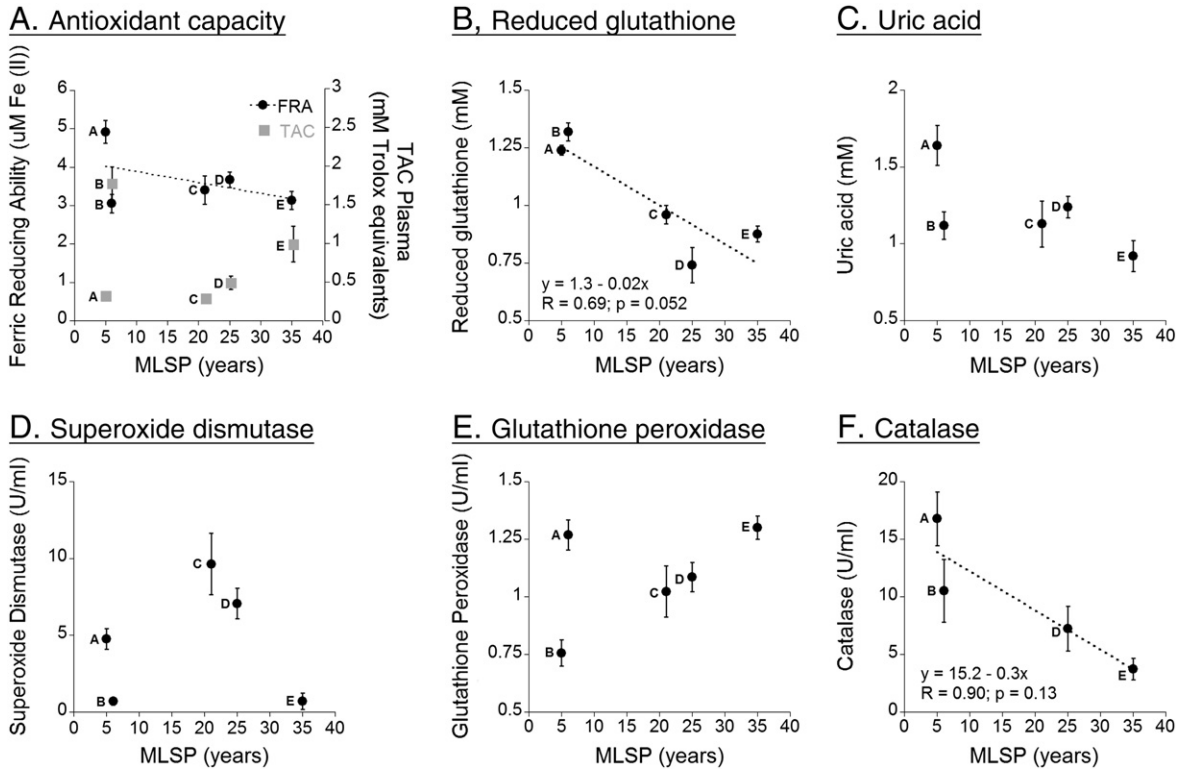

Fig. 1. The relationship between *plasma* antioxidant levels and maximum lifespan potential (MLSP) of quails and parrots is shown. (A) Total antioxidant capacity (TAC) and ferric reducing ability (FRA), (B) uric acid, (C) reduced glutathione, (D) superoxide dismutase, (E) glutathione peroxidase, and (F) catalase. 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. Linear regression was employed followed by a one-way ANOVA as an indication of fit. Letters next to data points: A = King quail, B = Japanese quail, C = Budgerigar, D = Lovebird, E = Cockatiel.

Table 1
Maximum lifespan (MLSP), body mass, tissue protein content, levels of enzymatic and non-enzymatic antioxidants and oxidative damage in parrots and quails. Values are expressed on a 'per g tissue' basis.

Parameter	Tissue	King quail	Japanese quail	Budgerigar	Lovebird	Cockatiel	Correlation with MLSP
MLSP (years)		5	6	21	25	35	
Body mass (g)		45.4 ± 1.0 ^{A}	221.2 ± 9.3 ^{B}	26.0 ± 0.7 ^{C}	48.9 ± 0.8 ^{A}	83.2 ± 2.3 ^{D}	
Number animals used		8	10	6	8	9	
Protein (mg total protein/g tissue)	Liver	231 ± 95 ^{A}	195 ± 26 ^{A}	149 ± 26 ^{A}	144 ± 25 ^{A}	99 ± 14 ^{A}	p = 0.006, R = 0.94)
	Heart	150 ± 24 ^{A}	106 ± 25 ^{A}	326 ± 24 ^{B}	159 ± 9 ^{A}	347 ± 62 ^{B}	N.S.
	Muscle	265 ± 115 ^{A}	164 ± 51 ^{A}	599 ± 92 ^{B}	197 ± 30 ^{A}	395 ± 52 ^{B}	N.S
Non-enzymatic antioxidants							
Ferric reducing ability (mmol Fe(II)/g tissue)	Liver	18.4 ± 2.4 ^{A}	4.2 ± 0.7 ^{BC}	11.3 ± 1.3 ^{ABC}	16.0 ± 3.7 ^{A}	2.7 ± 0.5 ^{BC}	N.S.
	Heart	6.0 ± 0.6 ^{AB}	1.6 ± 0.5 ^{A}	12.2 ± 2.7 ^{B}	8.1 ± 1.6 ^{AB}	6.9 ± 1.5 ^{B}	N.S.
	Muscle	2.3 ± 0.4 ^{A}	1.2 ± 0.2 ^{A}	6.2 ± 0.4 ^{B}	2.3 ± 0.3 ^{A}	4.0 ± 0.6 ^{C}	N.S
Reduced glutathione (μmol/g tissue)	Liver	2.7 ± 1.1 ^{A}	3.1 ± 0.7 ^{A}		1.4 ± 0.3 ^{B}	3.0 ± 0.3 ^{A}	N.S.
	Heart	2.3 ± 0.5^{A}	2.3 ± 0.4 ^{A}		1.7 ± 0.6 ^{A}	10.9 ± 2.5 ^{B}	N.S.
Enzymatic antioxidants							
Superoxide dismutase (U/g tissue)	Liver	6557 ± 1035 ^{A}	578.1 ± 123.7 ^{B}	3122 ± 1020 ^{C}	3613 ± 1158 ^{C}	431.2 ± 95.3 ^{B}	N.S.
	Heart	102.8 ± 59.5 ^{A}	1011 ± 154 ^{B}	37.5 ± 13.1 ^{A}	51.6 ± 17.2 ^{A}	1854 ± 594 ^{B}	N.S.
	Muscle	9444 ± 2042 ^{A}	394.2 ± 157.0 ^{B}	13188 ± 5661 ^{AC}	3116 ± 730 ^{D}	27051 ± 1137 ^{C}	N.S.
Glutathione peroxidase (U/g tissue)	Liver	612.8 ± 132.7 ^{A}	207.7 ± 24.6 ^{A}	1117 ± 267 ^{B}	533.2 ± 133.8^{A}	436.0 ± 21.7 ^{A}	N.S.
	Heart	4678 ± 1923^{A}	312.9 ± 36.6 ^{B}	9582 ± 2824 ^{C}	1059 ± 172 ^{D}	4653 ± 1552^{A}	N.S.
	Muscle	507.8 ± 172.2 ^{AB}	166.4 ± 15.4 ^{A}	1508 ± 235 ^{C}	345.8 ± 34.9 ^{A}	958.3 ± 152.1 ^{B}	N.S.
Catalase	Liver	7438 ± 1928 ^{A}	1544 ± 229 ^{B}		364.2 ± 54.4 ^{C}	620.4 ± 157.6 ^{C}	N.S.
(U/g tissue)	Heart	3983 ± 1591 ^{A}	694.2 ± 162.1 ^{B}		399.5 ± 51.4 ^{B}	2037 ± 509 ^{A}	N.S.
Oxidative damage							
Protein carbonyls (nmol/g tissue)	Liver	227.6 ± 105.3 ^{A}	211.8 ± 28.8 ^{A}	43.9 ± 8.0 ^{A}	144.3 ± 38.6 ^{A}	150.5 ± 23.4 ^{A}	N.S.
	Heart	352.6 ± 75.1 ^{AB}	56.1 ± 13.9 ^{B}	101.4 ± 31.9 ^{B}	118.0 ± 84.1 ^{B}	565.3 ± 141.3 ^{A}	N.S.
	Muscle	217.3 ± 42.0 ^{AB}	144.6 ± 17.3 ^{B}	177.0 ± 35.9 ^{AB}	301.0 ± 70.6 ^{AB}	324.8 ± 50.0 ^{A}	N.S.
TBARS (nmol/g tissue)	Liver	88.1 ± 34.5 ^{A}	21.7 ± 5.2 ^{B}	54.4 ± 15.4 ^{AB}	36.9 ± 5.9 ^{AB}	17.9 ± 4.1 ^{B}	N.S.
	Heart	40.9 ± 5.2 ^{AB}	12.4 ± 2.3 ^{B}	88.8 ± 22.1 ^{C}	62.4 ± 15.8 ^{AC}	33.7 ± 6.0 ^{AB}	N.S.
	Muscle	43.0 ± 10.4 ^{AB}	15.2 ± 2.7 ^{B}	143.3 ± 15.1 ^{C}	48.6 ± 4.4 ^{A}	16.9 ± 2.0 ^{B}	N.S.
Lipid hydroperoxide							
(pmol/g tissue)	Liver		18.0 ± 4.4 ^{A}			58.2 ± 12.8 ^{B}	

All values are expressed as means ± SEM. Values for each antioxidant without a common superscript are significantly different ($p<0.05$). See methods for statistical analysis.

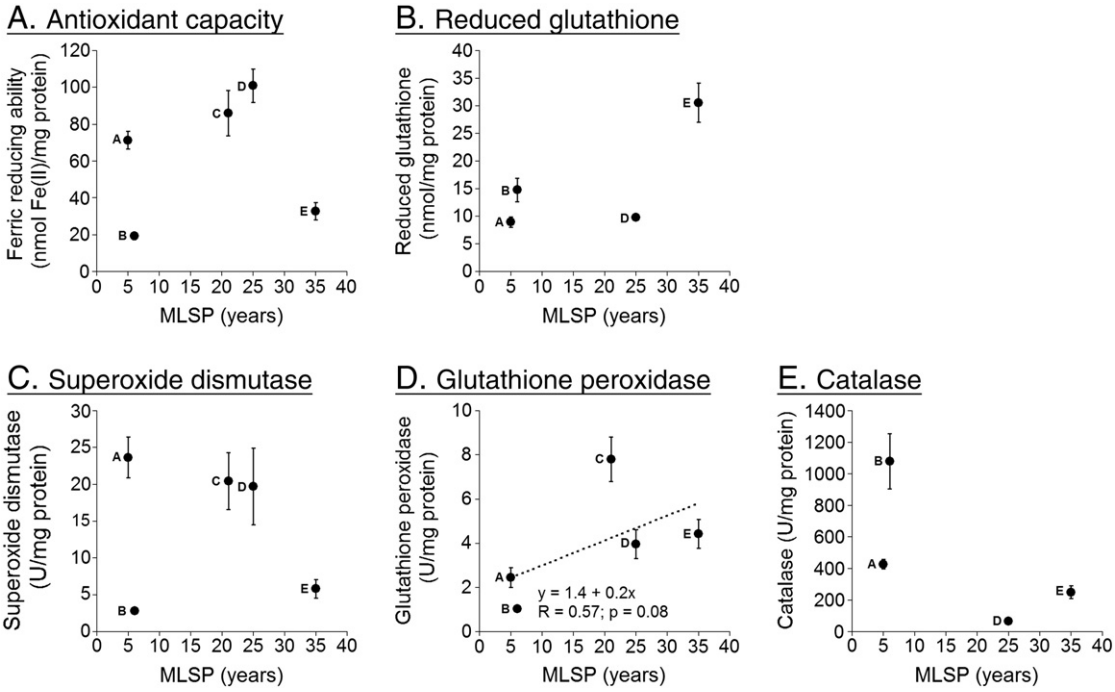

Fig. 2. The relationship between *liver* antioxidant levels and maximum lifespan potential (MLSP) of quails and parrots is shown. (A) Ferric reducing ability, (B) reduced glutathione, (C) superoxide dismutase, (D) glutathione peroxidase, and (E) catalase. Shown are means ± SEM, n = 6 for budgerigars, n = 8 for king quails and lovebirds, n = 9 for cockatiels and n = 10 for Japanese quails. Linear regression was employed followed by a one-way ANOVA as an indication of fit. Letters next to data points: A = King quail, B = Japanese quail, C = Budgerigar, D = Lovebird, E = Cockatiel.

determined by measuring lipid hydroperoxide (intermediate product of lipid peroxidation) and malondialdehyde levels (end product of lipid peroxidation), and mitochondrial 8-OHdG levels were determined as a marker for mitochondrial DNA damage. Due to the restricted size of some tissues, it was not possible to get a full set of oxidative damage measures in all species. Consequently, we have depicted these measurements as histograms instead of as correlations with MLSP. Oxidative damage was statistically compared (i) between each species (statistical differences highlighted by letters above bars), (ii) between short-living (the 2 quail species) and long-living (the 3 parrot species) bird species as one group each (statistical differences highlighted by asterisks), and (iii) as a correlation with MLSP when oxidative damage was determined in four or more bird species (all correlations with MLSP were non-significant).

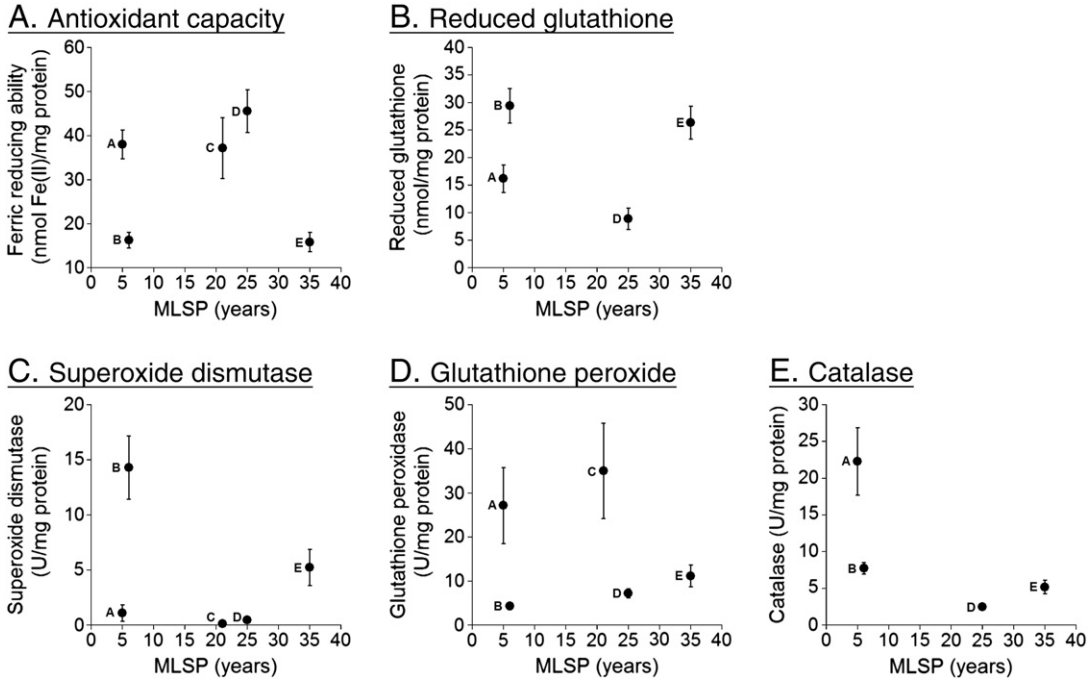

Fig. 3. The relationship between *heart* antioxidant levels and maximum lifespan potential (MLSP) of quails and parrots is shown. (A) Ferric reducing ability, (B) reduced glutathione, (C) superoxide dismutase, (D) glutathione peroxidase, and (E) catalase. Shown are means ± SEM, n = 6 for budgerigars, n = 8 for king quails and lovebirds, n = 9 for cockatiels and n = 10 for Japanese quails. Letters next to data points: A = King quail, B = Japanese quail, C = Budgerigar, D = Lovebird, E = Cockatiel.

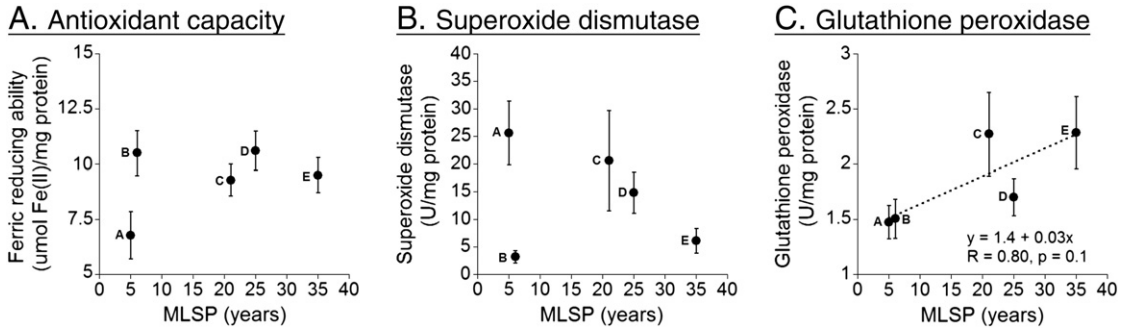

Fig. 4. The relationship between *skeletal muscle* antioxidant levels and maximum lifespan potential (MLSP) of quails and parrots is shown. (A) Ferric reducing ability, (B) superoxide dismutase, and (C) glutathione peroxidase. Shown are means ± SEM, n = 6 for budgerigars, n = 8 for king quails and lovebirds, n = 9 for cockatiels and n = 10 for Japanese quails. Letters next to data points: A = King quail, B = Japanese quail, C = Budgerigar, D = Lovebird, E = Cockatiel.

We have determined protein carbonyl levels in four tissues (plasma, liver, heart and skeletal muscle) in all five species. In all tissues, besides the plasma, we have calculated the protein carbonyl levels on a 'per mg of protein' (Fig. 5) and on a 'per g of tissue' basis (Table 1). Whereas using 'per mg of protein' as a denominator gives insights about the concentration of carbonylated proteins as a percentage of total protein content, expressing the results on a 'per g of tissue' basis highlights the differences in the intensity of protein damage within a tissue. On a 'per mg of protein' basis (or 'per ml' in the case of plasma), protein carbonyl levels differ between individual species and are lower in parrot plasma in comparison to quail plasma. In liver, heart and skeletal muscle, protein carbonyl levels differ between species, but are similar in short-living and long-living birds (Fig. 5). When expressed 'per g of tissue', carbonyl content of skeletal muscle shows a tendency to increase directly with MLSP ($p = 0.12$), whereas other tissues give no indication of lifespan influences.

Malondialdehyde (MDA) levels were measured using the TBARS assay. Despite the view that the TBARS assay is an imprecise measure

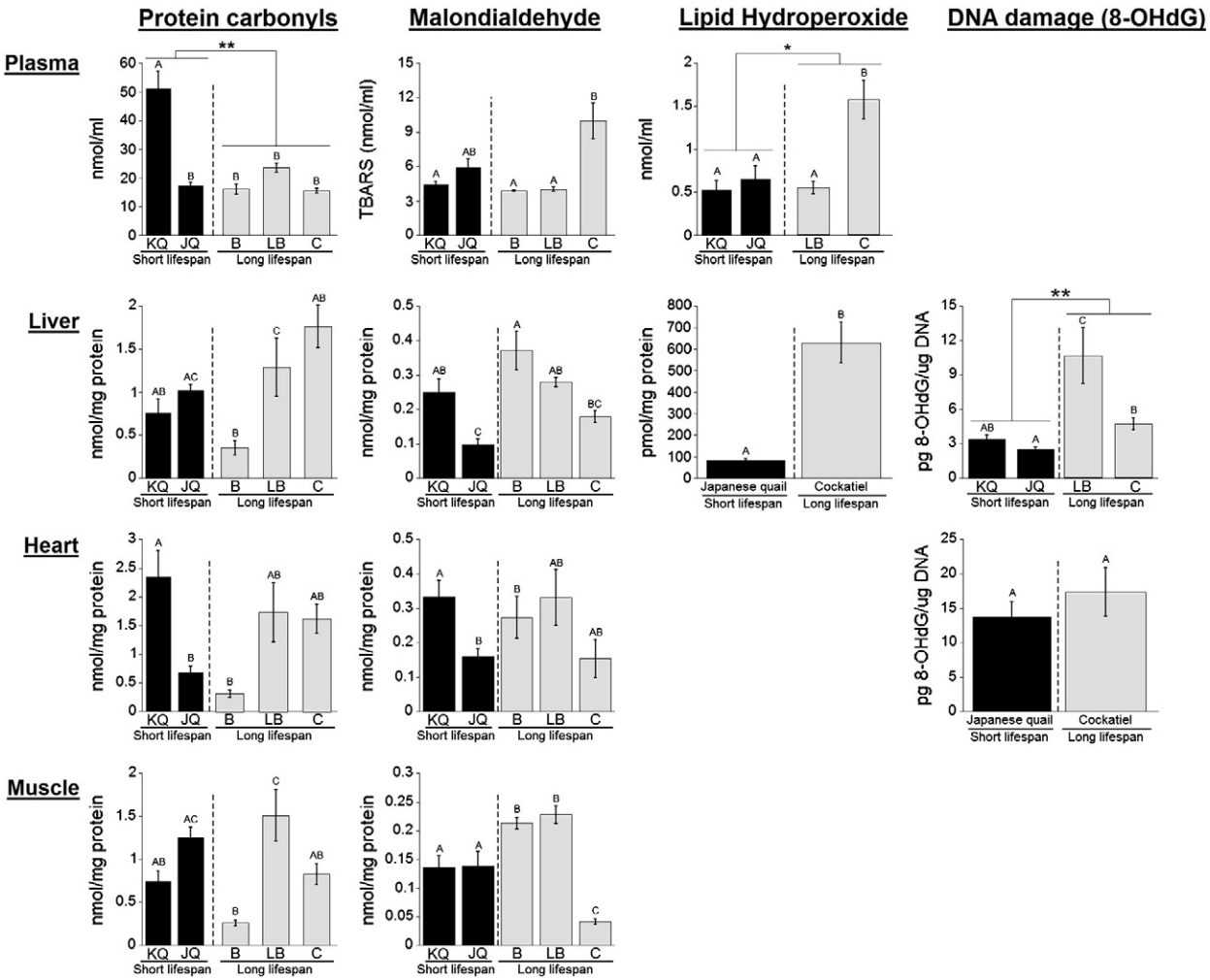

Fig. 5. Oxidative damage in parrot and quail tissues. Oxidative damage was determined as protein carbonyls (protein damage), lipid hydroperoxides and TBARS (lipid peroxidation) and mitochondrial 8-OHdG levels (mitochondrial DNA damage). Shown are means ± SEM, n = 6 for budgerigars, n = 8 for king quails and lovebirds, n = 9 for cockatiels and n = 10 for Japanese quails. Statistical differences are shown between individual species and between quails (black bars) and parrots (grey bars).

of lipid peroxidation due to complex formation of thiobarbituric acid with other compounds besides MDA, this assay is still viewed to be useful for providing a broad overview about differences in oxidative damage between species and tissues (Moore and Roberts, 1998). Because of the non-specificity of this assay, however, we will refer to TBARS levels instead of MDA levels. In all tissues, there is no difference in TBARS levels, either when short-living and long-living birds are compared as two groups, or when TBARS levels are compared as a correlation with MLSP (Fig. 5). The absence of differences in liver, heart and skeletal muscle is similarly apparent when results are compared on a 'per g tissue' basis (Table 1).

Lipid hydroperoxides were determined in plasma of four species and in the liver of two bird species (Japanese quails and cockatiels). In plasma, parrots exhibit significantly higher lipid hydroperoxide levels than quails (Fig. 5). In the liver, cockatiels have significantly more lipid hydroperoxide than Japanese quails, both a 'per mg protein' (Fig. 5) and on a 'per g tissue' basis (Table 1).

We measured 8-OHdG levels in isolated liver and heart mitochondria and calculated the results in pmol 8-OHdG/μg DNA (Fig. 5). In the liver, 8-OHdG levels are significantly higher in the long-living parrots in comparison to the short-living. In the heart, mitochondrial DNA damage was measured in only two species (Japanese quails and cockatiels) and is present in a similar concentration in both species.

4. Discussion

The overall conclusion from this study is that neither antioxidant defences nor the accumulation of oxidative damage vary in a manner predicted by the oxidative stress theory of aging. None of the antioxidant defences or the oxidative stress biomarkers we measured showed a statistically significant correlation with MLSP, demonstrating that neither superior antioxidant protection nor a high resistance to oxidative damage can account for the long lifespan of parrots. Importantly, the lack of a relationship between MLSP and levels of antioxidants and oxidative damage markers occurred whether expressed 'per mg protein' or 'per g tissue'. In many physiological comparisons, enzyme activities and tissue composition are commonly expressed using either 'per mg of protein' or 'per g of tissue', but rarely both. As protein content 'per g of tissue' can differ inter-specifically, as in our study, between species comparisons based on 'per unit protein content' may be misleading. Overall, it is physiologically more relevant to consider how highly concentrated an antioxidant is within a specific amount of tissue, as the more antioxidant 'molecules' that occur within a cell/tissue, the better the overall protection of that tissue against oxidants, irrespective of its protein content. Furthermore, the majority of antioxidants, especially the enzymatic antioxidants, are proteins themselves. Because we failed to find a consistently significant correspondence between antioxidant defences or amounts of oxidatively damaged products on a tissue mass-specific basis, we believe this casts further doubt on the relevance of the oxidative stress theory of aging.

Our results agree with findings in naked mole-rats that show low antioxidant protection (Andziak et al., 2005) and high levels of oxidative damage (Andziak et al., 2006; Pérez et al., 2009) in these extremely long-living rodents compared to same-sized short-living mice. In this regard, our study adds to a growing body of evidence that antioxidant defence mechanisms and the accumulation of oxidative damage are not involved in the determination of maximum lifespan as envisaged by the oxidative stress theory of aging (Buffenstein et al., 2008; Salmon et al., 2010). Because we have measured both oxidative damage and antioxidant activities in a range of tissues, we can examine how these variables differ individually as well as how they co-vary in the context of longevity differences.

The dismutation of mitochondrial superoxide by the SOD enzymes is likely the first line of antioxidant defence against damage by ROS (Halliwell, 1999). We found no correlation between SOD activities in any tissue and maximum lifespan. Our finding in birds agrees with research showing a lack of correlation between SOD activities in liver, brain and heart and MLSP in mammals (Tolmasoff et al., 1980). More recently, a study comparing 14 endothermic vertebrate species showed that the cytosolic Cu/Zn-SOD did not correlate with MLSP in any tissue, whereas the mitochondrial Mn-SOD showed a positive correlation with MLSP, but only in the brain (Page et al., 2010). This contrasts with studies of *Caenorhabditis elegans* that found only cytosolic Cu/Zn-SOD activities to have any effect on MLSP (Gems and Doonan, 2009). Our finding of lack of difference in SOD activities in relation to MLSP among bird species in our study is consistent with similar levels of overall oxidative damage in the same tissues.

The tripeptide GSH, being the co-substrate for both glutathione peroxidases and glutathione-S-transferases, is important in the detoxification of endogenous molecules and in the protection against oxidative damage (Kaplowitz, 1980; Wendel and Cikryt, 1980). The most consistent positive relationship with MLSP among birds in our study was for GPx. High GPx activities are expected to protect against the production of hydroxyl radicals from hydrogen peroxide via the Fenton reaction, whereby the non-enzymatic reaction of hydrogen peroxide and transition metals favours the formation of hydroxyl radicals, which are extremely reactive and will damage the first molecule they encounter (Halliwell and Gutteridge, 2007). The high GPx activities in the parrots might therefore provide precautionary mechanisms to guard against production of hydroxyl radicals, or be a response to inherently high levels of oxidative stress associated with high production rates of hydrogen peroxide and lipid hydroperoxides. Because the relatively higher GPx activities in liver and muscle of parrots were accompanied by high lipid hydroperoxide levels, we interpret this as further evidence of high oxidative stress levels in these tissues.

Catalase is a major antioxidant enzyme as it is involved in the decomposition of H_2O_2 (but not other hydroperoxides) and has one of the highest turnover rates of all enzymes (Evans, 1907). Unlike GPx activities, catalase tends towards a negative correlation with maximum lifespan among our birds, which raises the question as to whether this pattern has functional significance. In this respect, it is of interest that catalase is predominantly found in peroxisomes in both mammalian (De Duve and Baudhuin, 1966; Deisseroth and Dounce, 1970) and avian cells (Scott et al., 1969). Peroxisomes are the site of the final synthetic step of highly polyunsaturated fatty acids (PUFA), such as DHA (docosahexanoic acid). These 22-carbon PUFA result from a single cycle of beta-oxidation of a 24-carbon PUFA (Sprecher, 2000). The process of beta-oxidation in peroxisomes differs from beta-oxidation in mitochondria in that hydrogen peroxide is produced as a by-product in peroxisomes, whereas H_2O is the by-product in mitochondria (Alexson and Cannon, 1984). Therefore, differences in catalase levels between parrots and quails more likely mirror differences in rates of PUFA synthesis rather than differences in mitochondrial ROS production. A high PUFA synthesis rate would accompany high rates of membrane turnover, which, in turn, are expected to accompany elevated rates of lipid peroxidation. Although quail tissues showed lower lipid hydroperoxide levels than those of parrots, this might not be a result of lower oxidative stress, but instead reflect a faster rate of degradation and removal of lipoxidation end products.

Uric acid is an important scavenger of singlet oxygen and other radicals (Ames et al., 1981; Cutler, 1984) and is responsible for up to 90% of the variation in plasma total antioxidant capacity between bird species (Cohen et al., 2007). Furthermore, uric acid is able to form stable complexes with ferrous or ferric ions, protecting tissues and extracellular surroundings from these radical generators (Davies et al., 1986). We demonstrated a trend towards a negative relationship of uric acid with MLSP. Assuming that high antioxidant protection might be an indirect marker of high oxidative stress levels, this might indicate lower levels of oxidative stress in parrots. Indeed, parrots exhibit less protein damage in plasma in comparison to quails.

However, protein carbonyl content in plasma was the only oxidative stress marker that was lower in the longer-living parrots. In contrast, TBARS and lipid hydroperoxide levels were similar or higher in the parrots, respectively, perhaps again reflecting differences in capacity for repair and removal between these bird groups.

It is functionally appealing to measure total antioxidant capacity (TAC) rather than the activity of individual antioxidants, because TAC measures both the summation of single antioxidants effects as well as their synergistic and antagonistic interactions (Cohen et al., 2007). The FRA and TAC assays respond to different aspects of a tissue's total antioxidant system. The FRA assay measures mainly the activity of low-molecular weight molecules, such as uric acid, alpha-tocopherol, ascorbic acid and bilirubin (Benzie and Strain, 1996). It is arguable whether those assays measuring total antioxidant capacity that include damage to macromolecules are truly measuring biologically relevant total antioxidant defences. In the TAC assay we used (see Fig. 1A), the results are expressed as 'Trolox equivalents', a water-soluble form of Vitamin E. Proteins are the main antioxidant component determined in the TAC assay, especially in plasma where proteins are present in high concentrations, and it is suggested that the sulfhydryl groups of their amino acids are mainly responsible for their antioxidant function (Erel, 2004). Are such proteins really equivalent to vitamin E (an antioxidant that can be easily regenerated)? Should they be considered as biologically pertinent antioxidants? A broad definition of an antioxidant could be "any substance that, when present at low concentrations compared with those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell and Gutteridge, 2007). Taking this definition into account, proteins do indeed have antioxidant capacity, however they get damaged during the interaction with ROS, and have to be repaired or degraded, both being an energetically expensive process. Despite their incidental antioxidant function, proteins damaged by ROS potentially add to cumulative oxidative damage, which might, therefore, offset their antioxidant benefits. Furthermore, uric acid is a major component measured in the TAC assay. Although the TAC assay has the advantage of being a functional measure, different antioxidants (such as uric acid) exhibit different kinetics in the TAC reaction complicating the interpretation of the results, especially in birds where plasma uric acid content is high and varies dramatically between species (Cohen et al., 2007). We have tried to circumvent this problem by additionally measuring a variety of individual antioxidants.

Overall, our results on antioxidant activities in the species we studied show these measures to be poor predictors of MLSP. While there may be some concern that the diet we provided to all birds affected their antioxidant capabilities by not allowing them to choose foods they would encounter in free-living situations, we do not believe any of the birds in our study to be dietarily constrained. The experimental diet was common to all birds in this study and was carefully designed with a full complement of pertinent amino acids, trace minerals, and vitamins in amounts known to sustain both groups of birds. Furthermore, our findings are consistent with the outcomes of genetic manipulation of antioxidant capacity in mice (reviewed by Perez et al., 2009b) and *C. elegans* (reviewed by Gems and Doonan, 2009) that show minimal effect on MLSP. In contrast, improvement of the antioxidant defence systems has positive effects on age-related disease models by decreasing disease progression or severity. Oxidative stress and antioxidants may therefore have stronger effects on healthy aging rather than lifespan extension (see Salmon et al., 2010). In this context, rather than concentrating on how alteration of single antioxidants influences MLSP, it may be more beneficial to consider how oxidative stress generally affects physiological processes by examining the signalling networks that manage antioxidant and ROS levels in vivo (for review see Muller et al., 2007).

Given the vagaries of measuring pro-oxidation and anti-oxidant balance in animals, it is appealing to consider quantification of oxidative damage products to be a valid indicator of overall oxidative stress. It is important to recognise, however, that measurements of oxidative stress biomarkers in tissue samples collected at a specific age are the net consequence of a number of interacting processes involving ROS damage, damage repair, and removal of damaged products that precede the sampling point. Because oxidatively damaged molecules may be quickly repaired or removed from the cellular surroundings, interspecific variation in biomarker levels may not be indicative of differences in oxidative stress, but, instead, may reflect differences in repair and removal rates, such as differences in nuclease and proteasome activities. Portero-Otin et al. (2004) showed a higher protein carbonyl content in the long-living pigeon than in the short-living rat, which was due to the pigeons having a lower disposal rate of damaged protein because of lower proteasome activity. Our results suggest that the high MLSP of parrots is not associated with low levels of oxidative damage. However, as we did not determine repair or removal rates, the actual intensity of tissue damage by ROS is not known. Irrespective of the underlying reasons for their accumulation, measurements of oxidatively damaged products among species with variable MLSP provides a means to examine whether such accumulation affects lifespan, which is the main tenet of the oxidative stress theory of aging.

Our measurements of oxidative stress biomarkers refute the oxidative stress theory in showing most to be either unrelated or exactly opposite to predictions in relation to MLSP. This differs from observations that oxidative damage increases with age, both in invertebrates (Agarwal and Sohal, 1994a, b) and in vertebrates (Sohal et al., 1994), but agrees with conclusions reached by more recent studies. Examination of extremely long-living species such as pigeons (Portero-Otin et al., 2004), naked mole-rats (Andziak and Buffenstein, 2006; Pérez et al., 2009) and bats (Buffenstein et al., 2008; Wilhelm Filho et al., 2007) reveal these long-living species to have unexpectedly high levels of protein damage and lipid peroxidation. Due to such inconsistent findings, researchers in the aging field have started to question the importance of cumulative oxidative damage as a general determinant of lifespan (Buffenstein et al., 2008).

We have carefully chosen particular biomarkers to evaluate oxidative damage, but we are aware of their limitations. For example, although protein carbonyls are commonly used as an oxidative damage biomarker, not all protein modifications lead to the formation of carbonyl groups. In the case of methionine, because of its sulfhydryl (−SH) group, it does not produce a carbonyl product, but instead forms methionine sulfoxide (Brot and Weissbach, 1991; Moskovitz et al., 2001). We did not measure methionine sulfoxide in this study, however others have found a negative correlation between the methionine content in heart and brain and maximum lifespan (Pamplona and Barja, 2006; Pamplona et al., 2005). Although our measurements of protein damage do not explain longevity differences between the bird species we studied, it is possible that other markers of protein damage (alpha-keto acids, dimerization and cross-links) might be higher in the short-living bird species (Bourdon and Blache, 2001; Negre-Salvayre et al., 2008). The same uncertainty applies to our measured products of DNA damage and lipid peroxidation. For example, different methodological approaches exist to measure 8-OHdG and, dependent on the method used, 8-OHdG levels can vary up to 1000-fold, making comparisons about the actual rates of damage almost impossible (Collins, 2005). This is mainly due to DNA oxidation occurring during the process of sample preparation (ESCODD, 2002). As we have used the same methodological approach in all species and tissues, we can assume that a similar rate of background oxidation occurred in all samples. Furthermore, our results lie within the range of DNA damage reported previously (Sohal et al., 1994; Takasawa et al., 1993). Despite the limitations of our measurements, we emphasise that the substantial MLSP differences between the species we studied should have resulted in significant differences in most measures of oxidatively damaged products, irrespective of their specificity, if the oxidative stress theory is a robust explanation of longevity differences among species.

We unequivocally conclude from our results that antioxidant systems and oxidative damage alone do not explain the MLSP differences between parrots and quails. However, these are only two components of the oxidative stress theory, and there is potential for other oxidative stress parameters, such as mitochondrial ROS production, the susceptibility of tissues to oxidative damage, and unknown interactions between them, to contribute to the differences in lifespan between these groups. We recognise the complexity of the oxidative stress theory of aging and have consequently examined other components of oxidative stress in these five bird species. These include mitochondrial ROS production in three tissues (Montgomery et al., in press) and membrane fatty acid composition in seven tissues (Montgomery et al., 2011). ROS production was measured in intact erythrocytes (superoxide, hydroxyl radical and peroxynitrate) as well as in isolated heart, liver and skeletal muscle mitochondria as basal and maximum rates of ROS formation using two substrates for the respiration chain. Membrane fatty acid composition was determined and the peroxidation index (PI) was calculated to determine their susceptibility to oxidative damage. This combined analysis allows us to compare these components across the 5 species and better place the antioxidants and oxidative damage results into context.

The main strength of the parrot–quail comparison in examining the oxidative stress theory of aging is the 5-fold longevity difference between these groups. Consequently, we would expect substantial differences in the relative size of measured variables between the parrots and quails and not just statistical significance. With this reasoning, we have plotted "average quail value/average parrot value" ratios for the complete set of pertinent variables we measured

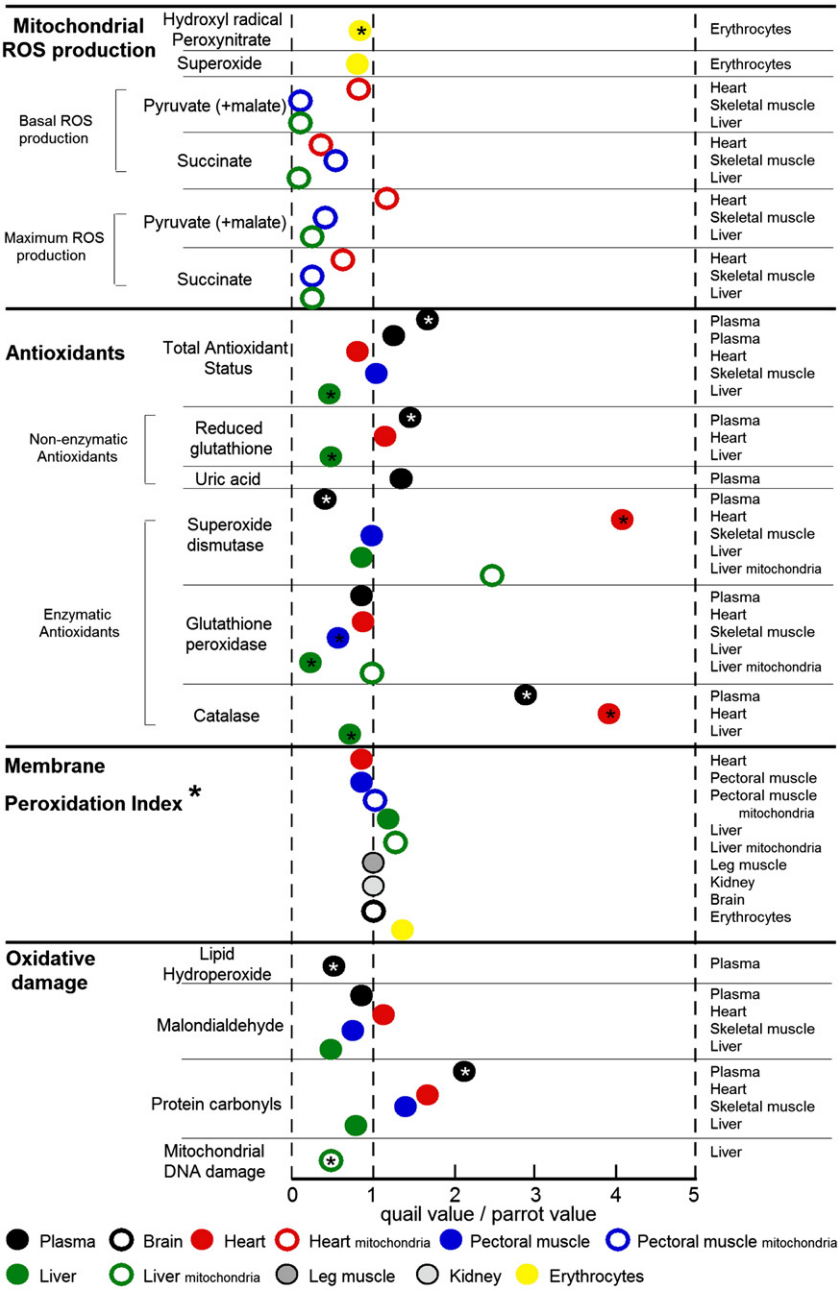

Fig. 6. "Average quail/average parrot" values for mitochondrial ROS production, antioxidants, membrane peroxidation index and markers of oxidative damage of the five quail and parrot species examined. A dotted line was added representing "average quail/average parrot" value = 1, and a second dotted line represents "average quail/average parrot" value = 5 because of the, on average, 5-fold longevity difference between quails and parrots. Significant differences between the average quail and the average parrot values are highlighted through an asterisk ($p<0.05$). * For membrane peroxidation index, the "average quail/average parrot" values were cubed (see Discussion).

(Fig. 6). For comparative purposes we have also plotted two reference lines in this figure; one for ratio = 1 (i.e., quail average = parrot average) and the other representing "average quail/average parrot" ratio = 5, which corresponds to the magnitude of MLSP difference between these groups. For the mitochondrial ROS production, antioxidant and oxidative damage categories, we have plotted the direct ratio of each "average quail/average parrot" value, because, in the absence of better knowledge, we have assumed a simple linear relationship between longevity and the particular parameter (e.g. doubling of ROS production might be associated with halving of longevity, etc.). By contrast, for the membrane PI category, the values plotted are (average quail/average parrot)^3. This transformation is justified because, unlike the other categories, we already know that the relationship between membrane PI and longevity is not simple and linear. In a wide variety of birds and mammals, the PI is proportional to the −0.30 power of MLSP for skeletal muscle phospholipids, and the −0.36 power of MLSP for liver mitochondrial phospholipids (Hulbert, 2010). If we assume for tissues in general that PI is proportional to MLSP$^{-0.33}$, then such a relationship can be transformed to MLSP being inversely proportional to PI^3. For this reason we have plotted the cube power of the "average quail/average parrot" ratio for the membrane PI values in Fig. 6.

It is obvious from examination of these ratios that none of the parameters associated with the oxidative stress theory is of similar magnitude to the 5-fold longevity difference between parrots and quails (Fig. 6). For the majority (71%) of the parameters, there is no statistically significant difference between short-living quails and long-living parrots. For others, some of the quail values are significantly higher than the parrot values (11%), while for the remainder (18%) of the comparisons, the parrot values are significantly greater than the quail values. Interestingly, the pattern of significant differences in the quail and parrot values point towards a higher ROS production and more oxidative damage in the long-living parrots than the short-living quails. Whereas early studies repeatedly suggested that differences in mitochondrial ROS production are associated with MLSP variation in mammals and birds (e.g. Herrero and Barja, 1997; Lambert et al., 2007), more recent studies raise doubts about the importance of oxidative damage in MLSP variation (Portero-Otin et al., 2004; Andziak et al., 2005). Our extensive examination of oxidative stress parameters in birds adds to a growing body of evidence that mitochondrial ROS production, the accumulation of oxidative damage and membrane susceptibility to peroxidation alone are not able to explain the long lifespan of parrots. The category in Fig. 6 that showed the greatest variation between quails and parrots was the antioxidants, and even these are not able to explain the long lifespan of parrots. Phospholipid composition of whole tissue and mitochondrial membranes, as seen in Fig. 6, was almost identical in quails and parrots (Montgomery et al., 2011).

We are left with the conclusion that the 5-fold differences in MSLP between quail and parrots are not explained by corresponding differences in antioxidant activities or cumulative levels of oxidatively damaged products. We are aware that each measure of oxidative damage carried out in this study lacks specificity and has low validity when analysed individually, but that the sum of all measures gives us a reasonable picture about oxidative damage and allows us to state without reservation that oxidative damage is not accumulating at different rates in birds with very different MLSP. Although this refutes an underlying tenet of the oxidative stress theory of aging, it does not necessarily eliminate oxidative stress as contributing to differences in MLSP among these or other species. Comparative analyses of individual variables pertaining to oxidative stress presume a direct and linear relationship between each variable being measured and its contribution to oxidative stress. Given the complex and dynamic interactions between multi-level antioxidant and pro-oxidant components of oxidative stress, it is unlikely that any single measure can be assumed to act in a stoichiometric manner. Furthermore, it is likely that rates of oxidative damage change over a species lifetime and that the onset of these changes may be sudden and differ chronologically among species with different MLSP. For example, there is evidence for age-related decreases in ROS stimulation of up-regulation of antioxidants in mammalian muscle (Jackson and McArdle, 2011), thus making these tissues more vulnerable to oxidative damage. Attention should now be directed toward examining age-related differences in oxidative stress and related damage among species differing in MLSP, with consideration of changes in homeostatic regulation of critically important modulators of oxidative stress, such as iron (Kell, 2009).

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References

Agarwal, S., Sohal, R.S., 1994a. DNA oxidative damage and life expectancy in houseflies. Proc. Natl. Acad. Sci. U. S. A. 91, 12332–12335.
Agarwal, S., Sohal, R.S., 1994b. Aging and protein oxidative damage. Mech. Ageing Dev. 75, 11–19.
Agarwal, S., Sohal, R.S., 1996. Relationship between susceptibility to protein oxidation, aging, and maximum life span potential of different species. Exp. Gerontol. 31, 365–372.
Alexson, S.E., Cannon, B., 1984. A direct comparison between peroxisomal and mitochondrial preferences for fatty-acyl beta-oxidation predicts channelling of medium-chain and very-long-chain unsaturated fatty acids to peroxisomes. Biochim. Biophys. Acta 796, 1–10.
Ames, B.N., Cathcart, R., Schwiers, E., Hochstein, P., 1981. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: a hypothesis. Proc. Natl. Acad. Sci. U. S. A. 78, 6858–6862.
Andziak, B., Buffenstein, R., 2006. Disparate patterns of age-related changes in lipid peroxidation in long-lived naked mole-rats and shorter-lived mice. Aging Cell 5, 525–532.
Andziak, B., O'Connor, T.P., Buffenstein, R., 2005. Antioxidants do not explain the disparate longevity between mice and the longest-living rodent, the naked mole-rat. Mech. Ageing Dev. 126, 1206–1212.
Andziak, B., O'Connor, T.P., Qi, W., DeWaal, E.M., Pierce, A., Chaudhuri, A.R., Van Remmen, H., Buffenstein, R., 2006. High oxidative damage levels in the longest-living rodent, the naked mole-rat. Aging Cell 5, 463–471.
Benzie, I.F., Strain, J.J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239, 70–76.
Bourdon, E., Blache, D., 2001. The importance of proteins in defense against oxidation. Antioxid. Redox Signal. 3, 293–311.
Brot, N., Weissbach, H., 1991. Biochemistry of methionine sulfoxide residues in proteins. Biofactors 3, 91–96.
Buffenstein, R., 2005. The naked mole-rat: a new long-living model for human aging research. J. Gerontol. A Biol. Sci. Med. Sci. 60, 1369–1377.
Buffenstein, R., Edrey, Y.H., Yang, T., Mele, J., 2008. The oxidative stress theory of aging: embattled or invincible? Insights from non-traditional model organisms. Age 30, 99–109.
Buttemer, W.A., Abele, D., Costantini, D., 2010. From bivalves to birds: oxidative stress and longevity. Funct. Ecol. 24, 971–983.
Cohen, A., Klasing, K., Ricklefs, R., 2007. Measuring circulating antioxidants in wild birds. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 147, 110–121.
Collins, A.R., 2005. Assays for oxidative stress and antioxidant status: applications to research into the biological effectiveness of polyphenols. Am. J. Clin. Nutr. 81, 261S–267S.
Cutler, R.G., 1984. Urate and ascorbate: their possible roles as antioxidants in determining longevity of mammalian species. Arch. Gerontol. Geriatr. 3, 321–348.
Davies, K.J., Sevanian, A., Muakkassah-Kelly, S.F., Hochstein, P., 1986. Uric acid-iron ion complexes. A new aspect of the antioxidant functions of uric acid. Biochem. J. 235, 747–754.
De Duve, C., Baudhuin, P., 1966. Peroxisomes (microbodies and related particles). Physiol. Rev. 46, 323–357.
Deisseroth, A., Dounce, A.L., 1970. Catalase: physical and chemical properties, mechanism of catalysis, and physiological role. Physiol. Rev. 50, 319–375.
Erel, O., 2004. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. Clin. Biochem. 37, 277–285.
ESCODD (European Standards Committee on oxidative DNA damage), 2002. Comparative analysis of baseline 8-oxo-7,8-dihydroguanine in mammalian cell DNA, by different methods in different laboratories: an approach to consensus. Carcinogenesis 23, 2129–2133.
Evans, C.A., 1907. On the catalytic decomposition of hydrogen peroxide by the catalase of blood. Biochem. J. 2, 133–155.

Felsenstein, J., 1985. Phylogenies and the comparative method. Am. Nat. 125, 1–15.
Gems, D., Doonan, R., 2009. Antioxidant defense and aging in *C. elegans*: is the oxidative damage theory of aging wrong? Cell Cycle 8, 1681–1687.
Gerschman, R., Gilbert, D.L., Nye, S.W., Dwyer, P., Fenn, W.O., 1954. Oxygen poisoning and x-irradiation: a mechanism in common. Science 119, 623–626.
Halliwell, B., 1999. Antioxidant defence mechanisms: from the beginning to the end (of the beginning). Free. Radic. Res. 31, 261–272.
Halliwell, B., Gutteridge, J.M.C., 2007. Free Radicals in Biology and Medicine. Oxford University Press, New York.
Harman, D., 1956. Aging: a theory based on free radical and radiation chemistry. J. Gerontol. 11, 298–300.
Harper, J.M., Wang, M., Galecki, A.T., Ro, J., Williams, J.B., Miller, R.A., 2011. Fibroblasts from long-lived bird species are resistant to multiple forms of stress. J. Exp. Biol. 214, 1902–1910.
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.
Ho, Y.S., Magnenat, J.L., Bronson, R.T., Cao, J., Gargano, M., Sugawara, M., Funk, C.D., 1997. Mice deficient in cellular glutathione peroxidase develop normally and show no increased sensitivity to hyperoxia. J. Biol. Chem. 272, 16644–16651.
Ho, Y.S., Gargano, M., Cao, J., Bronson, R.T., Heimler, I., Hutz, R.J., 1998. Reduced fertility in female mice lacking copper–zinc superoxide dismutase. J. Biol. Chem. 273, 7765–7769.
Holmes, D., Austad, S., 1995. Birds as animal models for the comparative biology of aging: a prospectus. J. Gerontol. A Biol. Sci. Med. Sci. 50, B59–B66.
Huang, T.T., Carlson, E.J., Gillespie, A.M., Shi, Y., Epstein, C.J., 2000. Ubiquitous overexpression of CuZn superoxide dismutase does not extend life span in mice. J. Gerontol. A Biol. Sci. Med. Sci. 55, B5–B9.
Hulbert, A.J., 2010. Metabolism and longevity: is there a role for membrane fatty acids? Integr. Comp. Biol. 50, 808–817.
Hulbert, A.J., Pamplona, R., Buffenstein, R., Buttemer, W.A., 2007. Life and death: metabolic rate, membrane composition, and life span of animals. Physiol. Rev. 87, 1175–1213.
Jackson, M.J., McArdle, A., 2011. Age-related changes in skeletal muscle reactive oxygen species generation and adaptive responses to reactive oxygen species. J. Physiol. Lond. 589, 2139–2145.
Janaszewska, A., Bartosz, G., 2002. Assay of total antioxidant capacity: comparison of four methods as applied to human blood plasma. Scand. J. Clin. Lab. Invest. 62, 231–236.
Jang, Y.C., Perez, V.I., Song, W., Lustgarten, M.S., Salmon, A.B., Mele, J., Qi, W.B., Liu, Y.H., Liang, H.Y., Chaudhuri, A., Ikeno, Y., Epstein, C.J., Van Remmen, H., Richardson, A., 2009. Overexpression of Mn superoxide dismutase does not increase life span in mice. J. Gerontol. Series A Biol. Sci. Med. Sci. 64, 1114–1125.
Jentzsch, A.M., Bachmann, H., Furst, P., Biesalski, H.K., 1996. Improved analysis of malondialdehyde in human body fluids. Free Radic. Biol. Med. 20, 251–256.
Kaplowitz, N., 1980. Physiological significance of glutathione S-transferases. Am. J. Physiol. 239, G439–G444.
Kell, D.B., 2009. Iron behaving badly: inappropriate iron chelation as a major contributor to the aetiology of vascular and other progressive inflammatory and degenerative diseases. BMC Med. Genomics 2, 2.
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.
Lebovitz, R.M., Zhang, H., Vogel, H., Cartwright Jr., J., Dionne, L., Lu, N., Huang, S., Matzuk, M.M., 1996. Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. Proc. Natl. Acad. Sci. U. S. A. 93, 9782–9787.
Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., Ahn, B.W., Shaltiel, S., Stadtman, E.R., 1990. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186, 464–478.
Lopez-Torres, M., Perez-Campo, R., Rojas, C., Cadenas, S., Barja, G., 1993. Maximum life span in vertebrates: relationship with liver antioxidant enzymes, glutathione system, ascorbate, urate, sensitivity to peroxidation, true malondialdehyde, in vivo H_2O_2, and basal and maximum aerobic capacity. Mech. Ageing Dev. 70, 177–199.
Maral, J., Puget, K., Michelson, A.M., 1977. Comparative study of superoxide dismutase, catalase and glutathione peroxidase levels in erythrocytes of different animals. Biochem. Biophys. Res. Commun. 77, 1525–1535.
McCord, J.M., Fridovich, I., 1969. Superoxide dismutase an enzymic function for erythrocuprein (hemocuprein). J. Biol. Chem. 244, 6049–6055.
Miller, R.A., Williams, J.B., Kiklevich, J.V., Austad, S., Harper, J.M., 2011. Comparative cellular biogerontology: primer and prospectus. Ageing Res. Rev. 10, 181–190.
Mockett, R.J., Orr, W.C., Rahmandar, J.J., Benes, J.J., Radyuk, S.N., Klichko, V.I., Sohal, R.S., 1999. Overexpression of Mn-containing superoxide dismutase in transgenic *Drosophila melanogaster*. Arch. Biochem. Biophys. 371, 260–269.
Monnier, V.M., 1990. Minireview. Nonenzymatic glycosylation: the Maillard reaction and the aging process. J. Gerontol. Biol. Sci. 45, B105–B111.
Monnier, V.M., Sell, D.R., Ramanakoppa, H.N., Miyata, S., 1991. Mechanisms of protection against damage mediated by the Maillard reaction in aging. Gerontology 37, 152–165.
Montgomery, M.K., Hulbert, A.J., Buttemer, W.A., 2011. Metabolic rate and membrane fatty acid composition in birds: a comparison between long-living parrots and short-living fowl. J. Comp. Physiol. 181, 807–816.
Montgomery, M.K., Hulbert, A.J., Buttemer, W.A., in press. Does the oxidative stress theory of aging explain longevity differences in birds? I. Mitochondrial ROS production. Exp Ger.
Moore, K., Roberts, L.J., 1998. Measurement of lipid peroxidation. Free. Radic. Res. 28, 659–671.
Moskovitz, J., Bar-Noy, S., Williams, W.M., Requena, J., Berlett, B.S., Stadtman, E.R., 2001. Methionine sulfoxide reductase (MsrA) is a regulator of antioxidant defense and lifespan in mammals. Proc. Natl. Acad. Sci. U. S. A. 98, 12920–12925.
Muller, F.L., Lustgarten, M.S., Jang, Y., Richardson, A., Van Remmen, H., 2007. Trends in oxidative aging theories. Free Radic. Biol. Med. 43, 477–503.
Murakami, K., Kondo, T., Kawase, M., Li, Y., Sato, S., Chen, S.F., Chan, P.H., 1998. Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with manganese superoxide dismutase deficiency. J. Neurosci. 18, 205–213.
Negre-Salvayre, A., Coatrieux, C., Ingueneau, C., Salvayre, R., 2008. Advanced lipid peroxidation end products in oxidative damage to proteins. Potential role in diseases and therapeutic prospects for the inhibitors. Br. J. Pharmacol. 153, 6–20.
Ogburn, C., Austad, S., Holmes, D., Kiklevich, J., Gollahon, K., Rabinovitch, P., Martin, G., 1998. Cultured renal epithelial cells from birds and mice: Enhanced resistance of avian cells to oxidative stress and DNA damage. J. Gerontol. Series A Biol. Sci. Med. Sci. 53, B287–B292.
Ogburn, C., Carlberg, K., Ottinger, M., Holmes, D., Martin, G., Austad, S., 2001. Exceptional cellular resistance to oxidative damage in long-lived birds requires active gene expression. J. Gerontol. Series A Biol. Sci. Med. Sci. 56, B468–B474.
Page, M.M., Richardson, J., Wiens, B.E., Tiedtke, E., Peters, C.W., Faure, P.A., Burness, G., Stuart, J.A., 2010. Antioxidant enzyme activities are not broadly correlated with longevity in 14 vertebrate endotherm species. Age 32, 255–270.
Pamplona, R., Barja, G., 2006. Mitochondrial oxidative stress, aging and caloric restriction: the protein and methionine connection. Biochim. Biophys. Acta-Bioenerg. 1757 (5-6), 496–508.
Pamplona, R., Prat, J., Cadenas, S., Rojas, C., Perez-Campo, R., Torres, M., Barja, G., 1996. Low fatty acid unsaturation protects against lipid peroxidation in liver mitochondria from long-lived species: the pigeon and human case. Mech. Ageing Dev. 86, 53–66.
Pamplona, R., Portero-Otin, M., Sanz, A., Ayala, V., Vasileva, E., Barja, G., 2005. Protein and lipid oxidative damage and complex I content are lower in the brain of budgerigar and canaries than in mice. Relation to aging rate. Age (Dordr) 27, 267–280.
Perez, V.I., Van Remmen, H., Bokov, A., Epstein, C.J., Vijg, J., Richardson, A., 2009a. The overexpression of major antioxidant enzymes does not extend the lifespan of mice. Aging Cell 8, 73–75.
Perez, V.I., Bokov, A., Van Remmen, H., Mele, J., Ran, Q.T., Ikeno, Y., Richardson, A., 2009b. Is the oxidative stress theory of aging dead? Biochim. Biophys. Acta-Gen. Subj. 1790, 1005–1014.
Pérez, V., Buffenstein, R., Masamsetti, V., Leonard, S., Salmon, A., Mele, J., Andziak, B., Yang, T., Edrey, Y., Friguet, B., Ward, W., Richardson, A., A., C., 2009. Protein stability and resistance to oxidative stress are determinants of longevity in the longest-living rodent, the naked mole-rat. Proc. Natl. Acad. Sci. U. S. A. 106, 3059–3064.
Portero-Otin, M., Requena, J.R., Bellmunt, M.J., Ayala, V., Pamplona, R., 2004. Protein nonenzymatic modifications and proteasome activity in skeletal muscle from the short-lived rat and long-lived pigeon. Exp. Gerontol. 39, 1527–1535.
Prior, R.L., Cao, G., 1999. In vivo total antioxidant capacity: comparison of different analytical methods. Free Radic. Biol. Med. 27, 1173–1181.
Ricklefs, R.E., 2010. Life-history connections to rates of aging in terrestrial vertebrates. Proc. Natl. Acad. Sci. 107, 10314–10319.
Salmon, A.B., Richardson, A., Perez, V.I., 2010. Update on the oxidative stress theory of aging: does oxidative stress play a role in aging or healthy aging? Free Radic. Biol. Med. 48, 642–655.
Scott, P.J., Visentin, L.P., Allen, J.M., 1969. The enzymatic characteristics of peroxisomes of amphibian and avian liver and kidney. Ann. N. Y. Acad. Sci. 168, 244–264.
Sentman, M.L., Granstrom, M., Jakobson, H., Reaume, A., Basu, S., Marklund, S.L., 2006. Phenotypes of mice lacking extracellular superoxide dismutase and copper- and zinc-containing superoxide dismutase. J. Biol. Chem. 281, 6904–6909.
Seto, N.O.L., Hayashi, S., Tener, G.M., 1990. Overexpression of Cu–Zn Superoxide-dismutase in Drosophila does not affect life-span. Proc. Natl. Acad. Sci. U. S. A. 87, 4270–4274.
Sohal, R.S., Agarwal, S., Candas, M., Forster, M.J., Lal, H., 1994. Effect of age and caloric restriction on DNA oxidative damage in different tissues of C57BL/6 mice. Mech. Ageing Dev. 76, 215–224.
Sprecher, H., 2000. Metabolism of highly unsaturated n−3 and n−6 fatty acids. Biochim. Biophys. Acta 1486, 219–231.
Staveley, B.E., Phillips, J.P., Hilliker, A.J., 1990. Phenotypic consequences of copper–zinc superoxide-dismutase overexpression in Drosophila-melanogaster. Genome 33, 867–872.
Takasawa, M., Hayakawa, M., Sugiyama, S., Hattori, K., Ito, T., Ozawa, T., 1993. Age-associated damage in mitochondrial function in rat hearts. Exp. Gerontol. 28, 269–280.
Tolmasoff, J.M., Ono, T., Cutler, R.G., 1980. Superoxide dismutase: correlation with life-span and specific metabolic rate in primate species. Proc. Natl. Acad. Sci. U. S. A. 77, 2777–2781.
Van Remmen, H., Ikeno, Y., Hamilton, M., Pahlavani, M., Wolf, N., Thorpe, S.R., Alderson, N.L., Baynes, J.W., Epstein, C.J., Huang, T.T., Nelson, J., Strong, R., Richardson, A., 2003. Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. Physiol. Genomics 16, 29–37.
Wasser, D.E., Sherman, P.W., 2010. Avian longevities and their interpretation under evolutionary theories of senescence. J. Zool. 280, 103–155.
Wendel, A., Cikryt, P., 1980. The level and half-life of glutathione in human plasma. FEBS Lett. 120, 209–211.
Wilhelm Filho, D., Althoff, S.L., Dafre, A.L., Boveris, A., 2007. Antioxidant defenses, longevity and ecophysiology of South American bats. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 146, 214–220.
Yant, L.J., Ran, Q., Rao, L., Van Remmen, H., Shibatani, T., Belter, J.G., Motta, L., Richardson, A., Prolla, T.A., 2003. The selenoprotein GPX4 is essential for mouse development and protects from radiation and oxidative damage insults. Free Radic. Biol. Med. 34, 496–502.