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THE ECOLOGY OF ANTIOXIDANTS & OXIDATIVE STRESS IN ANIMALS

From bivalves to birds: oxidative stress and longevity

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Summary

1. The oxidative stress theory of ageing predicts that animals living longer will have less cumulative oxidative damage together with structural characteristics that make them more resistant to oxidative damage itself.

2. Although a general relationship between body size, metabolism and longevity does not exist in marine invertebrates, they are generally characterized by low rates of metabolism and reactive oxygen species (ROS) formation associated with lower antioxidant enzyme activities compared to vertebrates.

3. Birds and mammals have very similar size-affected metabolic rates and their metabolic intensity explains only some of the variation in maximum lifespan potential (MLSP). Within each class, smaller animals have higher rates of metabolism and ROS production and membranes that are more susceptible to oxidative damage and autocatalytic propagation of free radicals than larger ones.

4. Although the high variation in life-history strategies is accompanied by substantial variation in MLSP, there is a consistent positive correlation between rates of ROS formation and antioxidant levels among most animals examined so far for these traits. The consensus of these studies is that ROS and antioxidant levels are inversely related to MLSP.

5. The lack of a clear stoichiometric relation between variables contributing to oxidative stress limits our capacity to infer longevity consequences from measures of pro-oxidant or antioxidant status among or within species.

Key-words: antioxidents, ectotherms, endotherms, life history influences, membrane fatty acids, peroxidation, reactive oxygen species, uncoupling proteins

Introduction

It is sometimes forgotten that Harman (1956) proposed ageing to be related to rate of free radical production by mitochondrial aerobic metabolism before there was proof that free radicals were a by-product of mitochondrial activities. His insight was confirmed with the discovery of a mitochondrial superoxide dismutase enzyme (McCord & Fridovich 1969), which converts superoxides to hydrogen peroxides, one of many reactive oxygen species (ROS). The earlier presumption that superoxide production was a constant proportion of mitochondrial oxygen consumption, however, has proven false (Barja 2007). Mitochondrial ROS production occurs at complexes I and III (Fig. 1), and varies markedly with mitochondrial membrane potential, oxygen level (partial pressure of oxygen, PO_2) and redox state of components of the electron transport chain (Murphy 2009). While there is no one-to-one correspondence between ROS production and oxygen consumption, ROS production rates are expected to be far lower in animals with inherently low rates of oxygen consumption that live in environments with low oxygen content than in those with high rates of oxygen uptake that live in oxygen-rich habitats. Marine bivalves provide excellent examples of animals comprising the former group, whereas the intense aerobic activities and high pulmonary PO_2 of birds means they have a much higher potential for ROS formation. Irrespective of these fundamental differences, the oxidative stress theory of ageing predicts that animals living long lives will have less cumulative damage resulting from mismatches in the production of ROS and antioxidants and may also have structural characteristics that make them more resistant to oxidative damage. We will explore these possibilities by examining what is known about oxidative stress and ageing in some representative marine ectothermic animals (low oxygen flux fauna), particularly bivalves, as well as among

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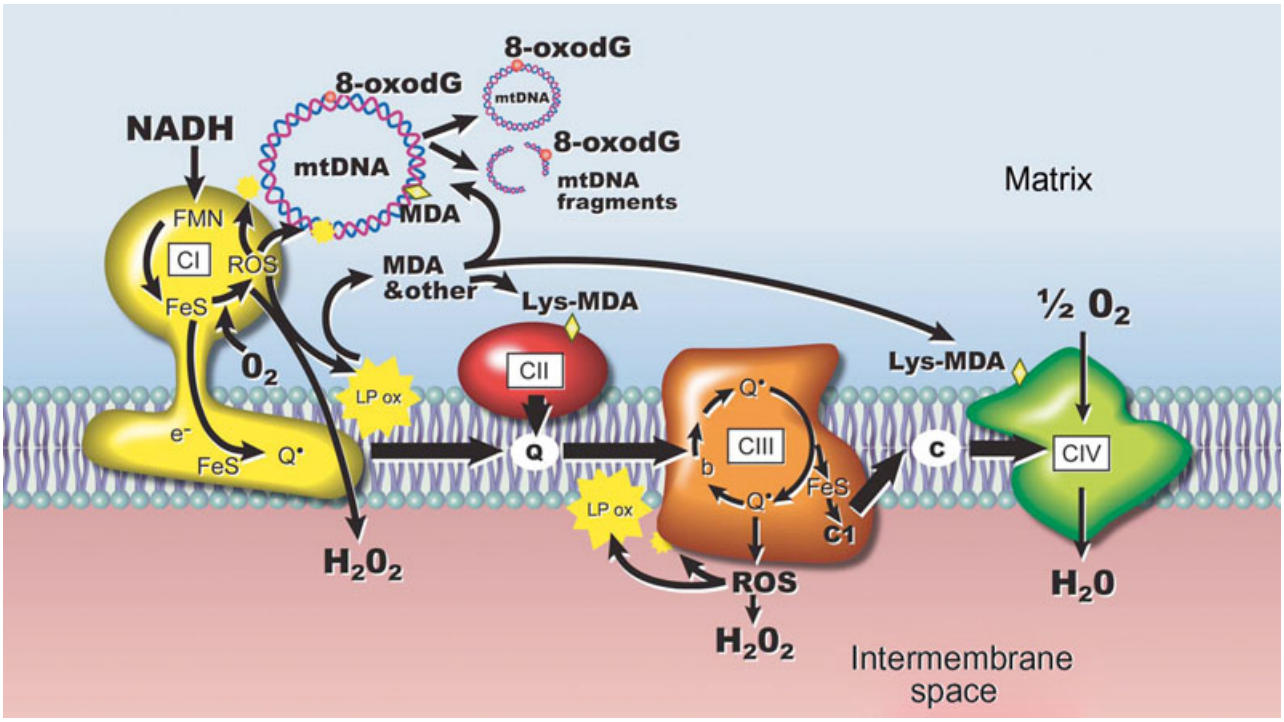

Fig. 1. Schematic representation of mitochondrial processes pertinent to reactive oxygen species (ROS) formation. The diagram identifies mitochondrial complexes I and III as the main sites of mitochondrial ROS generation, with complex I superoxide production being confined to the matrix side. Oxygen radicals attack lipids, carbohydrates, proteins and DNA. The products of lipid peroxidation include highly reactive molecules that can cause lipoxidation damage to mitochondrial DNA and proteins (from Hulbert *et al.* 2007, with permission of the American Physiological Society).

vertebrate endotherms (high oxygen flux fauna), particularly birds and mammals, which are common models for studying the links between oxidative stress and ageing. For ectothermic animals, we have mainly focused on bivalves because recent studies show that they are excellent models for ageing research (Abele, Brey & Philipp 2009; Philipp & Abele 2010). This taxonomically rich group includes the longest-living non-colonial metazoan (the Iceland clam *Arctica islandica* >400 years; Wanamaker *et al.* 2008), as well as surf clams (=Family Donacidae) with species of no more than 1 year lifespan. That bivalves age has been shown by studying senescent change in physiological parameters such as an increase in oxidative damage accumulation (Philipp *et al.* 2005a, 2006), as well as a decrease in antioxidant and respiratory capacities (Philipp *et al.* 2006, 2008) in aged individuals. Care has to be taken not to confound allometric and age effects, as some bivalves also exhibit lifelong interminable growth. Two major traits make bivalves ideal models for ageing research: first, bivalves from temperate and cold-water environments can be accurately aged by counting their annual shell growth rings. This makes it possible to relate physiological state to chronological age in wild populations. Secondly, bivalve molluscs are genetically intermediate to classical invertebrate models of ageing (e.g. worms and flies) and mammals. This provides a better opportunity to understand the evolution of stress response pathways and organismic ageing (Austad 2009; Philipp & Abele 2010).

Low oxygen flux fauna

Early ancestors of extant marine invertebrates evolved in the Cambrian (550 Ma) in a still relatively reduced atmosphere in which PO_2 ranged at or below 2 kPa, less than 10% of the present atmospheric oxygen level. Remnants of their evolutionary history are found in some extant benthic (=bottom dwelling) meio- and macrofauna species, which are extremely tolerant of environmental hypoxia and are known to behaviourally avoid fully oxygenated conditions. Similar to early atmospheric conditions, present-day low-oxygen marine sedimentary environments can be relatively rich in hydrogen sulphide (H_2S) from microbial breakdown of sedimentary organic matter, with H_2S reaching levels as high as 1000 μmol L^{-1} in pore water and in the sediment water interface at organically enriched sites (Oeschger & Pedersen 1994; Oeschger & Vismann 1994). Many marine infauna species (=animals living beneath the sediment surface) have mitochondria that have preserved a supreme capacity for anaerobic energy production (Thielens *et al.* 2002). For example, some extant polychaete worms (the lugworm *Arenicola marina* or the polychaete *Heteromastus filiformis*) and bivalves such as the soft shell clam *Mya arenaria* perform sulphide-induced anaerobiosis and have cyanide/sulphide-insensitive, sulphide-oxidizing electron transport systems that complement their 'classical' cytochrome *c* oxidase pathway. These 'microoxophilic' species avoid ROS formation from reduced electron transport system (ETS) intermediates when cyto-

chrome *c* oxidase is inhibited by H_2S by shunting electrons via alternative pathways to an 'alternative endoxidase' (Völkel & Grieshaber 1996; Abele *et al*. 2007).

Because oxygen, the alleged driver of oxidative tissue damage and ageing, is 30 times less concentrated in water than in air, water-breathing animals should be less prone to suffer oxidative stress than aerial invertebrates or vertebrate endotherms, and this may underlie the pronounced longevity recorded in several aquatic ectotherms. In addition, many marine species with open circulatory systems (molluscs and crustaceans) maintain oxygen levels at respiratory surfaces and associated arterial blood vessels at low and protective levels, even in normoxic environments (Tran, Boudou & Massabuau 2000; Davenport & Irwin 2003; Massabuau 2003). In gastropod and bivalve molluscs and in barnacles (Cirripedia), haemolymph PO_2 is usually adjusted to far below normoxic levels by shell closure and occasional pumping (Brand & Taylor 1974; Davenport & Irwin 2003; Morley *et al*. 2007; Weihe & Abele 2008).

The oxidative stress and rate of living theories and mitochondrial ROS formation in marine invertebrate models

Although the oxidative stress theory of ageing is generally applicable to invertebrates (Philipp *et al*. 2006; Abele, Brey & Philipp 2009; Begum *et al*. 2009), high phenotypic diversity and enormous genetic distance among invertebrate clades differentially shape the interactions between ROS production, antioxidant defences and maximum lifespan in different phyla. Two examples illustrate how antioxidant levels are adjusted to meet life stage and lifestyle requirements.

Antarctic midges (Diptera) have a lifespan of 2 years, much of which they spend in the surface of the ice sheets as cryo-resistant larvae. During the austral summer, short-lived non-feeding adults emerge with a lifespan limited to only 7–10 days. Compared to the longer-lived larvae, adults fail to express the antioxidant enzyme CuZn-SOD and express less strongly the antioxidant enzyme catalase (Lopez-Martinez *et al*. 2008). Also, the adult midges do not express either small heat shock proteins (smHsp) or Hsp70 under control conditions, but up-regulate Hsp70 in response to anoxia, a state that poses a stress on the midges. In contrast, larvae have inherently high constitutive heat shock protein levels (smHsp, Hsp70 and Hsp90) and do not further up-regulate these stress proteins upon exposure to anoxia, freezing or heat shock. The different capacities for gene regulation during stress may relate to the distinct durations of insect life stages (Lopez-Martinez *et al*. 2008), and illustrate the necessity to sustain cellular intactness in longer-lived larvae. Although the adult midges are more prone to suffer heat stress on warm rock surfaces than the larvae in their ice environment, physiological adaptive capacity in the short-duration adult life stage is absolutely restricted. This rather extreme example illustrates how investment in tissue protective functions meets the necessities posed by distinct microenvironments, but also is adjusted to life expectancy or, in midges, life stage duration, and no more. This pattern reflects a life-history strategy evolved by these insect species, consisting of a high

Table 1. Comparison of *in vitro* rates of mitochondrial ROS formation among different species and tissues

Species	Tissue	Substrate	Resp. state	*T* (°C)	ROS (nmol H_2O_2 mg^{-1} prot min^{-1})	Source
Bivalves						
Laternula elliptica	Mantle	Pyruvate	3	1	0·04–0·09	Heise *et al*. (2003)
L. elliptica	Mantle	Succinate	3	1	0·03	Philipp, Pörtner & Abele (2005)
Arctica islandica	Mantle	Malate	3	10	0·00	Unpubl. data*
A. islandica	Mantle	Malate	4+	10	0·00	Unpubl. data*
A. islandica	Mantle	Succinate	3	10	0·020 ± 0·01	Unpubl. data*
A. islandica	Mantle	Succinate	4+	10	0·073 ± 0·07	Unpubl. data*
Mya arenaria	Mantle	Malate	3	10	0·074 ± 0·03	Unpubl. data*
M. arenaria	Mantle	Malate	4+	10	0·223 ± 0·04	Unpubl. data*
M. arenaria	Mantle	Succinate	3	10	0·144 ± 0·14	Unpubl. data*
M. arenaria	Mantle	Succinate	4+	10	0·226 ± 0·20	Unpubl. data*
Aequipecten opercularis	Mantle	Succinate	2/4	10	0·002	Philipp *et al*. (2006)
Other invertebrates						
Arenicola marina (polychaete)	Body wall	Succinate	3	10	0·01	Keller *et al*. (2004)
A. marina (polychaete)	Body wall	Succinate	4	10	0·11	Keller *et al*. (2004)
Musca domestica	Flight muscle	α-Glycerophosphate	4	25	0·8–2·0	Sohal (1991)
Vertebrates						
Rat	Heart	Succinate	3	37	2·0–2·8	Lambert *et al*. (2007)
Pigeon	Heart	Succinate	3	37	1·4	Lambert *et al*. (2007)

Temperature of measurements in °C, substrate provided and respiratory state are included along with publication source. 0·00: below detection limit.
*Unpubl. data by S. Hardenberg and N. Fischer (Abele Laboratory, AWI, Bremerhaven, 2006).

investment of adult midges in reproduction during their very short life to the detriment of self-maintenance.

A similar example comes from the molluscan phylum, where bivalves can be grouped into active, energetically intensive and sometimes even semelparous (= only 1 reproduction cycle per lifetime) lifestyle as opposed to sessile, energetically economic, iteroparous species that have multiple reproductive periods over their protracted lifetime. Pectinids, or Jacobs clams, are burst swimmers with a high scope for activity, and a consequently high lifetime metabolic rate. To reduce oxidative stress during frequent exhaustive swimming, they maintain well-coupled mitochondria with very low oxygen radical output per mitochondrion (Table 1). Damaged mitochondria in the adductor muscle are eliminated apoptotically as the animals grow older (Philipp *et al.* 2008) combined with extremely high rates of cell proliferation in the adult animals (10% of nuclei dividing within 24 h, Strahl & Abele 2010). Such high rates of cell renewal are found in scallops with short lifespans, whereas longer-lived bivalves have much lower rates of apoptosis and cell proliferation. The short lifetime and high rate of cell renewal in scallops moreover seems to reduce their need for cell-protecting antioxidant activity as evidenced by their lower levels of antioxidants when compared with other molluscs (Strahl & Abele 2010).

The long-lived ocean quahog *A. islandica* has evolved a very different strategy. These bivalves exhibit low aerobic scope and moderate ROS production rates per mitochondrion but have the highest catalase activity among bivalves. Of the mud clams in Table 1, *A. islandica* has the highest maximum lifespan potential (MLSP) and the lowest rate of ROS formation, whereas *M. arenaria*, also a temperate but shorter-lived mud clam, produces between three (state 4) and five times (state 3) more oxygen radicals per unit of mitochondrial protein (Table 1: shaded grey). H_2O_2 formation with the complex 1 substrate malate is even below detection limit in *A. islandica* mitochondria (fluorometric assay using homovanillic acid and peroxidase) unless respiratory complex inhibitors (rotenone and antimycin) are added. On the contrary, *M. arenaria* mitochondria display moderate H_2O_2 formation in coupled state 3 and uncoupled state 4, and state 3 ROS output rate doubles with succinate. Repeated depression of metabolic rate may further form part of the quahog's longevity programme (Abele, Brey & Philipp 2009; Philipp & Abele 2009).

Generally, the low rates of metabolism and ROS formation of bivalves are also associated with lower antioxidant enzyme activities compared to vertebrates. Catalase activities between 8 and 40 U g^{-1} wet weight reported for vertebrates (Perez-Campo *et al.* 1998) are much higher than in bivalve and gastropod molluscs (0–6 U g^{-1} wet weight; Abele, Brey & Philipp 2009). Similar to that reported for mammals (for a review, see Sohal, Sohal & Brunk 1990; Barja 2004; but see Finch 1990), antioxidant capacity does not correlate with maximum lifespan in marine molluscan phyla (Abele, Brey & Philipp 2009).

The low metabolic rates at low water temperatures and slow oxygen diffusion in the respiratory medium water limit the basal rates of mitochondrial free radical formation in several cold-water marine molluscs to generally less than 0·1 nmol H_2O_2 mg^{-1} mitochondrial protein min^{-1}, whereas rates at least an order of magnitude higher are reported for mammalian mitochondria (Table 1).

It is important to note that the membrane potential is highest in the resting state when substrates are provided in the absence of ADP (state 4), and this is associated with marked increases in ROS production, primarily at complex I (Fig. 1). By contrast, when oxidative substrates are provided together with ADP (state 3 in actively 'working' animals), phosphorylation proceeds rapidly and is accompanied by a rapid fall in membrane potential and intracellular PO_2, both of which result in a substantial reduction in superoxide formation relative to oxygen uptake. This accounts for the exercise paradox, whereby animals experiencing ongoing exercise (Goodrick 1980; Herrero & Barja 1997) or cold-induced thermogenesis (Selman *et al.* 2008; Vaanholt *et al.* 2009) show significantly higher lifetime oxygen consumption, but no difference in MLSP compared to their sedentary or thermally comfortable counterparts. Because many tissues within the body vacillate regularly between states 3 and 4, ROS production per unit of oxygen consumed will usually be much lower than *in vitro* measurements under simulated rest conditions (state 4).

At the cellular level, mitochondrial ROS formation is modulated by tissue oxygenation (+: positive correlation with tissue PO_2), temperature which accelerates electron flow to oxygen (+), mitochondrial densities, inner membrane potential and amount and reduction state of respiratory electron carriers (+), and by the proton leak (−), which uncouples the membrane potential and reduces ROS formation (Abele *et al.* 2002; Heise *et al.* 2003; Keller *et al.* 2004; Philipp, Pörtner & Abele 2005b; Philipp *et al.* 2005).

Futile cycling of protons through uncoupling of the mitochondrial inner membrane in evolutionary early marine ectotherms (bivalves) amounts to between 30% and 40% of mitochondrial oxygen turnover in fully coupled state 3 respiration (Heise *et al.* 2003; Kern *et al.* 2009), values similar to those reported in other ectotherms (Brand *et al.* 1991; Hulbert *et al.* 2002a). In this context, proton leak can help to control cellular oxygen concentration simply through an increased consumption of O_2 under conditions of transient tissue over-oxygenation. A direct comparison of PO_2-dependent respiration of marine invertebrate mitochondrial isolates of the marine polychaete *Nereis diversicolor* and the mud clam *A. islandica* with mitochondria isolated from bovine heart clearly showed that oxyconforming respiration of the whole animal is based on oxyconformity in the mitochondria themselves (Tschischka, Abele & Pörtner 2000). Both kinds of invertebrate mitochondria increased respiration (with malate or succinate) linearly up to 47·5 kPa PO_2, the highest PO_2 applied in the experiment. Under the same exposure conditions, bovine heart mitochondria increased the respiration rate between 6 and 21 kPa, whereas at hyperoxic PO_2 (> 21 kPa) respiration rates declined. Oxyconforming respiration in marine invertebrate mitochondria is supported by an alternative end-oxidase with a lower oxygen affinity than

cytochrome *c* oxidase (cytox). This oxidase, which has recently been sequenced (McDonald & Vanlerberghe 2005), consumes excess oxygen within cells and tissues of some low-oxygen-adapted marine species without accelerating metabolism. Phosphorylation rates (measured as ADP/O ratios) were generally lower and, in the extremely hypoxia tolerant *A. islandica*, steeply declined with PO_2. By contrast, bovine heart mitochondria had higher ADP/O ratios (= better coupling) independent of experimental PO_2. The experimental comparison clearly indicates that marine mud-dwellers uncouple mitochondria at higher PO_2 using less and less oxygen to drive phosphorylation and, instead, consume more oxygen mainly through non-phosphorylating alternative electron transport (for a review, see Abele *et al.* 2007). Alternative end-oxidases and PO_2-dependent induction of futile proton cycling could therefore be another quality distinguishing 'hypoxia-tolerant' low oxygen flux species from 'hypoxia-sensitive' vertebrate mitochondria (next to the anaerobic capacities described above). In oxyconforming marine ectotherms, oxygen-sweeping mitochondria may represent a second line of defence against oxidative stress, following the control of tissue PO_2 by behaviour (see above).

Effects of temperature, size, growth and metabolic rates on lifespan

Growth and metabolic rates in ectotherms are principally governed by the environmental temperature a given species experiences. When comparing similar species from warm and cold water environments, the cold water populations usually exhibit slower growth and metabolic rates and longer lifespans (Philipp *et al.* 2005a, 2006). Given the wide range of life-history strategies, morphological complexity and group-specific physiological strategies in marine invertebrate phyla, an overall relationship between body mass (size), metabolism and longevity does not exist. Even in well-defined organismal groups such as the bivalves, long-lived species such as the Antarctic clam *Yoldia eightsi* (100 years, cf. Peck & Bullough 1993) can be limited to 2–4 cm shell length (ash free dry body mass: 0·01–0·1 g AFDM), whereas many larger bivalves, even from the same area, do not live to half this age. For example the Antarctic soft shell clam *Laternula elliptica* can reach 10 cm shell length but has a reported maximal lifespan of 36 years (Philipp *et al.* 2005; 0·1–3·2 g AFDM).

The maximum expected lifespan of a given population of marine ectotherms depends strongly on extrinsic modulators (mortality through predation or starvation, abiotic stress from fluctuating environmental parameters), which can select different life-history strategies that maximize reproductive potential during their lifetime. This results in differences among species, and among different populations of the same species, with respect to timing of gonad ripening and physiological stress tolerance and to evolution of different MLSP. An overall metabolic rate model for six different populations (sites) of the Iceland clam could not explain variation in site/population-specific MLSP (between 30 and > 100 years) (Begum *et al.* 2009). The extremely long lifespan in some populations seemed more related to the capability to enter into and emerge from a metabolically reduced state lasting a couple of days and occurring mostly in individuals from the fully saline Icelandic, Irish Sea and North Sea *Arctica* populations. The capacity for intermittent dormancy in *Arctica* is supreme, and animals reduce to 10% of normoxic energy turnover and heart beat during these periods (Taylor 1976), a behaviour also reported for other long-lived bivalves such as the pearl shell clam *Margaritifera margaritifera* (Ziuganov 2004). Importantly, the animals do not suffer oxidative damage on surfacing and reoxygenation, but the protective mechanisms they employ are still not fully understood. During metabolic shutdown, *Arctica* burrow a couple of cm deep into the sediment, the inside of the *Arctica* shell becomes anoxic (0 kPa), and metabolism functions anaerobically. Prolonged shell closure further leads to caloric restriction, the best studied life-prolonging intervention in animals, which has been proposed to promote longevity in bivalves having intermittent dormancy behaviour and other estivating invertebrates (Philipp & Abele 2009). The capacity for metabolic shutdown depends on environmental prerequisites such as constant low temperatures.

Antioxidant defences in marine ectotherms: effects of seasonal and regional temperature variation

In addition to the effect of habitat temperature fluctuation, animal life history in temperate and polar seas is largely governed by variations in other environmental factors such as seasonal light intensity, day length and food availability. In many marine invertebrates and fish, mitochondrial volume densities, as well as energetic and proton leak capacities of the mitochondria, are adjusted on time scales of weeks or months to seasonal changes (for reviews, see Guderley & St-Pierre 2002; Keller *et al.* 2004). For example, the rate of ROS formation is higher in mitochondrial isolates taken from the intertidal lugworm, *A. marina*, in summer compared with winter, but this is partly compensated by reduction in mitochondrial volume densities in summer acclimated animals. Membrane potential, proton leak and the rate of ROS formation are much more temperature sensitive in summer than in winter-isolated mitochondria. This means that rapid changes in the ambient temperature, common on intertidal mudflats during summer, can be met by rapid adjustment of proton leak to mitigate ROS formation. As a trade-off for this adjustment, mitochondrial phosphorylation efficiency is reduced during periods of rapid warming (mudflat exposure to sun during daytime). On a seasonal scale, proton leak is 30% higher in winter lugworms, which results in lower membrane potential and 50% less H_2O_2 formation by mitochondria respiring in state 4 (Fig. 2). This increased leakiness is presumably due to increased unsaturation of mitochondrial inner membrane fatty acid composition. The lugworm study gave the most comprehensive picture, especially with respect to flexible short-term adjustments of membrane potential and proton leak during thermal fluctuations in summer. The mode of

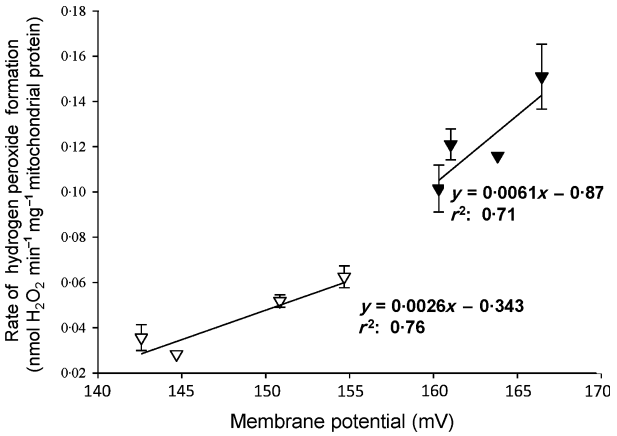

Fig. 2. Rate of hydrogen peroxide (H_2O_2) formation in relation to membrane potential in state 4 + respiration in mitochondrial isolates from the lugworm *Arenicola marina* in winter (white triangles) and summer (black triangles). Data are means ± SD from one to three isolations per point assayed at 10 °C (redrawn from Keller *et al.* 2004, with permission).

functioning seems to be widely applicable in different phyla and also across climatic ranges for similar species. The principle of seasonal and regional thermal adjustments including higher mitochondrial volume density and increased proton leakiness in cold-adapted marine animal ectotherms is conserved between invertebrates and fish (Guderley 2004).

Antioxidant enzyme activity (AOX: SOD, catalase, GPox, GR) and the levels of chemical antioxidants (especially vitamin E and C, glutathione) also vary with season in intertidal marine ectotherms from temperate to subpolar intertidal areas. Intertidal limpets and bivalves (Power & Sheehan 1996; Manduzio *et al.* 2004; Bocchettia & Regoli 2006; Malanga *et al.* 2007) and polychaetes (Abele-Oeschger, Oeschger & Theede 1994) are indeed experiencing thermal extremes from close to 0 in winter to 25 °C or even higher in the summer months and accordingly adjust their antioxidant capacities including both antioxidant enzyme induction and increased uptake of dietary antioxidants during summer.

Role of uncoupling proteins (UCPs) in marine invertebrates

Although UCPs are highly conserved in structure and are ubiquitously expressed in tissues of marine ectotherms including molluscs and fish (Mark, Lucassen & Pörtner 2006), they seem to be little involved in stress-induced mild uncoupling following ETS component autoxidation. A recent survey of UCP5 expression in oyster *Crassostrea gigas* tissues revealed the highest expression in adductor muscle (fast and slow fibres) and ganglia (Kern *et al.* 2009) with the lowest expression in the heart. However, overall, expression levels were much less variable in oyster than mammalian tissues (where UCP expression in brain can range orders of magnitude higher than other tissues). Oxidative stress conditions (Cd exposure and hypoxia-hyperoxic reoxygenation) failed to induce UCP5 in any of the oyster tissues, thus mitigation of oxidative stress does not appear to be a function of UCP5 in these bivalves (Kern *et al.* 2009).

High oxygen flux fauna

BODY SIZE INFLUENCES ON METABOLIC RATE AND MLSP

Although birds and mammals evolved endothermy independently, both have very similar size-affected metabolic rates. With each doubling of body mass, metabolic rate per unit of tissue decreases about 15–20% in both groups (Hulbert *et al.* 2007). At any given body mass, however, the metabolic rate of birds is about 1·5 times higher than that of mammals. If the rate of living theory accurately explains maximum lifespan, birds would be expected to live about two-thirds as long as mammals. Instead, the MLSP of birds averages about twice that of mammals, but shows a similar increase with increasing body mass (Hulbert *et al.* 2007). Significantly, there are greater differences between MLSP and basal metabolic rate (BMR) among same-sized species within the two classes, than the average differences between them. Although metabolic intensity appears to explain about 40% of the variation in MLSP in birds and 26% in mammals (Hulbert *et al.* 2007), it is important to note that BMR is affected by both size and phylogeny. When the effects of body mass and phylogeny on BMR are statistically corrected, there is no correlation between BMR and longevity in birds or mammals (de Magalhaes, Costa & Church 2007). This raises questions about the link between an animal's rate of aerobic metabolism and its level of oxidative stress.

BODY SIZE EFFECTS ON ROS FORMATION

Because mitochondrial membrane potential has such a strong effect on ROS production, membrane leakiness strongly influences the proton gradient and thus greatly affects the rate of superoxide formation. Comparative studies of mammals (Porter, Hulbert & Brand 1996) and birds (Brand *et al.* 2003) reveal that membrane leakiness is inversely related to body mass in both groups. Coincidentally, mitochondrial membrane lipid composition varies with body mass in a similar way in both classes of animals, containing proportionately less polyunsaturated fatty acids (PUFA), but more monounsaturated fatty acids (MUFA) with increasing size. The extent of membrane leakiness is not due directly to membrane fatty acid composition (Brookes, Hulbert & Brand 1997), but it is unclear if there might be an indirect catalytic influence of membrane acyl chain composition on membrane proteins associated with this process Although this leakiness will help limit the potential for ROS production, the rate of ROS formation will still be dominated by the aerobic metabolic intensity of tissues. This is shown by studies examining the rates of superoxide and hydrogen peroxide formation in mammalian liver mitochondria isolated from the size range of mice to horses (Sohal *et al.* 1989; Sohal, Sohal & Brunk 1990). An analysis of their data reveals that both superoxide

and hydrogen peroxide production per mg of mitochondrial protein scale inversely with body mass in these animals, with a scaling of mass$^{-0.15}$ for superoxide and mass$^{-0.29}$ for hydrogen peroxide. This implies that the potential for oxidative stress is inherently greater in smaller than in larger endotherms and, unless countered by other processes, would account for the relationship between body size and longevity. The wide range of MLSP at a given metabolic rate and size among birds and mammals indicates that animals differ in their capacity to limit size-affected oxidative stress, which has provoked a number of studies to address some obvious questions. Do animals that live longer decrease oxidative stress by having higher levels of antioxidants? Do similar-sized animals with divergent MLSP differ in the amount of ROS formed per unit of oxygen consumed? Do longer-living animals have vital structures that are less vulnerable to oxidative damage?

ANTIOXIDANT PATTERNS IN ENDOTHERMS

One of the ways that animals could offset their potential for oxidative damage is by increasing their levels of antioxidants. This has led some to believe that differences in longevity might be explained by variation in antioxidant capacity. The available evidence consistently shows that antioxidant levels vary inversely with MLSP in mammals and that treatment with exogenous antioxidants sometimes leads to extended median ages, but almost never extends MLSP (Perez-Campo *et al*. 1998; Sanz, Pamplona & Barja 2006; Pamplona & Barja 2007). Similarly, an extensive study evaluating plasma antioxidant capacity in 95 species of free-living birds concluded that longer-lived birds had lower levels than those with a faster pace of life (Cohen *et al*. 2008). The consensus of these studies was that antioxidant levels were aligned with rates of ROS production, which, in turn were inversely related to MLSP. If the oxidative stress theory is correct, this suggests that variation in rates of ROS importantly influences MLSP and that consequent oxidative damage is imperfectly countered by antioxidants.

This calls into serious question the relative contribution of dietary antioxidants on the capacity of organisms to cope with ROS. A number of recent ecological studies using experimental procedures show that carotenoids, a type of dietary antioxidants previously claimed to be 'vital', have minimal effect on the antioxidant defences in reptiles and birds (Costantini & Møller 2008; Costantini, Fanfani & Dell'Omo 2008; Isaksson & Andersson 2008; Olsson *et al*. 2008, 2009). Despite these limited effects, further studies are needed to see whether carotenoids may be important antioxidants for certain tissues at particular life cycle stages.

ROS formation in animals with divergent MLSP

The first studies to explore the mechanistic bases of how birds could live longer while having higher metabolic intensities than mammals were performed by Ku & Sohal (1993) and Barja *et al*. (1994). Both groups compared H_2O_2 formation in mitochondria extracted from pigeons (MLSP ~35 years) and similar sized rats (MLSP ~3·5 years), and Ku and Sohal also examined superoxide production in a preparation of submitochondrial particles. Their results demonstrated that pigeons had significantly lower rates of mitochondrial superoxide and H_2O_2 production than rats, a result consistent with the free radical theory of ageing. Significantly, the rate of ROS production per unit of oxygen consumed was lower in pigeons than in rats, thus reconciling their ability to have a higher metabolic rate than rats and yet live far longer (Barja *et al*. 1994). A recent study has greatly extended this comparative approach by examining mitochondrial ROS production in ten mammalian and two bird species chosen for their disparate MLSP and range of body sizes (Lambert *et al*. 2007). They found a significant inverse relation between MLSP and rate of mitochondrial H_2O_2 production (Fig. 3). Significantly, the influence of ROS production on MLSP was upheld after statistically eliminating size-related effects on longevity. The inverse relation between MLSP and rate of mitochondrial H_2O_2 production seems to be general across vertebrates as shown by a recent study on reptiles. Robert, Brunet-Rossinni & Bronikowski (2007) show that longer-lived colubrid snakes (> 15 years) produce less mitochondrial H_2O_2 than shorter-lived counterparts (< 10 years), while they do not differ in resting metabolic rate or in mitochondrial oxygen consumption.

Although the Lambert *et al*. (2007) study supports the oxidative stress theory of ageing, two aspects of this study deserve further comment. First, some of the interspecific differences in mitochondrial ROS production were far less distinct than some interspecific differences in MLSP, at times being statistically indistinguishable. For example, the Brazil-

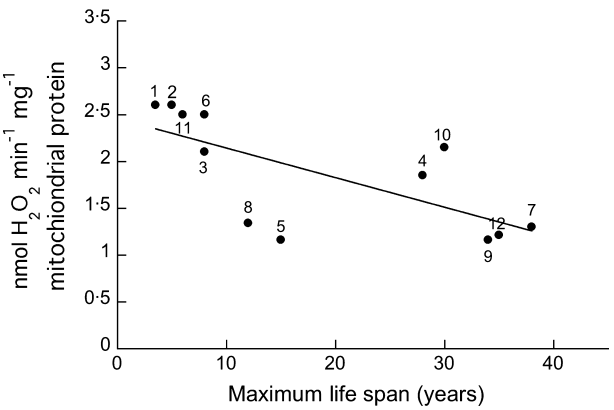

Fig. 3. Relation between hydrogen oxygen production by heart mitochondria of: (1) mice (*Mus musculus*), (2) rat (*Rattus norvegicus*), (3) white-footed mouse (*Peromyscus leucopus*), (4) naked mole-rat (*Heterocephalus glaber*), (5) Damara mole-rat (*Crypomis damarensis*), (6) guinea-pig (*Cavia porcellus*), (7) baboon (*Papio cynocephalus*), (8) Brazilian free-tailed bat (*Tadarida brasiliensis*), (9) little brown bat (*Myotis lucifugus*), (10) ox (*Bos taurus*), (11) Japanese quail (*Coturnix japonica*) and (12) domestic pigeon (*Columba livia*). The inverse relation is significant at $P = 0{\cdot}007$; $R^2 = 0{\cdot}54$ (redrawn from Lambert *et al*. 2007).

ian free-tailed bat (*Tadarida brasiliensis*) lives about three times longer than the little brown bat (*Myotis lucifugus*), yet had only a 10% lower ROS production rate. Similarly, the naked mole rat (*Heterocephalus glaber*) lives about eight times longer than mice (*Mus musculus*), but has about a 30% lower rate of mitochondrial ROS formation. This could be interpreted to mean that very small differences in rate of ROS formation have substantial effects on MLSP, but it also suggests that mitochondrial ROS production rates are but one influence on longevity.

The other issue to highlight is that the correspondence between mitochondrial ROS and MLSP depended on the type of metabolic substrate provided (Lambert *et al.* 2007). They found no relation between MLSP and complex III ROS generation (succinate in the presence of rotenone), but significant differences when mitochondria were provided saturating levels of succinate. Such levels are known to result in reverse electron flow from the Q pool to complex I, which, in turn, is associated with high rates of ROS production when the membrane potential is also high (Hansford, Hogue & Mildaziene 1997). While it is not known if reverse electron flux occurs naturally, fatty acids and α-glycerophosphate also enter the electron transport chain through the Q pool and may reduce it to levels provoking reverse electron flow (Schonfeld & Wojtczak 2008). Complex I is considered the dominant site of *in vivo* ROS formation (Miwa & Brand 2003) and also the site responsible for the reductions in rates of mitochondrial ROS production following the main treatment known to extend lifespan in mammals, calorie restriction (CR). In this regard, it is significant that birds examined thus far (pigeons, budgerigars, canaries) have proportionately less complex I in their mitochondria than mammals (rats, mice) (St-Pierre *et al.* 2002; Pamplona *et al.* 2005; Lambert *et al.* 2010). Furthermore, the maximal rates of complex I ROS production per mg of heart mitochondrial protein in pigeons was about half that of rat mitochondria, reflecting exactly the proportionate difference in complex I content of their mitochondria (St-Pierre *et al.* 2002). If this were a general characteristic of avian mitochondria, birds would be predisposed to a reduced mitochondrial ROS production compared to mammals.

Influence of membrane phospholipids on oxidative stress

In addition to their higher potential for ROS production, small endotherms are confronted with another size-related variable that places them further at risk of oxidative damage than their larger counterparts. The phospholipids comprising cell and mitochondrial membranes vary in a systematic, size-related manner among birds and mammals. In both groups, the phospholipid content of membranes from all tissues sampled, except brain, showed a significant decline in the proportion of PUFA, particularly docosahexaenoic acid (DHA), with increasing body mass, and a significant increase in the proportion of MUFA (Hulbert *et al.* 2002b; Hulbert, Rana & Couture 2002c; Brand *et al.* 2003). One major difference between the PUFA comprising bird and mammal membranes, however, was that bird membranes contained proportionately more n-6 PUFA and less n-3 PUFA than those of mammals.

It has long been known that PUFA and MUFA differ substantially in their vulnerability to peroxidative damage. This is due to the relative ease with which bis-allylic H atoms (those attached to the single-bonded C atoms situated between double-bonded C) are removed by free radicals (Halliwell & Gutteridge 2007). Because MUFA have only one double bond on their acyl chain, they lack bis-allylic H atoms and are consequently highly resistant to free radical attack. Conversely, because of the associated increase in the number of bis-allyic H atoms, PUFA susceptibility to peroxidation rises substantially with the degree of polyunsaturation.

The lipophilic nature of free radicals favours their presence in the lipid bilayer rather than the aqueous exterior (Gamliel, Afri & Frimer 2008). If these succeed in extracting a bis-allylic H atom, the C atom is left with an unpaired electron and, consequently, becomes a carbon-centred free radical that can interact with oxygen to form the highly reactive peroxyl radical. The latter radical can, in turn, attack membrane proteins and PUFA side chains which set about an autocatalytic chain reaction that will likely produce a number of reactive intermediates collectively called reactive carbonyl species (RCS). Because RCS have very long half-lives (minutes to hours) compared to ROS (fractions of a second), they can migrate considerable distances and penetrate membranes relatively far from the original lipoxidation event (Pamplona 2008). Thus, endotherms with high metabolic rates are associated with higher rates of ROS production and membranes that are more susceptible to peroxidative damage and autocatalytic propagation of free radicals. Conversely, those with lower metabolic intensities will form ROS at lower rates and have less vulnerability to membrane peroxidation. This scenario is the basis of the membrane-pacemaker modification to the oxidative stress theory of ageing (Hulbert 2005). A corollary of this hypothesis is that animals with higher MLSP than expected for their size are predicted to have membrane phospholipids with lower peroxidation vulnerability than their shorter-living counterparts.

Relation between MLSP and membrane phospholipids

Membrane susceptibility to peroxidation can be determined from its fatty acid composition and the relative susceptibility of each fatty acid group to peroxidation damage (Holman 1954), thus creating a scale called the Peroxidation Index (PI). If membrane susceptibility to peroxidation influences MLSP of vertebrate endotherms, there should be a correlation between membrane PI and longevity. Examination of PI in relation to MLSP of selected birds and mammals does indeed reveal a significant relation (Fig. 4), resulting in a doubling of MLSP for each 19% decrease in muscle membrane PI among birds and mammals. Similar proportionate changes in MSLP relative to membrane PI have been shown in mammalian liver

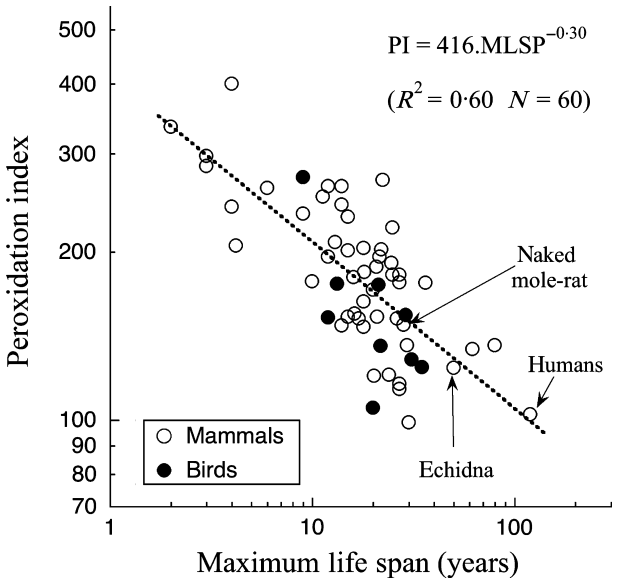

Fig. 4. Relation between skeletal muscle membrane fatty acid peroxidation index (PI) and maximum lifespan potential in selected birds and mammals (from Hulbert 2008, with permission).

mitochondria (Hulbert *et al.* 2007). Also highlighted in this figure are PI values in three species with much higher MLSP than expected for their size. The naked mole rat (*H. glaber*) lives about five times longer than predicted from its body mass (Buffenstein 2005), and although its basal metabolic rate is 30% lower than expected (O'Connor *et al.* 2002), this does not account for its high longevity. Its low PI is a result of it having proportionately large amounts of peroxidation-resistant MUFA, but low amounts of peroxidation-sensitive PUFA, particularly DHA (Hulbert *et al.* 2007). Similarly, humans and echidnas live about five times longer and have membrane PI values much lower than expected for their size (Pamplona *et al.* 1996; Hulbert, Beard & Grigg 2008).

Among birds, Procellariiformes (petrels and albatrosses) are exceptionally long living, whereas Galliformes (fowl) are associated with relatively short lifespans. A comparison of myocardial phospholipids from birds of each group revealed significantly lower PI values in the Procellariiformes than in the Galliformes, due mainly to the proportionately higher MUFA and reduced PUFA (particularly n-6 PUFA) in the seabirds compared with the fowl (Buttemer, Battam & Hulbert 2008). Interestingly, the 36% lower average PI in Procellariiformes was exactly the magnitude expected for the 4·5-fold greater average longevity in the petrels compared with the fowl (Buttemer, Battam & Hulbert 2008).

Because PI declines with body mass in birds and mammals, the correspondence between PI and MLSP will also be affected by any mass-related influences on longevity. Valencak & Ruf (2007) addressed this issue in their analysis of skeletal muscle phospholipid composition of 42 mammalian species. They statistically removed the effect of body mass on MLSP and various lipid measures and did not find any variation in PI to account for the residual differences in MLSP. They did, however, find a significant decrease in the proportion of n-3 to n-6 fatty acids with increasing MLSP that was independent of body mass or phylogeny (Valencak & Ruf 2007). It may be significant that birds, which live on average two times longer than same-sized mammals, also have consistently lower n-3/n-6 ratios than mammals (Hulbert *et al.* 2002b). Overall, membrane phospholipids appear to vary in a consistent manner with MLSP in vertebrate endotherms.

UCPs and MLSP

Following the discovery of genes encoding mitochondrial uncoupling proteins UCP2 and UCP3 in mammals (Boss *et al.* 1997; Fleury *et al.* 1997) and avian UCP in birds (Raimbault *et al.* 2001; Vianna *et al.* 2001), the search was on to identify their physiological functions. It is now well-established that protons outside the mitochondrial inner membrane can enter through these UCPs once they are activated, instead of via ATP synthase. When this occurs, the relatively low density of these UCPs in mitochondrial membranes results in a mild uncoupling along with a drop in membrane potential. During reverse electron flow, the ROS production at complex I is very sensitive to the inner membrane potential and a 10 mV decline in membrane potential, a value less than 10% of the state 4 maximum, can decrease ROS production by as much as 70% (Miwa & Brand 2003). Studies of *Caenorhabditis elegans* reveal that variation in membrane potential can significantly affect lifespan, with long-lived mutant strains displaying reduced mitochondrial membrane potentials compared with shorter living wild types (Lemire *et al.* 2009). Because all superoxide production of complex I is directed to the matrix side of the membrane (St-Pierre *et al.* 2002), mild uncoupling has the potential to significantly reduce mitochondrial DNA exposure to superoxides and ROS. Following the discoveries that mitochondrial lipid peroxidation products such as 4-hydroxy-trans-2-nonenal (HNE) and superoxides can activate these UCPs (Echtay *et al.* 2002, 2003; Talbot, Lambert & Brand 2004), especially when the mitochondrial membrane potential is high (Parker, Vidal-Puig & Brand 2008), attention has turned to the protective role that these inducible uncouplers could play in reducing ROS-related damage and, by extension, in increasing MLSP. Experimental evidence that UCPs afford oxidative protection to mitochondria *in vivo* comes from studies of genetically modified mice. Crosses between mice that were heterozygous for mitochondrial superoxide dismutase production ($SOD2^{+/-}$) and others heterozygous for UCP2 expression ($UCP2^{+/-}$) produced progeny with various combinations of SOD and UCP expression (Andrews & Horvath 2009). SOD2-deficient animals are unable to convert matrix-generated superoxide into membrane-permeable H_2O_2 and oxygen, resulting in the formation of high levels of complex I-generated superoxide in the mitochondrial matrix. Mice lacking SOD2 usually do not survive more than a few weeks of age due to high levels of mitochondrial oxidative damage in metabolically active tissues (Lebovitz *et al.* 1996). Andrews & Horvath (2009) confirmed the lethal consequence of SOD2 deficiency, but noted the time of death depended on UCP2

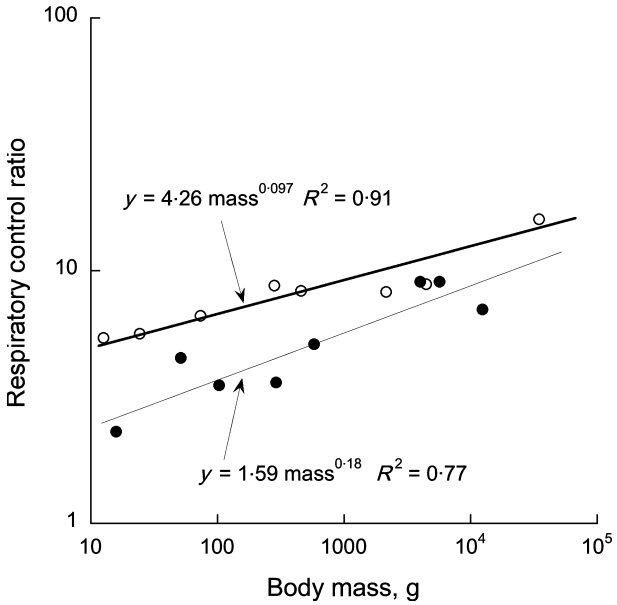

Fig. 5. The relation between respiratory control ratio (RCR = state 3 respiration rate/state 4 respiration rate with ATPase inhibition) and body mass in mammals (filled circles; from Porter & Brand 1993) and birds (open circles; from Brand *et al.* 2003). The correlation between RCR and body mass is significant for both mammals ($P < 0{\cdot}02$) and birds ($P < 0{\cdot}001$).

expression, with SOD2-deficient mice surviving only 7 days if lacking UCP2 expression ($UCP2^{-/-}$), 14 days if heterozygous for UCP2 and 21 days if homozygous ($UCP2^{+/+}$). This confirms both the capacity for mitochondrial oxidative damage to affect longevity and the ability of UCPs to mitigate its deleterious potential.

The possibility that increases in UCP expression might explain the difference in MLSP has been inferred from a study examining the mitochondrial characteristics of two metabolically distinct cohorts from a population of outbred mice that differed in lifespan (Speakman *et al.* 2004). Because the longer living mice had a higher UCP3 content than the other cohort, an active debate has followed regarding the involvement of UCPs in increasing uncoupling of longer-lived species, with the assumption that increases in uncoupling necessarily lead to longer lifespans. It is generally forgotten that measurements of basal proton flux rates in mitochondrial membranes in mammals (Porter & Brand 1993) and birds (Brand *et al.* 2003) show a significant increase in respiratory control ratio (RCR) as body mass increases (Fig. 5). The RCR is the ratio of the oxygen consumption rate at maximum ATP synthesis (state 3 respiration with non-limiting ADP) to the non-phosphorylating respiration rate (state 4 in the presence of ATPase inhibition; therefore, proton leak). Thus, the mitochondrial membranes of smaller species are more uncoupled than those of larger species as the non-phosphorylating respiration rates decrease more strongly than the phosphorylating respiration rates as body mass increases, yet larger species usually live longer.

Critical assessment of the extent to which interspecific differences in MLSP is accounted for by UCP variation will require quantification of mitochondrial UCP content and how this affects ROS formation under physiological conditions known to provoke high rates of superoxide production. In this regard, it is important to note that Lambert *et al.* (2007) examined mitochondrial ROS formation under the same conditions that Talbot, Lambert & Brand (2004) activated UCP3 expression in rats. If mitochondrial UCP activation did occur in the species examined by Lambert *et al.* (2007), then the lack of statistically significant differences in ROS formation among some species with very different MLSPs raises doubt about the contribution of UCPs in effecting interspecific differences in longevity.

General conclusions

The consistent correlation between rates of ROS formation and antioxidant levels among most animals examined for these traits reveals the limitations of inferring relative oxidative stress from limited measures of pro-oxidant or antioxidant status among or within species. There is also a tendency to treat measurements made from a few individuals of a given species as reflecting lifelong characteristics of that species. Serial sampling of individuals over their lifetime has revealed dramatic differences in ROS-damaged proteins among short-living and long-living rodents (Pérez *et al.* 2009b). It is important to stress here that almost all evidence supporting the oxidative stress theory of ageing is mainly of a correlative nature and comparative studies are still limited to a few species. It is clear that we need carefully designed long-term studies of animals selected from a wide range of taxa and MLSP before we can understand the extent to which oxidative stress directly affects ageing. Further insights could be gained by experimentally perturbing the oxidative stress experienced by animals through manipulation of reactive species production. In addition, more extensive use of knock-in and knock-out animal models could provide further experimental capacity to delve into the underlying mechanisms of ageing. This approach was taken in long-term studies of mice by Pérez *et al.* (2009a) to show the absence of life-extending effects of antioxidant over-expression and the very limited effect of under-expression on shortening of MLSP. Finally, it would be fruitful to carry out parallel experiments on captive and free-living animals of the same species to better understand how oxidative stress influences rates of ageing in the context of natural and sexual selection. Robert & Bronikowski (2010) demonstrated the insights deriving from this approach in their study of oxidative stress relations among populations of a snake species with very different life histories. They showed that neonates from the long-lived ecotype (from low extrinsic mortality environments) of western terrestrial garter snakes (*Thamnophis elegans*) are smaller, consume equal amounts of oxygen when corrected for body mass, have DNA that damages more readily but repairs more efficiently, and have more efficient mitochondria and more efficient cellular antioxidant defences than short-lived snakes (from high extrinsic mortality environments). Future studies targeting other species with similar differences in life history and MLSP will

greatly improve our understanding of the relationship between oxidative stress and longevity.

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