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Walking the Oxidative Stress Tightrope: A Perspective from the Naked Mole-Rat, the Longest-Living Rodent

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Abstract

Reactive oxygen species (ROS), by-products of aerobic metabolism, cause oxidative damage to cells and tissue and not surprisingly many theories have arisen to link ROS-induced oxidative stress to aging and health. While studies clearly link ROS to a plethora of divergent diseases, their role in aging is still debatable. Genetic knock-down manipulations of antioxidants alter the levels of accrued oxidative damage, however, the resultant effect of increased oxidative stress on lifespan are equivocal. Similarly the impact of elevating antioxidant levels through transgenic manipulations yield inconsistent effects on longevity. Furthermore, comparative data from a wide range of endotherms with disparate longevity remain inconclusive. Many long-living species such as birds, bats and mole-rats exhibit high-levels of oxidative damage, evident already at young ages. Clearly, neither the amount of ROS *per se* nor the sensitivity in neutralizing ROS are as important as whether or not the accrued oxidative stress leads to oxidative-damage-linked age-associated diseases. In this review we examine the literature on ROS, its relation to disease and the lessons gleaned from a comparative approach based upon species with widely divergent responses. We specifically focus on the longest lived rodent, the naked mole-rat, which maintains good health and provides novel insights into the paradox of maintaining both an extended healthspan and lifespan despite high oxidative stress from a young age.

Keywords

Comparative biology of aging; mitochondria; naked mole-rat; oxidative stress; proteasome; autophagy; reactive oxygen species

1. INTRODUCTION

The oxidative stress theory of aging asserts that the decline in functionality that characterizes the aging process is due to progressively accumulating levels of oxidative damage. This intuitively logical theory has been around for more than a century and despite an equivocal array of data, this invincible theory has held steadfast as the key proximate mechanism that determines species maximum lifespan potential (MLSP) [1]. More than a century ago, Max Rubner first noted the inverse correlation between the mass specific rate of oxygen consumption and longevity in mammals. He further coupled MLSP with body size and metabolism and determined that the lifetime energy expenditure (LEE) was relatively constant [2, 3]. Twenty years later Raymond Pearl expanded upon this idea and proposed that differences in metabolic rate explained the lifespan extension of *Drosophila melanogaster* maintained at different temperatures and through this observation defined the “rate of living theory of aging” [4].

The toxic nature of oxygen was already a well-known phenomenon since the seminal work of Lavoisier in 1781 [5]. However, free radicals were first regarded as the cause of oxygen toxicity in 1954 [6] and soon afterward in 1956 Denham Harman proposed that physiological metals would cause reactive oxygen species (ROS) to form in cells potentially damaging nearby molecules, including DNA. These would cause mutations, and based on the belief at the time, such induction of mutations could cause both cancer and aging. Harman also proposed that administering compounds that could oxidize easily and absorb the ROS in the cell could slow down this mutation-induced aging [7]. Since that time, the free radical theory of aging has been repeatedly modified and renamed to the “oxidative stress theory of aging [8–11]. As such, more than 50 years later, it remains a key focal area for aging research. Research has focused on two broad categories to validate and expand upon the notion that oxidative stress is an integral component of aging: testing the levels of oxidatively damaged biomolecules in aging tissue and manipulating—either biochemically, genetically or behaviorally—various stressors to determine their effects on lifespan (Fig. 1; reviewed in [12] and [13]). The boldest version of the oxidative stress theory of aging makes the all-encompassing prediction that lifespan is determined by oxidative damage and, thus, that an increase in oxidative damage will contribute to a shorter lifespan. While a shortened lifespan may not be the product of accelerated aging, determining the lifespan of animal or plant species with compromised antioxidant pathways can be used further to explore this theory: it follows that if an organism has increased oxidative damage but exhibits no change in lifespan, the result falsifies the hypothesis.

Here, we offer new insights into oxidative stress, longevity, and the role of oxidative stress in species longevity. We base our insights on research primarily using mammals and birds, and in particular, highlight research on the longest-lived rodent known, the naked mole-rat. This strictly subterranean, eusocial rodent, found in the northeast horn of Africa lives more than 30 years in captivity while maintaining cancer-free, good health well into its third decade of life [14]. The lack of spontaneous neoplasia is most unusual among captive wild-caught rodents (such as *Mus musculus* and *Peromyscus* species) as well as domesticated laboratory strains of mice and rats. Approximately 70% of domesticated laboratory rodent deaths are attributed to various types of cancers [15]. Understanding the mechanisms that facilitate cancer resistance in captive naked mole-rats may reveal important insights into

cancer prevention. We hypothesized that this extraordinary longevity relative to its shorter-lived rodent cousins may be explained by the production of less ROS and/or extremely efficient mechanisms to protect this species against oxidative damage.

2. OXIDATIVE STRESS, DISEASE AND LONGEVITY

ROS are formed during oxidative phosphorylation in the mitochondria. During this process, electrons from NADH or FADH₂ are transferred through the electron transport chain (ETC) to oxygen and the energy released in the process is used to power proton transport across the mitochondrial inner membrane at complexes I, III, and IV. The proton motive force generated is used to drive the ATP-synthesis reactions. During this process, however, electron leakage may occur predominantly in complex I and III, leading to the generation of superoxide [16, 17] and it is estimated that up to 3%, of oxygen consumed during respiration ends up as oxygen radicals [18]. This is likely to be an overestimate as other studies report less than 0.15% of the products of maximum oxygen consumption become ROS [16]. The rate of superoxide production is primarily dependent upon the local oxygen concentration and the presence of reducing equivalents, although the exact mechanisms that lead to superoxide formation are still not fully understood. At physiological concentrations, ROS may have important intracellular signaling functions affecting cell function, growth and development [19–21]. However, excessive mitochondrial ROS production is detrimental. Oxygen radicals may react with numerous other compounds to form a variety of other reactive species all of which are capable of indiscriminately damaging nearby macromolecules including phospholipids, proteins and DNA. Oxidized lipids are themselves potent ROS, autocatalyzing and self-propagating this unregulated process. Genotoxic intermediates of lipid peroxidation may also play a role in eliciting age associated DNA mutation [22]. ROS cause oxidative damage to proteins by showing that oxidative damage to a histidine in glutamate synthetase resulted in enzyme inactivation [23]. The damage also modified the residue to a carbonyl group. Additionally, carbonyl groups reportedly increase with age and are resistant to normal proteolytic degradation, making them the preferred marker of protein damage as a result of ROS [24]. The harmful effects of ROS have led to the evolution of numerous antioxidant and cytoprotective mechanisms that neutralize and detoxify the free radicals before they can induce substantial damage.

A complex suite of mechanisms has evolved to eliminate ROS and thereby offset their potential for accruing oxidative damage. The primary lines of defense include a) enzymatic antioxidants including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (Gpx) b) hydrophilic scavengers such as reduced glutathione (GSH) c) lipophilic scavengers such as tocopherols, flavonoids and carotenoids as well as d) the proteins involved in reducing oxidized antioxidants and thereby recycling them for further use (e.g., GSH reductase, peroxiredoxins). As a second line of defense against free radicals, cells possess a number of detection and repair mechanisms for the different types of DNA damage (nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), double-strand break repair (DSBR)) and also rely on proteasome degradation and autophagy to remove damaged proteins and organelles [25]. Likewise lipid peroxidation can trigger autophagic response leading to a degradation of oxidized products by lysosomes [26, 27]. Despite the plethora of antioxidants mechanisms, some ROS always evade detection and subsequent neutralization, inducing damage that is seldom completely repaired. As a consequence, according to the oxidative stress theory, damage accrues and accumulates with age. Furthermore, ROS have been implicated in the causation of several age-related diseases from cancer to diabetes. Better processing of these free radicals resulting in less damage could ameliorate these diseases (reviewed in [28]).

Research has persistently tried to understand how antioxidant defenses work, either by overexpressing key genes or knocking them out in various species. Manipulation of antioxidant genes shows a myriad of phenotypes in different animal models from yeast to mice. The invertebrate phenotypes have been described extensively (reviewed in [12, 13]). Here we focus specifically on the investigations conducted in vertebrate (mainly mouse) models.

Knockout Models

Glutathione peroxidase 1 (Gpx1) is ubiquitous in almost all mammalian tissues and is thought to be the main cellular scavenger of hydrogen peroxide (H_2O_2) [29]. The most significant pathological finding to date for Gpx1 knockouts is that the null mice develop cataracts, potentially resulting from increased ROS damage in the eye, at a considerably younger age than wild-type mice [30, 31]. Also, Gpx1^{-/-} mice are very sensitive to paraquat and diquat, both potentiators of ROS which cause fatal oxidative damage to tissues in these animals [32]. Unfortunately, glutathione peroxidase 4 (Gpx 4) knockout mice are embryonically lethal indicating a vital developmental function [33], but recent studies using a spermatocyte-specific knockout show that the lack of Gpx4 leads to infertility possibly due to an increase of ROS in the germ cells [34]. Survival curves of Gpx1^{-/-} and Gpx4^{+/-} mice have not shown a difference in lifespan when compared to wild-type mice [13, 35].

Cu/Zn superoxide dismutase (SOD1) is a highly conserved enzyme responsible for the scavenging of the superoxide radical (O_2^-) in the cytosol and in the mitochondrial inter-membrane space. Formerly called erythrocuprein, the enzymatic function was first described by McCord and Fridovich (1969). They reported that CuZnSOD1 catalyzes the reaction where the first superoxide molecule is oxidized and the second molecule is reduced, turning two molecules of superoxide into O_2 and H_2O_2 [36]. In 1993, Rosen *et al.*, determined that mutations in the SOD1 gene correlated with familial amyotrophic lateral sclerosis (ALS) [37]. Reportedly, SOD1 is ubiquitously expressed in all tissues with highest concentrations found in neural tissue [38]. Mutations in this gene are the most common known cause of ALS, comprising close to 10% of all cases [39]. Copper is rapidly incorporated into both newly translated and pre-formed SOD1. Mouse fibroblasts labeled with radioactive copper (^{64}Cu) show that the concentration of apo-SOD1 has an inverse relationship with copper levels [40]. It has been shown that SOD1 activity is induced under conditions of oxidative stress [41, 42].

Female mice without CuZnSOD are prone to infertility due to a dysfunction in ovarian development, in addition to signs of early, age-related hearing-loss and early onset of cataracts [43–47]. Furthermore, mice lacking SOD1 have an increased risk of developing hepatocellular carcinoma [48] and this predisposition to cancer may be the main reason for the 30% reduction in maximum lifespan of these animals [48]. Congenic SOD1 knockout animals have high levels of oxidative damage to all macromolecules [48]. Sod1^{-/-} mice also exhibit a pronounced loss of muscle mass as they age, such that by 20 months of age they have ~50% of the muscle mass of that of their wild-type counterparts and accrue a significant amount of oxidative damage in skeletal muscle compared to wild-type mice [49]. More recently, Sod1^{-/-} mice were found to exhibit an increase in blood glucose and blocked glucose-stimulated insulin production [50]. These collective differences in Sod1^{-/-} mice phenotype relative to wild type suggest that the lack of an accelerated enzymatic method to remove superoxide anions results in biological toxicity with a long-term pathogenesis of oxidative stress and damage to biomolecules.

Mn superoxide dismutase (SOD2) is the main scavenger of superoxide in the mitochondrial matrix. Mutations in this gene may cause idiopathic cardiomyopathy and diabetes and increase the risk of breast cancer [51–53]. Depending on the genetic background, removal of

the SOD2 gene in mice results in neonatal lethality predominantly via neurodegeneration within the first 24 days after birth [54–56]. These mice also have a significant increase in DNA oxidative damage and are very sensitive to hypoxia [57, 58]. Heterozygous transgenic mice show that a non-lethal decrease in Sod2 nevertheless increases oxidative damage and the sensitivity to oxidative stressors. However, while the animals have a higher incidence of cancer, their lifespans were indistinguishable from those of wild-type mice [59–61].

The third, though least abundant SOD enzyme is extracellular SOD (ECSOD) which is encoded by the Sod3 gene. ECSOD is located in the extracellular fluids including plasma, lymph and synovial fluid. It also has a substantial presence in the lung and a strong affinity to heparin [62, 63]. Polymorphisms in the SOD3 gene in human populations lead to increased susceptibility to type II diabetes [64]. Sod3 knockout mice are viable, can reproduce normally and do not show a decrease in lifespan. However, they do show sensitivity to hyperoxia [65].

Genetic manipulations of methionine sulfoxide reductase (MsrA; a reducing enzyme found in the cytoplasm and mitochondria that selectively reduces the sulfoxide of methionine) reveal a possible impact on longevity [66]. One group found that deletion of MsrA decreased longevity by 40% and increased sensitivity to oxidative stressors such as paraquat [67]. These knockout animals have also shown compromised cardiomyocyte activity following oxidative stress [68]. A more recent study also observed sensitivity to oxidative stress, however, no decrease in either median or maximum lifespan was observed [69].

Knockout studies of five of the six known peroxiredoxins--antioxidants that scavenge H₂O₂, organic peroxides and peroxynitrite--in mice all show some signs of oxidative damage related pathology and increased cancer risk [70, 71]. For instance peroxiredoxin 1 (Prx1) knockout mice have increased oxidative damage to both DNA and proteins [72, 73] and suffer from hemolytic anemia, an increase in cancer incidence and a 15% reduction in lifespan [73], but aside from Prx1, no data on lifespan are yet available regarding other peroxiredoxin knockout animals.

Although many of these animal models are more susceptible to oxidative stress and may even show similar dysfunction to that observed in human disease (See Table 1) clearly, there is no clear link between genetically reduced antioxidant expression and a decrease in lifespan.

Overexpression of Antioxidant Enzymes

An alternative approach to studying the impact of antioxidants on longevity is to genetically overexpress antioxidant genes with the intention of extending lifespan. In mice, only a handful of studies examining lifespan in response to overexpression of antioxidant enzymes have been published. A key question in these studies is whether or not an increase of activity in one antioxidant enzyme will in fact reduce endogenous oxidative stress, cause down-regulations of other antioxidants or even upset the balance of oxidant removal leading ultimately to a counterintuitive increase in oxidative damage. A large cohort study showed that overexpression of CuZnSOD did not increase lifespan [74]. High overexpression of both CuZnSOD and MnSOD leads to harmful phenotypes such as muscular dystrophy, neuronal degeneration and infertility, with at least the muscular dystrophy correlated ironically with higher levels of oxidative damage such as lipid peroxidation, [75–77]. Furthermore, although overexpression of CAT showed protection in a cell culture model, at the whole organism level it actually led to an increase in gamma irradiation sensitivity [78, 79]. Mice overexpressing CAT (2- to 4-fold) in the peroxisome did not show a significant extension of maximal lifespan, although median lifespan showed a modest 10% increase [79]. Transgenic mice that overexpress human CAT in mitochondria (MCAT) showed a

statistically significant extension of both median and maximum lifespan of approximately 20% and equivocal changes in lifespan when expressed in other organelles [80]. No increases in lifespan was evident with overexpression of MnSOD [81]. Age-related changes in oxidative damage (8-Oxo-2'-deoxyguanosine and mitochondrial DNA deletions in skeletal muscle), H₂O₂ production (in heart-specific mitochondria) and H₂O₂-induced aconitase inactivation (in heart tissue) were attenuated in MCAT animals [80]. In a more recent study, mice cardiomyocyte-targeted overexpression of CAT or endothelial nitric oxide synthase (eNOS) showed a reduction in vascular H₂O₂ and an increased expression of EC-SOD potentially leading to improved cardiac aging [82, 83]. However, whether this finding can lead to life extension is not yet known. Thioredoxin (Trx) facilitates the reduction of other proteins by cysteine thiol-disulfide exchange [84]. Overexpression of human thioredoxin (Hs Trx1) in mice reportedly leads to a 20% increase in lifespan, as well as increased resistance to cerebral ischemia and to UV-induced oxidative stress [85, 86]. In these animals HsTrx1 was three to six times more prevalent in tissues than endogenous Trx1 [85]. Metallothionein (MT), a scavenger of metal ions OH⁻ is also highly induced by oxidative stress [87]. Transgenic mice overexpressing metallothionein (MT-TG) showed a 15% increase in mean lifespan relative to control mice [88]. Furthermore, their cardiomyocytes have attenuated age-related increases in superoxide generation, cytochrome c release, p47phox (Neutrophil cytosol factor-1) expression and higher aconitase activity [89]. In contrast, knockout mouse models of MT show enhanced toxicity from ROS especially in cardiomyocytes [90, 91]. However, a lifespan study of MT null mice has not been conducted. The Mastushima group reported that overexpression of Prx3 protects the heart against post-myocardial infarction remodeling and failure in mice. Prx3 overexpression reduced left ventricular dilatation and dysfunction and attenuated myocyte hypertrophy, interstitial fibrosis and apoptosis of the non-infarcted myocardium. These beneficial effects of the Prx3 gene overexpression were also associated with attenuation in oxidative stress and mtDNA decline and dysfunction [92].

Clearly, overexpression of the antioxidants discussed above enhance protective mechanisms that could contribute to extended healthspan and lifespan. While lifespan studies have not been conducted on every antioxidant gene that is overexpressed, despite the many signs of enhanced protection, the available data show that the impact on lifespan is modest.

Studies in Humans

In humans, natural variation, measurable by genetic polymorphisms is a useful modality to investigate the effects of antioxidant enzyme alterations on lifespan. An abundant amount of literature exists on whether or not certain polymorphisms may present risk factors for disease where a subset of those risk factors includes oxidative stress. Studies in aged human cells containing mutated mtDNA or defective mitochondria show that a compromised metabolic function creates a higher rate of ROS production [93–95]. In contrast, a study on Japanese centenarians reported that 20% of this population possessed a mitochondrial gene variant in a coding a subunit of NADH dehydrogenase, Mt5178A [96] associated with low ROS production. Not only did the people in this study survive to be over 100 years of age, but they were 50% less likely to be hospitalized for age-related disease [96, 97].

A polymorphism in the mitochondrial localization sequence of MnSOD (the Val/Ala polymorphism at position -9 from the mature protein or 16 from the full-length precursor; V16A) is a known risk factor in many oxidative-stress-related pathologies such as diabetic nephropathy, hypertension and certain, but not all cancers [98–101]. However while the Ala allele has been found by some studies to increase the risk of breast cancer [102, 103] others cannot find a correlation [104, 105].

The cumulative indices of oxidative markers and oxidative stress in healthy humans related to aging and gender have been measured in multiple biological fluids and in many instances confounding results have been reported [106–115]. In several of these investigations some of the parameters tested showed significant correlations between age and an increase of pro-oxidative capacity together with a decrease of antioxidants [109, 110, 112, 113]. This was demonstrated by the observations of increases of plasma malondialdehyde (MDA) and 4-hydroxynonenal (HNE), erythrocytic glutathione disulfide (GSSG), cysteinylcysteine (CysCys) and by a slight decrease of erythrocytic GSH with age [108–113]. However, not all markers behaved predictably and the conclusions varied among the studies. For example the plasma protein carbonyls reached the lowest value in aged individuals in the Gil *et al.*, (2006) study. Indeed that study reported that metabolic and nutritional influences play a greater role than does the balance between anti-oxidants and oxidants. Age-related changes in antioxidants (SOD, GPx and CAT, carotenoids and tocopherols) in humans do not show a consistent pattern; generally erythrocytic activity of SOD and GPx increased while plasma values were unchanged [108, 110–112, 114, 115]. Similarly oxidative damage indices are also equivocal. Some studies report positive and linear correlations with increasing age were significant with regards to MDA, HNE, protein carbonyls and GSSG [108, 110, 113, 114]; levels of urinary isoprostanes reportedly are unchanged with age; and assessment of DNA damage by comet assay and the FLARE (Fragment Length Analysis using Repair Enzymes) method are contradictory with the former increasing while the latter remains unchanged [114]. Lastly, there does not appear to be any sexual dimorphism with regards to the selected measures of oxidative stress in age-matched men or women [112–114]. One of the limitations of these studies is the cross-sectional nature. The subjects cannot be followed to track whether more favorable markers of oxidative stress (low oxidative damage; high antioxidant capacity) lead to increased longevity and no study has examined ROS production. Direct measures of antioxidants, the results of their manipulations or correlations with disease and oxidant polymorphisms have not shown unequivocal support for the theory. More than likely, the successful aging seen in these populations is not only linked to their antioxidant/oxidative damage profile, but also how the body responds to the damage to maintain a “survivable” set point for homeostasis and thereby avoiding the common debilitating age-associated diseases that ultimately increase the chances of dying.

Oxidative Damage and Alzheimer’s Disease

Oxidative damage has also been implicated in Alzheimer’s disease (AD) (reviewed in [116]), the most common age-related neurodegenerative disease currently affecting ~30 million people (www.alz.org). Accumulation of neurotoxic amyloid beta (A β) is considered one of the pathological hallmarks of AD. The greatest risk factor for AD is age and to date there is no therapy for this devastating disease. Lipid peroxidation has been extensively studied because of the abundance of fatty acyl groups which account for ~10% of neural tissue. Moreover, the brain is an energetically expensive organ, with high oxygen consumption and having a high iron content along with ascorbates, which may further damage proteins by binding to them under pro-oxidant conditions. Finally, the limited antioxidant defenses yield the brain prone to oxidative stress and damage (reviewed in [117]).

Clinical assessments and postmortem studies have shown an inverse relationship between docosahexaenoic acid (DHA) and AD (reviewed in [118]) linking oxidative damaged lipids and AD. Furthermore, a clear association has been made between overproduction of free radicals arising from dysfunctional mitochondria and from A β with oxidative damage in brain regions with AD pathology (hippocampus and cortex). However, the cerebellum which remains clear of AD pathology, shows low oxidative stress and no neuronal loss [117]. An increase in antioxidant levels has also been reported in AD brains and dietary manipulations

increasing antioxidant activity have been investigated as a therapeutic targets for AD (reviewed in [117]). A promising clinical trial recently showed that attenuating metal proteins was effective in improving cognition in patients with AD [119] further highlighting the role of oxidative damage in the pathologies observed in this disease.

3. THE COMPARATIVE BIOLOGY OF OXIDATIVE STRESS; A POTENTIAL DETERMINANT OF SPECIES LONGEVITY

As reiterated above, the oxidative stress theory of ageing asserts that as an inevitable by-product of aerobic metabolism, the electron transport chain in the mitochondria produce ROS and that those species capable of living long lives will accrue less cumulative damage as a result of more complete neutralization by antioxidants. Furthermore, one would predict that long-lived species may also have structural macromolecule characteristics and/or better repair processes that make them more resistant to the cumulative effects of oxidative damage.

Although there is considerable support for this proximate mechanism of aging in invertebrates, [120–122], there are several contradictory studies even when using the same species (e.g. *Drosophila*, [123, 124]). Furthermore, the high phenotypic diversity and reproductive modality among invertebrates, coupled with the metabolic dependence upon temperature and terrestrial or aquatic lifestyle differentially influence the interactions between ROS production, antioxidant defense and maximum species lifespan potential. Endothermic vertebrate classes (birds and mammals) exhibit markedly higher oxygen flux than aquatic and terrestrial invertebrates and the ectothermic vertebrates (fish, amphibians and reptiles). Given their high metabolic flux, endotherms ought to be more susceptible to the harmful effects of potentially higher levels of unchecked ROS. However, birds exhibit a 1.5-fold higher mass specific basal metabolic rate (BMR) than do similar-sized mammals and in contrast to the predicted detrimental effects on longevity caused by the inevitable ROS-induced damage due to oxygen consumption, some live approximately twice as long as do similar-sized mammals [125].

Most biologists are aware of the importance of scaling biological traits with body size [126] and the famous “mouse-to-elephant curve” produced by Benedict [127] which profoundly demonstrated mammalian metabolic intensity to be inversely related to body mass. A very similar allometric relationship exists for birds, from humming birds to ostriches [128]. The allometric relationships for metabolic rate between birds and mammals are indistinguishable, despite the fact that endothermy arose independently in these two evolutionarily distinct vertebrate classes. In both birds and mammals, doubling of body mass, leads to a 15–20% decline in mass specific metabolic rate [129, 130]. Lindstedt and Calder [131] showed that longevity in birds and mammals increased similarly with increasing body size, although birds generally live twice as long as similar-sized mammals [129]. BMR is affected by several variables including body mass and phylogeny. When these effects of size and phylogeny on BMR are statistically corrected, the relationship between BMR and MLSP no longer holds in either birds or mammals [132]. The combination of a relatively higher metabolic rate and a longer lifespan also means that birds, on average, will have three-fold greater lifetime energy expenditure (LEE) than mammals. This raises critical questions about how aerobic metabolism and susceptibility to oxidative damage might differ between birds and mammals and if exceptionally long-lived mammals exhibit a profile more in keeping with that of long-lived birds. Both these outcomes refute the explanatory power of the rate of living theory for MLSP. Furthermore, when a more comprehensive assessment of >240 mammals is used to determine LEE, calculated values are not constant, but rather MLSP is negatively correlated with LEE. Furthermore BMR, the integral component upon which LEE is determined, is specifically measured at rest in post

absorptive, non-growing, non-breeding, healthy adults housed in their thermoneutral zone and is not an accurate indicator of total daily energy expenditure; it does not take into account the energy costs associated with daily activity, foraging, digestion, growth or reproduction. If average daily metabolic rates (ADMR) or field metabolic rates (FMR) are used instead of BMR, LEE among mammals declines with increasing body mass [129, 130]. There are also multiple exceptions to the presumed constant relationship between metabolic rate and lifespan posited by the rate of living theory. For example voluntary exercise and its associated increase in metabolic rate does not shorten lifespan of rats [133] or humans [134] and is generally thought to extend healthy lifespan. Dietary caloric restriction (CR) is well known to extend lifespan in a wide range of species [135], yet this process is not accompanied by attenuations in mass specific metabolic rate resulting in elevated LEE [136]. Also, there is no inverse relationship between lifespan and mass-specific metabolic rates of individual mice, dogs or flies [137–139]. Intra-specific data also provide compelling exceptions to this theory showing that within a species those individuals with the highest metabolic rates live longest and this is attributed to a decline in mitochondrial efficiency and metabolic uncoupling processes [139]. Similarly significant interspecific differences in MLSP within both groups of endotherms cannot be explained by metabolic rate differences. Rather, interspecific differences in metabolic rate generally reflect climatic zones and geographical regions such as desert or mesic habitats [140–142], the season during the year when the measurements were taken [143] and/or whether animals live above or below ground [144, 145]. Generally all subterranean rodents have low basal metabolic rates relative to that expected allometrically [144, 146]. However the low metabolic rates of African mole-rats species (Bathyergidae) with known MLSP data do not correlate with species longevity or other life history traits (Table 2) such as time to sexual maturity, gestation length or litter size, [147]). Furthermore, while BMR of naked mole-rats is 75% that of age-matched mice their greater longevity would result in the highest mass specific LEE of any known mammal [148].

Membrane Composition

Membrane composition, and in particular mitochondrial membrane lipid composition, also varies in a systematic manner with body mass in both endothermic vertebrate classes (see [130] for review). Phospholipids in membranes of larger species contain proportionately less easily oxidized polyunsaturated fatty acids (PUFA), but rather have more monounsaturated fatty acids (MUFA) that are more resistant to oxidation. The products of lipid peroxidation are powerful ROS, and initiate a self-propagating autocatalytic process of oxidative damage to adjacent macromolecules. Interclass variability in membrane composition could explain the seven-fold difference in metabolism between reptiles and mammals as well as differences in metabolic rate attributed to intra-class variability in body size [130]. Differences in membrane composition in both mammals and birds also are correlated with their maximum lifespans such that shorter-lived smaller mammals have membranes rich in n-3 PUFAs (such as DHA) that are more susceptible to lipid peroxidation [149, 150]. These findings have given rise to the “membrane pacemaker theory of aging” (see [130] for review). Comparative studies assessing membrane composition of species with disparate longevity provide support for this theory. For instance long-lived naked mole-rats [151], echidnas [152] and humans [153] all have membranes with a lower peroxidation index than predicted by body size. Similarly, longer-lived strains within a species have membranes with less DHA [151] and while CR does not influence BMR, membrane fatty acid composition varies with the degree of restriction [154]. Membrane leakiness and concomitant mitochondrial membrane potential influences the proton gradient and thus the rate of ROS production. Membrane leakiness, like BMR is inversely related to body mass in both mammals and birds [155, 156]. The extent of membrane leakiness however is reportedly not directly due directly to membrane fatty acid composition [157].

ROS Production

Both superoxide and H_2O_2 production per mg of mitochondrial protein scale inversely with body mass in a number of animals, from mice to horses [158, 159] further revealing that the potential for oxidative stress is inherently greater in smaller than in larger endotherms. Unless countered by other processes, oxidative stress could account for the relationship between body size and longevity. 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 [160]. A similar inverse correlation between longevity and mitochondrial H_2O_2 production was found in a comparative study among three similar-sized rodents with widely divergent longevity [161] and in a larger comparative study using mitochondria from heart tissue of ten mammalian and two bird species that differed substantially in both body size and MLSP [162]. Significantly, even after statistically correcting for the influence of body size on longevity and ROS production, this relationship was upheld. Furthermore recent studies in reptiles also exhibit a similar relationship between MLSP and rate of mitochondrial H_2O_2 production [163]. Despite this generalized strong support, several inter-specific data sets do not back the oxidative stress theory of aging and these discrepancies lead us to be wary of fully supporting this august theory. For example, in a comparative study among long-lived little-brown bats (*Myotis lucifugus*, MLSP 34y); short-tailed shrews (*Blarina brevicauda*, MLSP 2 years) and white-footed mice (*Peromyscus leucopus*, MLSP 8 years), while ROS production in heart tissue was lowest in the long-lived species, ROS production in brain tissue was considerably higher than that of shorter-lived, white-footed mice and the species differences in ROS production did not correlate with the 17-fold and 4-fold difference in MLSP. Furthermore, in the Lambert study described above, in which rates of H_2O_2 production were measured under identical conditions, statistically indistinguishable levels of ROS production were evident among the two short- and long-lived rodent pairs, namely white-footed mice and wild-derived mice despite a two-fold difference in longevity; and among the naked mole-rats and the C57Bl/6 mice despite an eight-fold difference in MLSP. Similarly, Brazilian free-tailed bats (*Tadarida brasiliensis*, MLSP 12y) are approximately three times shorter-lived than the little brown bat (*Myotis lucifugus*, MLSP 34 y), yet have only a 10% lower ROS production rate. While ROS production in heart tissue was statistically similar among the long-lived naked mole-rats and C57Bl/6 mice ROS production in naked mole-rat skeletal muscle and kidneys showed significantly lower levels (Fig. 2) in the longer-lived species (unpublished data Lambert, Brand and Buffenstein). This tissue difference among the heart and other tissues may reflect the fact that the naked mole-rat heart is a smaller and slower pump than that of the mouse [$139\text{mg} \pm 0.6$ for the naked mole-rat heart mass, or 0.36% of average body weight ($n=15$) and $143\text{mg} \pm 0.3$ or 0.56% of the average body weight ($n = 10$) for that of the mouse]; also, the naked mole-rat anesthetized heart operates between 150–220 beats per minute whereas that of mice under similar conditions beats at 450–500 beats per minute (Grimes, Lindsey, Lewis, and Buffenstein, unpublished data). Given the large difference in heart function, it is likely that the heart may not be the most appropriate organ for comparing mitochondrial function among species. 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 tissue specific and are only one of many factors that may influence species longevity and that these are not the main determinants of longevity.

It is worth mentioning that the detected inverse relationship between mitochondrial ROS and MLSP we observed is only evident when one substrate (succinate) is supplied in abundance [162]. This study suggests the reverse electron transfer through complex I may be an indicator of longevity. The substrate succinate is known to induce significant amounts of reverse electron transfer 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 [164]. There is

considerable debate regarding whether or not this stalled electron transport occurs naturally and it is also not known why the correlation is not evident when multiple other substrates representative of forward electron transport through the various components of the ETC were employed. However, 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 [165]. Complex I is considered the dominant site of *in vivo* ROS formation [166] and also the site responsible for the reductions in rates of mitochondrial ROS production following CR in mammals [167]. In this regard, it is significant that birds examined thus far (pigeons, budgerigars, and canaries) have proportionately less complex I in their mitochondria than mammals (rats, mice) [16, 168, 169]. 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 [16]. If this were a general characteristic of avian mitochondria, birds ought to be predisposed to a reduced mitochondrial ROS production when compared to mammals.

In addition, it is important to determine whether this phenomenon would be observed in mitochondria from other vital tissues such as brain, liver, kidney or skeletal muscle. Mitochondria from different tissues of the same species have different phenotypes in physiological and pathological situations [170, 171], (Lambert, Brand and Buffenstein unpublished data). In addition, interspecies differences in mitochondrial organization may influence the efficiency of mitochondrial substrate utilization as well as proton leak. Furthermore, whether mitochondrial ROS would cause similar extent of damage in intact cells containing endogenous antioxidant systems is still an open question and probably more meaningful. Interestingly, targeted disruption of the mitochondrial gene *SURF1*, causes an increase in longevity in laboratory mice [172]. However, the cause of the phenotype may neither be dependent on membrane potential nor be related to ROS control, but instead depend on the handling of Ca^{2+} uptake [172].

Antioxidants

A complex suite of mechanisms have evolved to eliminate ROS and thereby offset their potential for accruing oxidative damage. Antioxidant activity as a determinant of MLS remains controversial with reports of a positive, negative and no correlation between antioxidant activity and MLSP [173–178]. CAT and GPx activities reportedly negatively correlated with MLSP [179] whereas Sohal *et al.*, [158] found that SOD and CAT activities were positively correlated and indeed in the case of GPx, levels correlated positively with MLSP in brain samples but negatively in the liver and heart [176]. When birds, fish and amphibians were included for comparison for antioxidants activities and MLSP, strong negative correlations for some but not all antioxidants were obtained and results were tissue specific [160, 180, 181]. An often-touted explanation for this negative correlation is that antioxidant defenses are not the reason for extended longevity of long-living animals, but rather the lower antioxidant levels in long-living species may be indicative of lower levels of oxidative stress [173, 182–184]. Similarly, we too have found that total antioxidant power varies among tissues and is responsive to dietary manipulation, as seen by the increase in total antioxidants following supplementation with peroxidation prone omega n-3 fatty acids (Fig. 3; Wywiał and Buffenstein, unpublished data).

One difficulty in studying the role of antioxidants in longevity is that there is no consistent stoichiometric relation between pro-oxidation or antioxidant measures, making inferences between species from limited measures of only a few antioxidants essentially meaningless. Many members of the antioxidant defense system share redundant roles and are rapidly induced in response to elevated oxidative stress rather than maintained at high basal levels.

In order to estimate the overall ability to defend against ROS, it is necessary to evaluate the activities of all antioxidants present in tissues and their interactions and not simply focus on a few key antioxidant enzymes.

No consistent differences in known processes that remove radicals and repair the damage have been found to correlate with MLSP [162, 175, 185, 186]. Many long-lived species, such as the humans, and naked mole-rat, show unremarkable antioxidant defenses [186, 187]. Clearly levels of antioxidant defense cannot account for lifespan difference across the animal kingdom and as such the anti-oxidant arsenal are not integral determinants of species longevity. These findings concur with studies showing that the effects of anti-oxidants on human health are equivocal [188] as are the efficacy of pharmacological mimetics of antioxidants [189]. Genetic overexpression of various antioxidants and their effects on lifespan in various animal models similarly yield conflicting results and knocking down antioxidant protection, while leading to more oxidative damage, does not impact upon longevity [35]. This observation correlates with the negative data from various dietary treatments with exogenous antioxidants that sometimes lead to extended median ages, but almost never extends MLSP [176, 183, 184]. If the oxidative stress theory holds true it would suggest that variation in rates of ROS importantly influences MLSP and that consequent oxidative damage is imperfectly countered by antioxidants.

Oxidative Damage

The oxidative stress theory of aging asserts that the decline in functionality that characterizes the aging process is due to progressively increasing oxidative damage. Most comparative studies compare data from young healthy adults providing a snap shot of oxidative damage at a given age of each species. The rationale behind this approach is that traits influencing rates of aging ought to be present throughout life and if these traits facilitate long life, even at an early age their impact should be evident. Accrued oxidative damage may not only influence aging but also may impact considerably on many life history characteristics dependent upon the apportioning of energy metabolism into fecundity, development, somatic maintenance and maximum species lifespan.

Several comparative studies show an inverse relationship between oxidative damage and longevity, already evident in young healthy adults [173, 190, 191]. However, many exceptions to this premise have been reported. For example within flies, the longer-lived *Drosophila* species has more protein carbonyls than the shorter-lived blow flies (*Calliphora vicina*) [192]. Similarly longer-lived birds have higher levels of oxidative damage to DNA than do shorter-lived mammals [193], and within mammals both long-lived vampire bats and naked mole-rats have higher levels of protein carbonyls than short-lived rats and mice measured under identical conditions [1, 175, 182, 194]. The level of oxidative damage, such as MDA differs substantially among tissues. Despite lower levels of ROS production in muscle than in heart tissues (Fig. 2), muscle tissue exhibits twice as much lipid peroxidation as observed in heart tissue (Fig. 4) and exhibits a similar level of antioxidant protection (Fig. 3). The degree of oxidative damage is depended upon dietary fatty acid content, as is evidenced by the significantly greater MDA levels following chronic dietary supplementation with n-3 rich flaxseed oil. High levels of oxidative damage in mole-rats and bats has been attributed to their high intracellular iron content wreaking havoc with redox [182, 195]. Such interspecific and ontogenetic differences in iron homeostasis are likely to have a huge impact on rates of aging and sudden increases in ROS-related damage leading to death.

Oxidative damage measurements made in tissue samples are the net result of several interacting processes associated with primary rates of ROS generation in mitochondria including autocatalytic secondary ROS production as a result of phospholipid peroxidation,

neutralization of ROS by antioxidants as well as damage repair and the removal of damaged products by nucleases, proteases and autophagy that precede the sampling point. These net levels of oxidative damage as such may represent a “steady-state” level of damage tolerance rather than indicative of rates of oxidative stress. Steady-state levels of oxidative damage, based upon these premises reveal a lack of consistent patterns, with higher and lower levels of net damage reported in longer-lived birds, bats, and rodents [178, 186, 196, 197]. While it is possible that all the exceptions to the oxidative stress theory reflect unusual species that have been subjected to a different collage of evolutionary pressures and thus present with their own suite of “private mechanisms” to deal with aging, given the broad range of exceptions both within species, among species and among vertebrate classes, this seems unlikely [14]. Rather the lack of a consistent pattern in these measures of oxidative damage and lifespan challenge the validity of this theory.

This mode of comparative approach, however, is not without limitations and ignores the rates of age-related changes in oxidative stress and thereby fails to address the dynamics of aging and how this differs among species of disparate longevity, even though there is convincing evidence from numerous studies that aging is associated with increased oxidative stress and oxidative macromolecular damage in various tissues studied [193, 198]. Indeed age-dependent accumulation of oxidative damage is considered an intrinsic factor determining the rate of aging, with both damage to protein carbonyls, lipids and DNA increasing more markedly in the latter third of life [199–201]. For example oxidative damage to DNA gradually increases in a non-linear modality with age in rats, however levels appear to be tissue-specific such that DNA damage in the brain is greater than in liver and kidney [199]. The few studies that utilized both approaches are obviously more powerful for testing the oxidative stress theory of aging [186, 202–204]. These studies often yield contrary results to those simply using young adults. For example although naked mole-rats show high levels of oxidative damage, evident already at a young age, both cysteine oxidation and lipid peroxidation are maintained at those high steady state levels for at least two thirds of the extraordinary lifespan of naked mole-rats, providing some support for the oxidative stress theory of aging [205]. Clearly, this species has a higher threshold of damage tolerance and has mechanisms in place to facilitate structural resistance to the harmful effects of oxidative damage and still maintain cellular integrity and function.

Stress Resistance

Although antioxidant status and accrual of oxidative damage show mixed correlations with MLSP, stress and toxin resistance appears to play a significant role in the determination of lifespan in naturally long-lived species, as well as genetically modified models of longevity [206–209]. In worms and flies, manipulation of genes resulting in longer lifespan often result in increased resistance to exogenous stressors or toxins including heat shock, H_2O_2 and paraquat [210–212]. This toxin resistance is observed in rodent models of longevity as well. Dwarf mice, which can be drastically long-lived compared to wild-type littermates, show increased resistance *in vitro* and *in vivo* to an array of stressors [207, 213].

Most notably, fibroblasts cultured from naked mole-rats prove to be incredibly resistant to a variety of toxins, including oxidative stressors, when compared to fibroblasts from standard laboratory mice [205, 209]. However, while naked mole-rat fibroblasts showed resistance to toxicity from paraquat, consistent with the model of oxidative stress and longevity, the cells were acutely sensitive to H_2O_2 [209]. Interestingly, the toxicity of H_2O_2 differs from that of paraquat. Paraquat causes cellular toxicity through the production of O_2^- in the mitochondria disrupting NADPH-dependent biochemistry and eventually leading to tissue damage and compromised organismal function [214]. H_2O_2 , on the other hand, causes oxidative stress through the production of hydroxyl radicals that cause damage to membranes, lipids and

nucleic acids [215]. Aside from the different mechanisms of radical formation, the standard culture conditions of 20% oxygen, or the use of serum-free conditions (affecting membranes and lipids as well) may have contributed to this seemingly paradoxical result pushing the naked mole-rat fibroblasts, already containing high levels of oxidative damage compared to age-typed laboratory mice beyond their ability to mitigate the damage [205, 209, 216, 217]. In any case, because the naked mole-rat appears to be resistant to a diverse range of toxins that have different modes of action, a pathway that may serve as a key regulator of cytoprotection may serve as the main mechanism for the profound resistance to toxins observed not only in the naked mole-rat, but other naturally and genetically-modified long-lived species. One such pathway is the nuclear factor erythroid-2 related factor-2 (Nrf2) signaling pathway, which regulates the transcription of 230 genes [218]. These genes include those involved in GSH synthesis and conjugation [219], free radical scavengers [220] as well as proteasomal subunits [221]. Nrf2 is a transcription factor expressed in all tissues of an organism and is conserved from worms to humans [210]. Under constitutive (non-stressful) conditions, Nrf2 is basally expressed at low levels and has a relatively short half-life (~15 minutes). Nrf2 is largely regulated by kelch-like ECH-associated protein 1 (Keap1), which targets Nrf2 for ubiquitination and degradation via the proteasome [222, 223]. Under stressful conditions (i.e. an increase in ROS or xenobiotic stressor), cysteines within Keap1 are modified, impeding the interaction with Nrf2. Levels of free Nrf2 increase resulting in an increase in half-life (~60 minutes) and increased nuclear levels of Nrf2. Nrf2 binds to the antioxidant response element to promote the transcription of cytoprotective molecules [224, 225].

In *C. elegans*, the Nrf2 homolog, Skn-1, was shown to be a mediator for the life-extending effects of CR [210]. Similar results were observed in *Drosophila*, in addition to a lifespan extension observed in flies that had decreased Keap1 expression [212]. In both of these invertebrate models, long-lived organisms show an increase in the signaling of Nrf2/Skn-1-regulated molecules, possibly contributing to their longevity. These trends are also observed in long-lived rodent models including the naked mole-rat [226]. Caloric and methionine-restricted mice, as well as dwarf mice, show signs of increased Nrf2-signalling activity further supported by increased toxin resistance [227–229].

Repair Processes

Repair processes play a vital role in maintaining cellular homeostasis and not surprisingly there are numerous cellular pathways to ensure quality control and the rapid removal of damaged molecules before these can impair functionality [230]. A gradual decline in some of these pathways occur during aging and the malfunction of these quality control mechanism form the basis of many age-associated diseases such as neurodegeneration, metabolic disorders and cancers [230]. Cells rely on various molecular chaperones to maintain the three dimensional structure of their proteins. Chaperones selectively binding to non-native protein conformations to form stable complexes and prevent the irreversible aggregation of non-native protein conformations [231]. Not surprisingly many of these chaperones are induced under stressful conditions to assist in the maintenance of protein quality. Expression of chaperones declines with age and manipulations that retard aging enhance chaperone expression, including the heat-shock response [230, 232, 233]. Previously we have shown that the proteome of naked mole-rats and bats is more resistant to both urea-induced and heat-induced unfolding than that of shorter-lived mice [205, 234]. This implies that long-lived species might have evolved enhanced chaperone-like activities to preserve protein structure and prevent misfolding/aggregation. If the damaged proteins cannot be repaired by these chaperones, the proteins are directed towards proteolytic degradation pathways either via the ubiquitin-mediated proteasomal system or via lysosomal

mediated autophagy. Both of these systems are subject to age-related declines in functionality [235, 236].

Proteasome Activity

Amongst the many biological substrates of proteasome are oxidatively damaged proteins, the accumulation of which is one of the hallmarks of aging [237, 238]. Such proteins are suspected to inhibit the proteasome machinery, either by overwhelming the proteasome or by a noncompetitive inhibition [24]. In parallel, free radicals causing oxidative damage of substrates may modify the components of the proteasome system, effectively changing (most likely inhibiting) its actions [24]. Aging is also accompanied by the increase of protein carbonyl groups and aggregated cross-linked material which are resistant to proteolytic degradation and may act as inhibitors of the proteasome [24, 239–241]. Consequently, a model with reduced oxidative damage as observed in caloric restricted animals might reduce these potential inhibitors of the proteasome system [242, 243].

Data based upon the proteasome in fission yeast (*Schizosaccharomyces pombe*) reveal three basic principals of proteasome function [244]. 1) G0 cells require proteasome function during the maintenance of G0 quiescence. Temperature sensitive mutants of 26S proteasome subunits perturb this maintenance. 2) proteasome functions differ between G0 and vegetative proliferation phase (VEG). This conclusion comes from the examination of defective phenotypes of proteasome mutants in G0 phase, leading to a huge increase of oxygen stress responsive compounds and the massive decrease of mitochondria that did not occur in VEG. 3) proteasome dysfunction in G0 elicits defensive responses both by triggering the production of antioxidant components and inducing the degradation of mitochondria by autophagy. These defensive responses were not found in VEG cells, suggesting that proteasome functions in G0 are directly or indirectly involved in minimizing ROS [244]. Oxidative stress biomarkers, of which 20S proteasome was included, showed different responses depending on age in brown trout (*Salmo trutta*) exposed to paraquat. Fish aged five months showed no changes in carbonylation in liver tissue and 20S proteasome when exposed to the drug while the 12-month-old fish had both an increase in protein carbonylation in liver tissue and a decrease in 20S proteasome activity in brain tissue [245].

In mammalian studies, the influence of oxidative stress on the proteasome is strongly evident. Following injury to the brain or retina, the immunoproteasome activity is upregulated [246]. Furthermore, following immunoproteasome deficiency, the proteasome in the mouse retina could not respond to stress and as such became more susceptible to “oxidation-induced cell death” [247]. In rat brain and liver tissue preparations, exposure to oxidative damage-inducing agents, H₂O₂ and HNE, inhibited peptidase activity from the proteasome in a dose-dependent manner [248]. The effect was more pronounced in the brain. Interestingly, neither age of the animal or dietary restriction produced a significant effect on the oxidative-stress induced decline of proteasome activity observed in that study [248]. When proteasome activity is inhibited in neuronal cell crude lysates, an increase in both ubiquitinated and oxidized proteins is observed, as well as a selective increase in newly synthesized but insoluble proteins [249], again implicating the proteasome in oxidative-stress related protein homeostasis. An exception to the age-related decline of the proteasome is seen in the case of the naked mole-rat [205]. Perez *et al.*, postulated that the robust maintenance of the proteasome in older mole rats (20yr) when compared to old mice (2yr), may contribute to a better preservation of the proteome and thus contribute to the increased longevity seen in the naked mole-rat [205]. Also, old naked mole-rats have significantly lower levels of ubiquitinated proteins than do old and young mice suggesting less accrual of oxidized or misfolded proteins. Chymotrypsin-like activity from the proteasome is higher in

both young and old and naked mole-rats than that observed in their similarly aged mouse counterparts, supporting their hypothesis [205]. Naked mole-rat cytosolic and nuclear liver preparations also showed a more than two-fold increase in trypsin-like activity when compared to the activity from liver preparations of the same subcellular fractions obtained from livers of age-matched mice (Rodriguez KA and Buffenstein R, unpublished data) (Fig. 5).

Oxidative stress also links the proteasome to human disease. In a mouse model of ALS (SOD1G93A), a decrease in both the mRNA and protein expression in catalytic subunits $\beta 1$ and $\beta 5$ is observed during the progression of the disease in spinal cord tissue. A simultaneous increase is also noted in Lmp7 ($\beta 5i$), an immunoproteasome subunit [250]. When these mice were crossed with ALS-mice possessing a fluorescently-tagged UPP substrate, revealed both an accumulation of the substrate, and ubiquitin suggesting an impairment of motor neuron UPP in the mutant SOD1-linked ALS mouse [250]. Furthermore, Sod1^{-/-} samples show a relative decline of proteasome activity with both age and oxidative stress especially in the cytosolic and microsomal fractions of mouse liver tissue and a significant reduction in the contribution of activity from 26S in favor of 20S activity in tissue from young oxidatively stressed animals and from old animals (Rodriguez KA, Osmulski PA, and Gaczynska M, unpublished data). If indeed naked mole-rats have a higher steady-state level of oxidative stress as suggested by the studies referenced above [1, 175, 182, 194, 205], they might also have a higher contribution of activity from their 20S proteasomes and better maintenance of that activity with age.

Autophagy

Autophagy is an evolutionarily conserved catabolic pathway, present in all eukaryotic cells. This lysosomal system involves degradation of intact proteins and protein aggregates [251]. There are three pathways by which proteins and cellular organelles are delivered to the lysosome: microautophagy, macroautophagy, and chaperone-mediated autophagy. Surprisingly little research compares species differences regarding autophagy. Studies conducted on senescent avian and mammalian fibroblasts reveal that cells from longer-lived birds better maintain autophagosomal and lysosomal enzymes whereas these activities are down regulated with age in mammalian cell lines [252, 253]. Furthermore, our unpublished data reveal that this may indeed play an important role in the maintenance of protein quality in the naked mole-rat (Perez VI and Buffenstein R, unpublished data) as macroautophagy (degradation sensitive to 3-methyladenine, 3-MA) is substantially higher in fibroblasts maintained under serum starvation from a naked mole-rat compared to those from shorter-lived mice. Similarly when autophagy is assessed by monitoring markers of vacuole development (i.e., the conversion of LC3-I to LC3-II), the LC3-II/LC3-I ratio induced by serum deprivation was ~ 2-fold greater in naked mole-rat cells (Perez VI and Buffenstein R, unpublished data) (Fig. 5). This suggests perhaps that this exceptionally long-lived rodent, under basal conditions maintains higher levels of autophagy, thereby removing potentially toxic proteins and possibly its high ferritin load, before these can negatively impact upon organ functionality. Nevertheless exceptionally old (>29 yr) naked mole-rats accrue the age-associated yellowish-brown pigment lipofuscin which accumulates in various organs including skin, heart and liver, as a result of incomplete lysosomal digestion of cell products [254].

4. CONCLUSION

The plethora of contradictory results together with the lack of a general consensus among multiple species across the animal kingdom as well as among genetically manipulated mice in which oxidative stress is altered strongly suggest that species MLSP is unrelated to any of the oxidative stress parameters currently being measured. Certainly current data from

genetically modified mice that exhibit high levels of oxidative damage in response to experimentally impaired antioxidant defenses show no concomitant changes in age-related mortality. Similarly high levels of oxidative damage to lipids, proteins and DNA in the naturally long-lived naked mole-rat coupled with low levels of Gpx that are not compensated for by other enzymatic antioxidants, do not negatively impact on their exceptional longevity. Instead they seem to have a higher set point of equilibrium for oxidative damage. In fact there may be great differences in tolerance and structural resistance to oxidative damage that may underlie the insignificant differences in ROS production and antioxidant protection, despite the large differences in species longevity. This may limit the role of oxidative stress in leading to diseases including cancer, myopathy, vascular disease and neurodegeneration known to result from unchecked ROS. Through the mitigation of damage naked mole-rats may have arrived at an adaptive state of flux. Finally, we hope that the studies reviewed here can act as a springboard to gain insights into the mechanisms on how certain species, like the naked mole-rat, are able to tolerate high levels of oxidative stress without an endpoint of cancer and neurodegenerative disease. Such information in the future can translate to a better understanding of oxidative stress and its relation to a healthy lifespan free of age-related disease in our own species.

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Fig. 1.

Schematic diagram outlining the components of the oxidative-stress theory of aging. The theory predicts that, as an inevitable byproduct of metabolic activity, reactive oxygen species (ROS) are produced. If these are not neutralized, oxidative damage to proteins, DNA and lipids may occur. However, as some damage may occur, repair mechanisms are in place to mitigate the damage. Without repair, accelerated aging occurs. With repair, we may have a slow-down in the aging process.

Fig. 2.

Hydrogen peroxide (H_2O_2) production by heart, skeletal muscle, and kidney mitochondria in mice and naked mole-rats. To assess ROS production by complex I, succinate was added to the assay and H_2O_2 generation rate was determined by monitoring the oxidation of either *p*-hydroxyphenylacetic acid or amplex red with the reaction being coupled to the enzymatic reduction of H_2O_2 by horseradish peroxidase as described [162]. While there was no significant difference in H_2O_2 production in heart tissue, naked mole-rats showed significantly less H_2O_2 in both skeletal muscle and kidney. (Lambert, Buffenstein and Brand, unpublished data).

Fig. 3.

Total antioxidant power in naked mole-rats varied substantially among tissues. Furthermore, dietary supplementation for 6 weeks with flaxseed oil, an omega-3 fatty acid (7g PUFA per 100g ProNutro) [267] resulted in augmented total antioxidant activity (Wywiał and Buffenstein, unpublished data). Total antioxidant power was measured quantitatively using a commercial colorimetric microplate assay for total antioxidants (Oxford Biomedical Research, Oxford, MI).

Fig. 4.

Lipid peroxidation, as indicated by levels of malondialdehyde (MDA) in various tissues of naked mole rats. Note that MDA varies significantly within tissues (see Control profile), with skeletal muscle showing the highest levels. The degree of lipid peroxidation is influenced by dietary manipulations. After only six weeks of dietary supplementation with the omega-3 rich fatty acids present in ground flaxseeds, all tissues showed a significant increase in MDA. Wywiał and Buffenstein, unpublished data). LPO was measured quantitatively using a commercial colorimetric assay (Oxford Biomedical Research, Oxford, MI).

Fig. 5.

Both autophagy and proteasome activity show an increase in repair capacity in naked mole-rats when compared to mice. A) In serum-free media, the ratio of LC3II/LC3I increases significantly in naked mole-rats suggesting greater sensitivity in activating autophagy processes (Perez and Buffenstein, unpublished data). Data were collected using a Western blot technique and the ratios were calculated using Storm Image Quant software package (Molecular Dynamics, Sunnyvale, CA). B) Species differences in chymotrypsin-like (ChT-L) activity in liver cytosol lysates of young mice (4 m) and naked mole-rats (2y) in response to varying doses of proteasome inhibitor (MG132, N-(benzyl-oxycarbonyl) leucinyll-leucinal) (Rodriguez and Buffenstein, unpublished data). Data (presented as mean $\pm$ standard deviation) are based on three different experiments, run in triplicate, using a modification of the methods described by Rogers and Dean using the release of AMC from 100 μ M of the fluorogenic peptide Succ-LLVY-AMC (Boston Biochem, Boston MA) to measure proteasome ChT-L activity [268, 269]. Naked mole-rats not only show greater levels of proteasome activity compared to mice but also appear to be markedly resistant to a known inhibitor of proteasome activity.

Table 1
Diseases and Disorders Associated with Antioxidant Defense Genes and the Effect of Genetic Manipulations of These Genes.

Antioxidant Enzyme	Antioxidant Function	Genetic Manipulation	Phenotype(s)	Disorder/Disease Association	Ref(s).
Glutathione Peroxidase 1	Scavenges H_2O_2	Gpx1 ^{-/-} Gpx TG in PD model	Increased myo-carditis after viral infection. Protection against insulin resistance under high fat diet Lifespan not reported Reduced neural death, improved locomotion	Cardiovascular Disease, Diabetes, Neurodegeneration	[255] [256] [257]
Glutathione Peroxidase 4	Scavenges LOOH	Gpx4 ^{-/-}	Embryonic Lethal. Infertile in spermatocyte-specific knockout	Unknown	[33] [34]
Cu/Zn Superoxide Dismutase	Scavenger of $O_2^{\cdot-}$	Sod1 ^{-/-}	Multiple pathologies, 30% shortened lifespan	Neurodegeneration (ALS), Diabetes, Cancer	[48]
Mn Superoxide Dismutase	Scavenger of $O_2^{\cdot-}$	Sod2 ^{-/-} Sod2 TG	Neonatal lethal Protection from insulin resistance under high fat diet	Cardiovascular Disease, Cancer, Diabetes	[54] [258]
Extra-Cellular Superoxide Dismutase	Scavenger of $O_2^{\cdot-}$	Sod3 ^{-/-} Sod3 TG	"Normal" Protection against LPS-induced inflammation in lungs	Inflammation, Diabetes	[62] [64] [259]
Catalase	Scavenges H_2O_2	Cat ^{-/-} M-Cat TG	"Normal" Increase lifespan, protection against insulin resistance	Cardiovascular Disease, Diabetes	[260] [79] [261]
Peroxioredoxin 1	Scavenges peroxide ^a	Prdx1 ^{-/-}	Hemolytic anemia	Cancer	[73]
Peroxioredoxin 3	Scavenges peroxide ^a	Prx3 TG	protects the heart against post-MI remodeling and failure in mice	Cardiovascular disease	[92]
Thioredoxin 1	Reduction of proteins by cysteine thiol-disulfide exchange	Trx1 ^{-/-} Trx1 TG	Embryonic lethal 20% increased lifespan, increased resistance to cerebral ischemia	Inflammation, Stroke	[262] [85]
Methionine Sulfoxide Reductase	Repairs oxidized methionine	MrsA ^{-/-}	40% shortened lifespan	Cardiovascular disease	[67]
Metallothionein	Metal and OH ⁻ scavenger	Mt1 ^{-/-} MT-TG	Sensitivity to ROS 15% increase in lifespan	Cardiovascular disease	[91] [88]

^a Peroxioredoxins scavenge H_2O_2 , short chain organic peroxides, fatty acid alkyl hydroperoxides, and in the case of Prdx6, also lipid hydroperoxides

Bathyergid Key Life History and Metabolic Traits.

Table 2

Species	Common Name	Avg Body Mass (g)	MLSP (years)	% MLSP Expected#	BMR (mL O ₂ /g/h)	% BMR Expected*	Core Body Temp (°C)	Time to Sexual Maturity (days)	Gest. (Days)	Litter Size	Ref.
<i>Cryptomys hottentus</i>	Common Mole Rat	75	11	116	0.68	62	34	450 (f)	81	3.5	[263]
<i>Fukomys damarensis</i>	Damaraland mole-rat	152	15.5	147	0.57	60	35	511 (f)	93	2.5	[263, 264]
<i>Fukomys mechowii</i>	Giant mole-rat	272	20	174	0.60	75	36	unknown	112	2.6	[265]
<i>Georychus capensis</i>	Cape mole-rat	197	11.2	100	0.60	68	36	304(f), 304(m)	44	5	[141]
<i>Heterocephalus glaber</i>	Naked mole-rat	35	31	381	1.00	66	33	228 (f)	77	16	[195, 266]

Information not specifically cited taken from the AnAge Database (<http://genomics.senescence.info/species/>) [147]

% predicted MLSP based upon the allometric equation of JP de Magalhaes [132]

* % BMR expected based upon the allometric equations of BK McNab [144]