A TEST OF THE OXIDATIVE STRESS THEORY: HIGHER LEVELS OF OXIDATIVE DAMAGE IN LONG-LIVING NAKED MOLE-RATS THAN IN MICE

by

BLAZEJ ANDZIAK

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

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Abstract

A Test of the Oxidative Stress Theory: Higher levels of oxidative damage in long-living naked mole-rats than in mice

by

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Advisor: Rochelle Buffenstein

The Oxidative Stress Theory proposes that aging results from a progressive accrual of oxidative damage to cellular components, which reflects an imbalance between endogenous Reactive Oxygen Species generation and the capacity of organismal defenses to prevent damage accumulation. Inter-specific longevity variation may thus result from disparate oxidative damage accrual. Naked mole-rats (*Heterocephalus glaber*; NMRs) are the longest-living rodent species known (>28.3y) and live 9-times longer than mass-allometric predictions. I tested the Oxidative Stress Theory by comparing various biochemical markers in NMRs and in similar-sized (~35g), yet much shorter-living mice (~3.5y), at physiologically equivalent ages. Activities of four enzymatic antioxidants were compared to assess if NMRs posses superior antioxidant defenses than mice. Tissue non-heme-iron content and ratios of glutathione redox pair (GSH/GSSG) were determined to establish if NMRs experience attenuated levels of oxidative stress. Urinary and tissue markers of lipid (isoprostanes, malondialdehyde), protein (protein carbonyls) and DNA oxidation (8-hydroxy-2'-deoxyguanosine) were assayed to asses if long-living NMRs generate and accrue less oxidative damage than mice. NMRs do not posses superior antioxidant defenses, since their glutathione peroxidase activity was 0.014-times that of mice, with only a moderate up-regulation of other enzymatic antioxidants. Higher

iron levels and lower GSH/GSSG ratios in NMRs suggest a more pro-oxidative cellular environment in NMRs. A 10-fold greater urinary isoprostane excretion in NMRs indicates high oxidative damage generation in this rodent, while lower urinary 8-hydroxy-2'-deoxyguanosine excretion imply attenuated oxidative damage repair. Contrary to the predictions of the Oxidative Stress Theory, NMRs had more accrued oxidative lipid, protein and DNA damage than mice, even at a young age. Age-related patterns of lipid damage generation and accrual differed between mice and NMRs. Isoprostanes declined in NMRs and remained unchanged in mice, while malondialdehyde increased in mice but not in NMRs. Together these results demonstrate that quantitative differences in oxidative damage accrual do not explain the 9-fold difference in longevity between mice and NMRs, and thus do not support the Oxidative Stress Theory. Although oxidative damage may be a component of organismal aging, age-related changes in its levels, as well as their impact on physiological function are likely to be species specific.

Acknowledgements

I wish to thank my advisor Rochelle Buffenstein for her guidance, patience and encouragement during my doctoral studies. I would like to also thank Tim P. O'Connor for his co-mentorship during the early years of my project, as well as his continued input and support throughout. In addition, I thank Karen Hubbard for her comments and advice with my work, as well as Carol Boozer, James Greenberg and Mark Pezzano for sitting on my doctoral committee. For helping me cope with the day-to-day life of graduate school, I would like to thank Gina Cantone. Finally, I wish to express my fullest gratitude to my parents for their guidance and for always believing in me.

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CHAPTER 1: BACKGROUND

Aging is a process by which with increasing age after maturity, animals experience progressive declines in physiological functions that eventually lead to death (Kirkwood and Austad, 2000). The aging process does not kill organisms directly, but makes them more frail and increasingly susceptible to pathological processes (Kasapoglu and Ozben, 2001). Considerable variation exists in rates of aging among species, as reflected by the observed differences in species longevity. The widely held Oxidative Stress Theory of Aging offers a proximate mechanism for both physiological declines during aging (Harman, 1956), as well as for the natural variation in lifespan between species (Sohal and Weindruch, 1996). This theory ascribes age-associated physiological declines to a progressive accrual of oxidative damage, and variation in species longevity to disparate rates of damage accrual.

Most traditional model species in aging research are chosen partially because of their short lifespan, allowing investigators to conduct longitudinal studies over the entire lifetime of the model organism. Austad (2001) suggested that an attractive alternative approach to discerning mechanisms of aging is to examine species with exceptional longevity that demonstrate retarded senescence, since these species may possess exceptional anti-aging defenses. One such animal is the naked mole-rat (*Heterocephalus glaber*). These mouse-sized animals are the longest-lived rodent species known, surviving in captivity for over twenty-eight years (Buffenstein and Jarvis, 2002).

1.1 Naked Mole-Rat Biology

Naked mole-rats (NMRs) are the longest-living rodent species on record surviving in captivity for over 28 years (Buffenstein and Jarvis, 2002). Because NMRs have evolved physiological mechanisms that enable them to delay the aging process, they are valuable models for research on aging (Austad, 1993). At the ultimate level, NMR longevity can be explained by the Evolutionary Theory of Aging, which postulates that increased longevity can evolve in species or populations that are subject to low levels of extrinsic mortality (Austad, 1997*a*; Partridge and Mangel, 1999). NMRs live in safe and climatically stable environments, and have evolved specialized physiological adaptations that enhance their ability to survive in their subterranean milieu (Brett, 1991; Buffenstein, 1996). Interest in elucidating the proximate mechanisms responsible for NMR longevity has only recently become the subject of extensive study. A comparative study involving these long-living rodents could be relevant to advancing our understanding of the physiological changes that occur with increasing age, and may reveal the causes for natural longevity variation among species.

Longevity

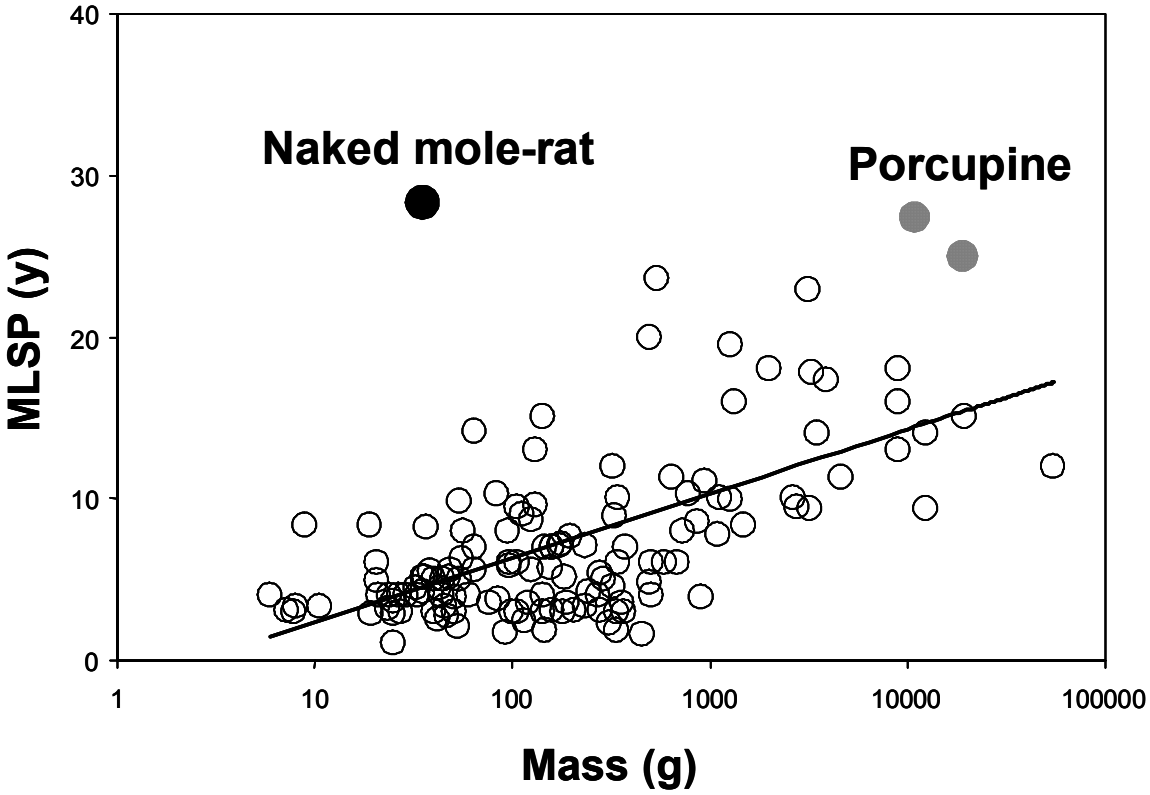
NMRs routinely survive in captivity well in to third decade of life, while in the wild their lifespan reportedly exceeds 17 years (Braude, pers. comm.). Aging studies often utilize the ratio of a species' maximum known lifespan to that predicted by body mass (Longevity Quotient, LQ) to compare the longevities of different species (Austad, 1997*b*). The LQ of NMRs ranges between 5 and 10. These LQ values are similar to those calculated for humans and far greater those of all non-flying mammals (Table 1).

NMRs are the longest-living rodents known. Their longevity is more than two standard deviations greater than predicted by body mass, and only is rivaled by that of much larger porcupines (Buffenstein, 2005, Figure 1).

Table 1) Life-history traits of naked mole-rats compared to those of common mammalian models for aging research (humans, rats and mice), and other small mammals. The Longevity Quotient (LQ) represents observed longevity divided by that predicted by body mass. Two LQ values were calculated for each species based on existing allometric equations [y = 8.15*$M_{kg}^{(0.187)}$ and; b) y = 5.3*$M_{kg}^{(0.174)}$]. Naked mole-rats are only surpassed by certain bat species [(1) McArdle et al., 1991; (2) Prothero and Jurgens, 1987; (3) Hayssen and Lacey, 1985; (4) Jurgens and Prothero, 1987; (5) Hinds et.al., 1993; (6) Haim and Izhaki, 1993; (7) Speakman, 1997; (8) Hinds and Rice-Warner, 1982; (9) Zepelin and Rachtschaffen, 1974; (10) Nowak, 1999; (11) Bozinovic, 1992]

SPECIES	Body Mass (kg)	Maximum Longevity (yrs.)	Longevity Quotient (LQ) [2]
Naked Mole-Rats	**0.040**	**28.3**	**5.0 - 9.6**
Lab Mouse (*M. musculus*)[8,10]	0.032	3.5	0.6 - 1.2
Rat (*R. norvegigus*)[9,11]	0.253	4.7	0.6 - 1.1
Human[1]	62	120	5.2 - 11.2
Pipistrelle[7,10]	0.007	14.8	3.5 - 6.6
Little Brown Bat[4,10]	0.008	30	7.0 - 13.1
Silky Pocket Mouse[3,10]	0.008	8.3	1.9 - 3.6
Deer Mouse[7,10]	0.019	8.3	1.6 - 3.1
Bank vole[8,10]	0.023	4.9	0.9 - 1.8
Red-backed Mouse[7,10]	0.023	4.9	0.9 - 1.8
Mouse-eared Bat[4,10]	0.025	18.6	3.5 - 6.7
Brown Antechinus[3,10]	0.030	2	0.4 - 0.7
Greater Egyptian Gerbil[7,10]	0.032	8.2	1.5 - 2.8
Jamaican Fruit-eating bat[4,10]	0.038	7	1.2 - 2.3
Jerboa Mouse[5,10]	0.039	5.2	0.9 - 1.7
Eastern American Mole[6,10]	0.040	3.5	0.6 - 1.2
Four-striped Grass Mouse[6,10]	0.040	2.9	0.5 - 1.0
Collard Lemming[8,10]	0.047	3.3	0.6 - 1.1
Kangaroo Rat[8,10]	0.049	9.8	1.6 - 3.1
Bush-tailed Jird[6,10]	0.057	5.4	0.9 - 1.7
Brown Lemming[7,10]	0.062	5	0.8 - 1.5
Water Vole[3,10]	0.092	5	0.7 - 1.4
Eastern Am. Chipmunk[7,10]	0.092	8	1.2 - 2.3
Antelope Ground Squirrel[7,10]	0.092	5.8	0.9 - 1.7
Ground Squirrel[9,10]	0.101	9	1.3 - 2.5
Common Mole-Rat[6,10]	0.102	9.6	1.4 - 2.7
Pigmy Marmoset[3,10]	0.117	11.5	1.6 - 3.2
Golden Hamster[9,10]	0.120	3.9	0.5 - 1.1
Sugar Glider[6,10]	0.124	14	1.9 - 3.8
Balding's Squirrel[7,10]	0.290	11	1.3 - 2.6
Eastern Gray Squirrel[3,10]	0.440	23.5	2.6 - 5.1
African Ground Squirrel[6,10]	0.542	6	0.6 - 1.3
European Hedgehog[9,10]	0.785	6	0.6 - 1.2
Squirrel Monkey[3,10]	0.830	20	1.9 - 3.9
Guinea Pig[9,10]	1.040	7.6	0.7 - 1.4
Domestic Rabbit[8,10]	1.242	9	0.8 - 1.6
N. American Oppossum[9,10]	1.700	5	0.4 - 0.9

Figure 1) Relationship between body weight and lifespan of rodents. Figure from Buffenstein (2005).



As they get older, NMRs become more frail and exhibit both morphological and behavioral changes commonly observed in other species (Figure 2). Older individuals are less active than their younger counterparts and spend the majority of their time sleeping (personal observation). The very oldest mole-rats (>26 years) display declines in motor co-ordination that impairs their ability to efficiently move within their systems (Buffenstein, 2005). Age-related morphological changes include reduced skin elasticity that manifests itself in the form of wrinkles (Figure 2; Buffenstein, 2005). However, NMRs fail to display age-related physiological changes commonly observed in other models of aging research (Fukagawa et al., 1990, Cartee, 1995). Gross components of mole-rat body composition do not change with age in a typical mammalian pattern, such that the relative proportions of lean tissue do not decline and those of fat fail to increase, while both mass-specific and lean mass-specific basal metabolic rates appear to remain unaltered throughout the lifetime of this species (Figure 3, O'Connor et al., 2002). In addition, both bone composition and bone density do not change throughout life in these rodents (Buffenstein, 2005). This implies that although naked mole-rats succumb to the aging process, it is slower in this species relative to most other mammals.

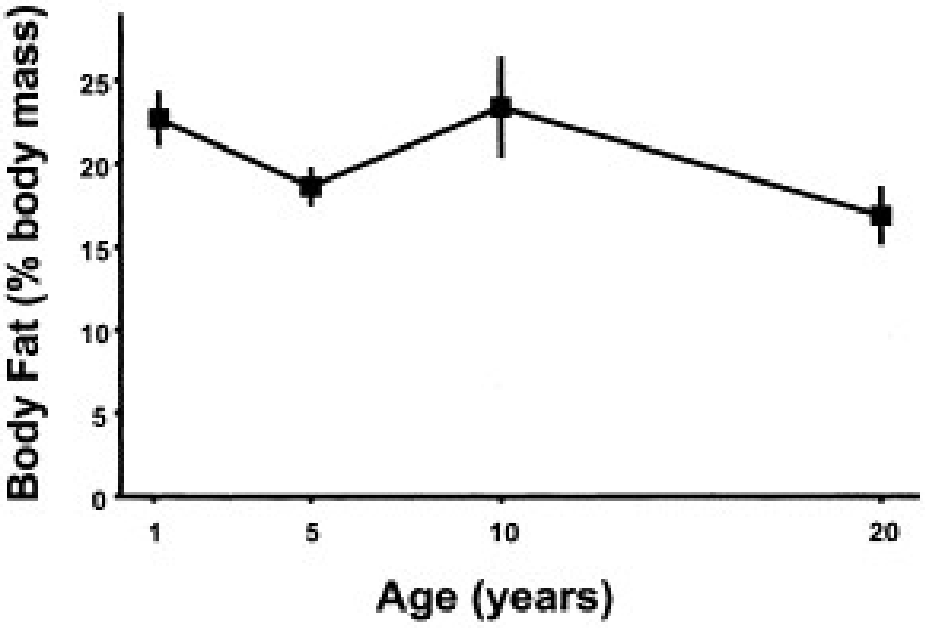
Figure 2) Morphological differences in NMRs of different ages. a) A photograph of a breeding NMR female (5-10 year old) with her juvenile (~0.5-1 year old) offspring. The skin of young and adult animals is taut and hydrated. b) A photograph of a 28 year-old naked mole-rat. This animal is extremely frail and exhibits morphological and behavioral changes commonly associated with aging. His skin is wrinkled and dehydrated and is relatively inactive.

A

B

Figure 3) Absence of age-related changes in body composition and metabolism in NMRs. A) The effect of age on percent body fat of NMRs. No significant differences were detected for the three older (5, 10 & 20 year olds) cohorts. B) The effect of age on basal metabolic rate of NMRs. Both mass-specific (solid) and lean mass-specific (dashed) rates remain unchanged throughout life. Both figures are from O'Connor et al., (2002).

A



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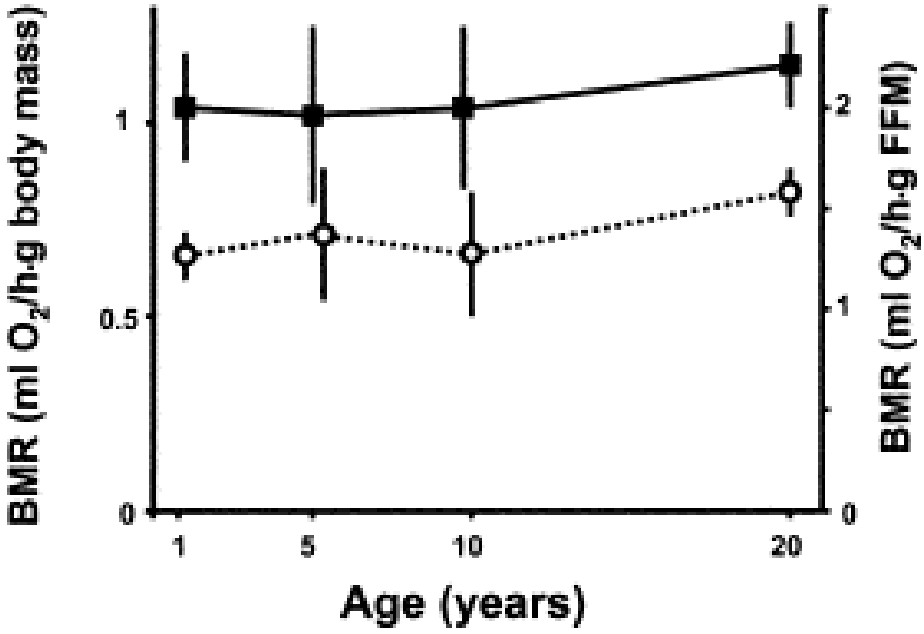

BMR (ml O2/h·g body mass)
BMR (ml O2/h·g FFM)
0
0.5
1
0
1
2
1
5
10
20
Age (years)

Taxonomy and Ecology

NMRs are hystricognath rodents native to the arid and semi-arid regions of tropical Eastern Africa. They are one of the smallest (~40 g) of 18 species that comprise the African mole-rat family Bathyergidae (Buffenstein, 1996; Faulkes and Bennett, 2001). NMRs are eusocial and live in large colonies within extensive tunnel systems that offer protection from predators and climatic extremes (Jarvis, 1981; Brett, 1991). These animals feed exclusively underground, eating patchily distributed roots, tubers and geophytes. This species has evolved a number of adaptations, such as a low basal metabolic rate and low body temperature that allow it to thrive in a physiologically hostile subterranean environment.

Physiology

Life underground poses numerous physiological challenges for NMRs. Their burrows, despite being thermally stable (~29ºC ± 1.5ºC) are humid (RH~90%), hypoxic and hypercapnic, thus impeding gas and heat exchange (Brett, 1991; Jarvis and Bennett, 1991). This species successfully overcomes these respiratory challenges by a reduced body temperature and a metabolic rate two-thirds that predicted by allometry, reducing the need for gas exchange in a hypoxic habitat (McNab, 1988; Buffenstein and Yahav, 1991; Buffenstein, 1996). Both are common features of subterranean mammals and are regarded as well suited to an environment where gas and heat exchange are impaired (McNab, 1988).

NMRs further extend this pattern of a reduced body temperature and metabolic rate by displaying unconventional metabolic and thermoregulatory profiles. Regardless of

ambient temperature, the body temperature of NMRs tracks that of their surroundings. This species shows 2 distinct metabolic patterns in response to changing ambient conditions. At temperatures above 28°C the thermoregulatory profile of NMRs resembles that of other endotherms. They have a distinct thermoneutral zone (31°C to 34°C), below which their metabolic rate increases with decreasing ambient temperature (Buffenstein and Yahav, 1991). NMRs employ non-shivering thermogenesis and do so in response to both cold exposure and pharmacological injection with norepinephrine (Hislop and Buffenstein, 1994). As ambient temperatures decline below 28°C, the thermoregulatory profile of NMRs more closely resembles that of ectotherms (Buffenstein and Yahav, 1991). Regardless of the thermoregulatory profile NMRs are unable to effectively regulate their body temperature, which exceeds that of their environment by only 1°C across a broad range of temperatures (McNab, 1988; Buffenstein and Yahav, 1991). This occurs primarily because naked mole-rats lack insulation and lose most endogenously generated heat across their skin (Daly and Buffenstein, 1998). Consequently, this species must rely on behavioral (e.g. huddling and thigmothermy) strategies to effectively maintain body temperature (Withers and Jarvis, 1980; Yahav and Buffenstein, 1991; Hislop and Buffenstein, 1994; Daly and Buffenstein, 1998). Because of the relative thermal stability of naked mole-rat burrows their metabolic profiles can be considered adaptive by decreasing their resting oxygen, energy and water requirements, as well as by reducing the possibility of thermal death in their sealed burrows (Buffenstein, 1996).

Despite very low resting metabolic rates, naked mole-rats have high metabolic scopes during activity. Energetic demands imposed on them by burrowing in soil result in increases in metabolic rates that are five times greater than resting (Lovegrove, 1986).

Burrowing underground is not only energetically costly but is a random process exacerbated by patchy distribution of food. When food is found, however, NMRs fully exploit all available nutrients as indicated by digestive efficiencies in excess of 90% (Buffenstein, 2000)

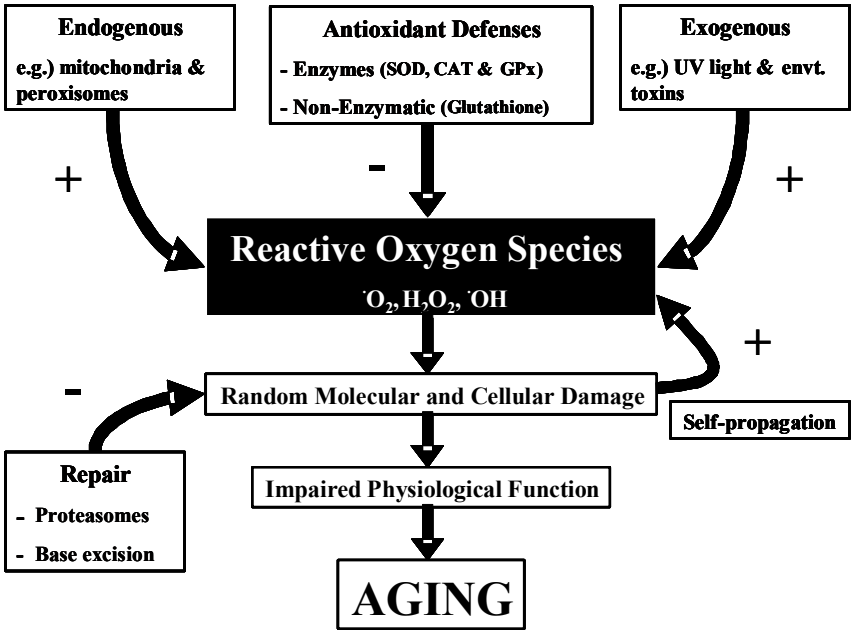
Eusociality

NMRs are eusocial (Jarvis, 1981), living in highly inbred colonies of up to 300 individuals (Honeycutt et al., 1991; Jarvis et al., 1994). There is a single breeding "queen" that behaviorally suppresses reproduction in all other colony members, with the exception of one to three males with whom she breeds (Jarvis et al., 1994). Division of labor is another characteristic of eusociality. Individuals participate in maintenance and defense of the burrow system, as well as in caring for the young (Lacey and Sherman, 1991). Once mature, the offspring are philopatric remaining in their native colonies, such that the colony is comprised of individuals from many generations (Spinks et al., 2000). The evolution of this social system has been explained by the "Aridity Food Distribution" hypothesis, which states that ecological constraints on dispersal and food location should favor philopatry. Therefore, through co-operation individuals decrease the time and energy needed to locate food and thus increase their chances for survival (Spinks et al., 2000).

1.2 Oxidative Stress Theory

The Oxidative Stress Theory offers a testable biochemical mechanism of the aging process (Harman, 1956). This theory postulates that declines in physiological and reproductive function that are characteristic of the aging process result from a progressive accumulation of oxidative damage to various biological molecules (Figure 4). Although the aged phenotype is thought to reflect lifelong oxidative damage accrual, the process that leads to this state involves a number of factors that either favor or prevent damage generation and accumulation.

Figure 4) Mechanism responsible for aging, as proposed by the Oxidative Stress Theory. Aging is thought to result from an accumulation of oxidative damage caused by Reactive Oxygen Species (ROS). Levels of accumulated oxidative damage reflect an imbalance between ROS generation rates and the capacity of organismal defenses to prevent damage formation and accrual. Figure modified from Finkel and Holbrook, (2000).



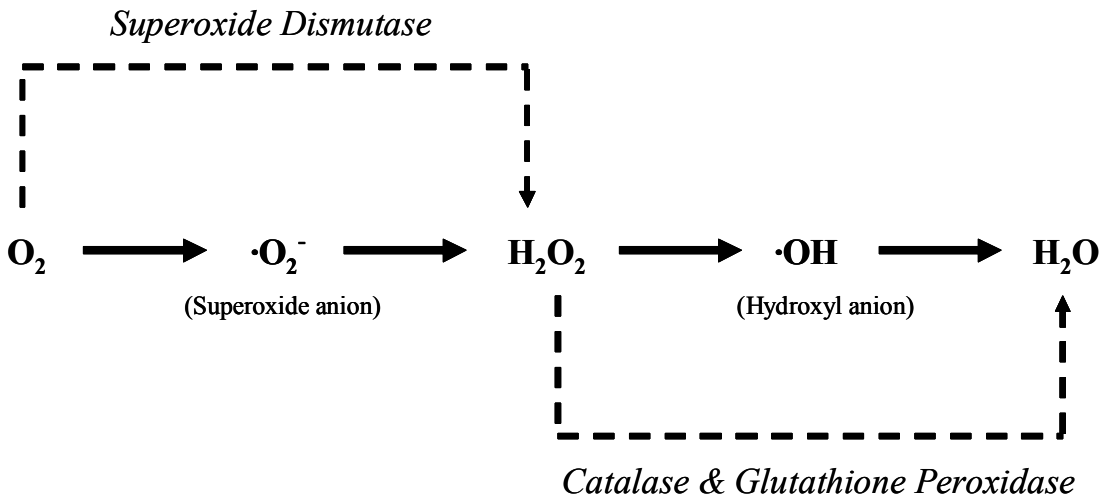
Oxidative damage is caused by endogenously formed reactive oxygen species (ROS), which primarily originate as by-products of normal metabolic activity (Davies, 2000). Specifically, both complex I (NADH Dehydrogenase) and complex III (ubiquinone-cytochrome *c* reductase) of the electron transport chain are considered as the predominant generators of superoxide anions, which are the initial ROS within the oxidative stress pathway (Turrens, 1997). Superoxide is subsequently dismutated by superoxide dismutase into hydrogen peroxide, which may undergo iron or copper catalyzed Fenton-type reaction to form the highly reactive and thus damaging hydroxyl radical (Halliwell and Chirico, 1993). While hydrogen peroxide is sufficiently stable to permeate into the cytosol, ROS concentrations are greatest within the mitochondria. For this reason, it has been suggested that ROS-induced modifications of mitochondrial biological molecules and the resultant impairment of mitochondrial function may be central to organismal declines during aging (Miquel et al., 1980; Harman, 1981; De Gray, 2000; Barja, 2002).

The physiological impact of ROS can be substantial and may involve modifications of lipids, proteins and DNA, as well as result in the altering of intracellular red-ox state. Together these changes may lead to impaired cellular and physiological function with increasing age (Beckman and Ames, 1998a). ROS damage lipids through a sequence of self-propagating peroxidative chain reactions. These modifications can alter phospholipid membrane properties by decreasing membrane fluidity and through disruption of membrane-bound proteins (Halliwell and Chirico, 1993). In addition, lipid peroxidation derivatives (e.g., lipid hydroperoxide, hydroxyalkenals and malondialdehyde) are highly reactive and can further propagate oxidative damage of

various biological molecules (Esterbauer et al., 1981; Marnett, 1999). DNA is modified by ROS through backbone strand breaks and adducts of base and sugar groups (Henle et al., 1996; Helbock et al., 1998). Damage to DNA is significant since its oxidation can ultimately alter the message that it codes for (Bohr, 2002). Mitochondrial DNA damage may lead to mitochondrial defects, and the impaired function that follows results in heightened ROS production (Finkel and Holbrook, 2000). The impact of ROS on proteins is extensive and affects a broad range of their normal functions (Stadtman, 1992; Floyd et al., 2001). ROS-induced protein oxidation can affect enzymatic activities and reportedly lead to age-related declines in activities of both cytosolic (e.g., glucose-6-phosphate dehydrogenase) and mitochondrial (e.g., aconitase) enzymes (Friguet et al., 1994; Yan et al., 1997). The consequence of unchecked reactive oxygen species is thus impaired tissue maintenance and repair, with a concomitant and irreversible decline of physiological processes.

Organisms are equipped with a diverse antioxidant system that serves to counter the generation and activities of ROS (Beckman and Ames, 1998*a*). Both enzymatic and non-enzymatic in nature, antioxidants restrict ROS generation or scavenge ROS, converting them into less reactive molecules (Figure 5) (Navarro et al., 1998). Examples of enzymatic antioxidants include superoxide dismutase (SOD), which converts superoxide anions into hydrogen peroxide (McCord et al., 1971). The hydrogen peroxide can then be metabolized by two other antioxidant enzymes, catalase (CAT) and glutathione peroxidase (GPx), back to oxygen and water (Chiu et al., 1976; Fridovich, 1978). Rates of damage generation thus reflect the imbalance between ROS production and antioxidant defenses (Sohal et al., 2002).

Figure 5) Modes of action of enzymatic antioxidants. Three main enzymatic antioxidants (superoxide dismutases, catalases and glutathione peroxidases) convert reactive oxygen species into more benign forms. Antioxidant action is both parallel and serial. Figure from Del Maestro, (1981).



Studies on individual species have failed to produce a consensus regarding trends in antioxidant activities with increasing age (Sohal et al., 1995; Sohal et al., 2002). Results appear contingent on the particular species studied, as well as on the individual tissues assayed. The activities of catalase and glutathione peroxidase reportedly increase, decline or remain unchanged with increasing age in different mammalian species (Ji et al., 1990; Carrillo et al., 1992; Lass et al., 1998; Panserasa et al., 1999; Sverko et al., 2002). Age-associated trends in superoxide dismutase (SOD) depend on whether this enzyme is assayed in its mitochondrial (manganese) form, its cytosolic (copper/zinc) form or as a total of the two (Ji et al., 1990; Mo et al., 1995; Sohal et al., 1995; Panserasa et al., 1999). Despite the inconsistencies in antioxidant data, levels of oxidative damage to biological molecules have been reported to increase with age in humans, mice, rats and gerbils (Salminen et al., 1988; Sohal et al., 1995; Panserasa et al., 1999; Miro et al., 2000; Hamilton et al., 2001*b*).

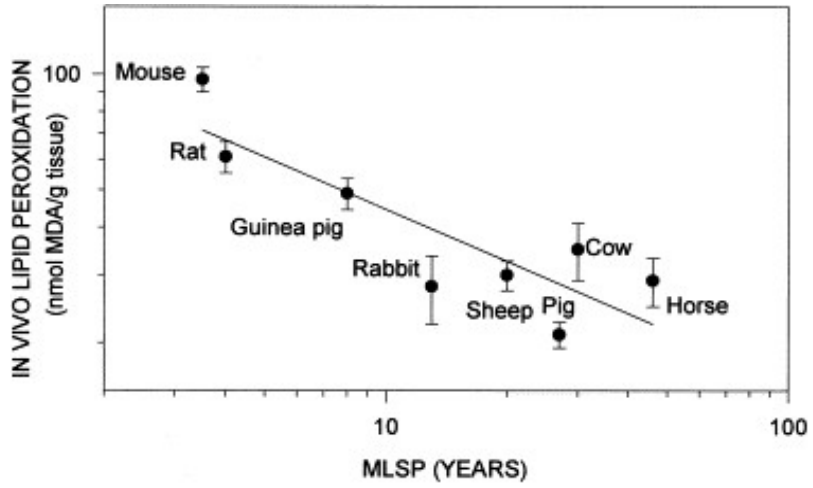
Organismal repair mechanisms breakdown and remove incurred oxidative modifications, thus limiting levels of accumulated damage in tissues (Davies, 2000). Damaged proteins are removed through the enzymatic degradation by lysosomal/autophagic and the ubiquitin proteasome systems (Martinez-Vicente et al., 2005). Base Excision Repair (BER) and Nucleotide Excision Repair (NER) pathways are responsible for removing oxidatively-induced DNA lesions (Bohr et al., 2002; Cooke et al., 2005). The DNA repair systems rely on a variety of enzymes (e.g., DNA N-glycosylases, such as oxoguanine DNA glycolase 1 or its mitochondrial isoform mitochondrial Oxidative Damage Endonuclease), to recognize, cleave and replace the damage. Capacity for protein and DNA damage repair reportedly declines with age

(Agarval and Sohal, 1994; Stevnsner at al., 2002; Szczesny et al., 2004; Ferrington et al., 2005). Therefore, even in the absence of an attenuated antioxidant suite, age-associated declines in damage repair may be responsible for rapid increases in accrued oxidative damage levels, which reportedly occur during the last third of the lifespan (Levine and Stadtman, 2001; Hamilton et al., 2001*a*).

Within the context of the Oxidative Stress Theory, lifespan differences between species result from unequal oxidative damage accumulation. Indeed, comparative studies have shown that oxidative damage accrual is negatively correlated with species longevity (Figure 6, Barja and Herrero, 2000; Pamplona et al., 2000; Ruiz et al., 2005). Causes for differences in damage accumulation can include disparate rates of ROS generation, disparate antioxidant suite and/or differential damage repair capacity (Sohal and Weindruch, 1996).

Figure 6) Relationship between levels of accrued oxidative products and species longevity (MLSP) in the heart of eight mammalian species. Data from previous comparative studies suggest that there is an inverse relationship between levels of accrued oxidative damage in tissues and species longevity. This relationship appears to hold true for assays of; A) lipid peroxidation (malondialdehyde, MDA; Pamplona et al., 2000), B) protein oxidation (malondialdehydelysine, MDAL; Ruiz et al., 2005) and C) DNA damage (8-hydroxy-deoxyguanosine, 8-oxodG; Barja and Herrero, 2000). All comparisons were made between physiologically young individuals.

A



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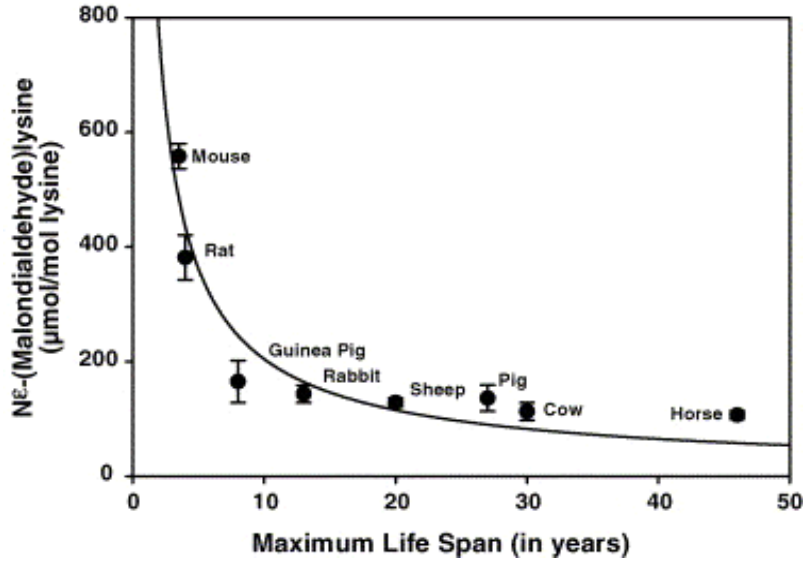
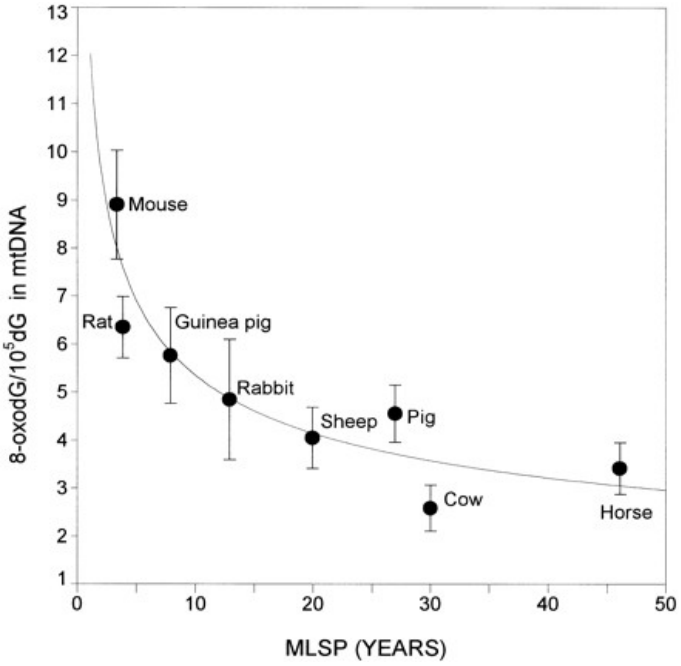

800
600
400
200
0
Nε-(Malondialdehyde)lysine (µmol/mol lysine)
Mouse
Rat
Guinea Pig
Rabbit
Sheep
Pig
Cow
Horse
0
10
20
30
40
50
Maximum Life Span (in years)

C


13
12
11
10
9
8
7
6
5
4
3
2
1
8-oxodG/10^5dG in mtDNA
Mouse
Rat
Guinea pig
Rabbit
Sheep
Pig
Cow
Horse
0
10
20
30
40
50
MLSP (YEARS)

Differences in species longevity could reflect disparate rates of oxidant generation. Long-lived species have a higher total metabolic output and thus higher lifetime energy expenditures, and thus over the course of their lifetime they would be expected to generate considerably more ROS than shorter-lived species (Perez-Campo et al., 1998). Broad comparative studies have shown, however, that per unit time, long-lived species generate fewer ROS than their short-lived counterparts (Sohal et al., 1990; Ku and Sohal, 1993; Perez-Campo et al., 1994; Brunet-Rossinni, 2004). These results have led some to postulate that reduced oxidative damage accumulation and thus extended longevity, is primarily a consequence of low ROS generation rates (Perez-Campo et al., 1998; Barja, 2002).

Differences in oxidative stress resistance may also account for lifespan variation among species. This notion has been supported by data showing that cells of long-lived species (e.g., humans or budgerigars) display a greater resistance to oxidative stress when compared to those of shorter-lived species like mice (Ogburn et al., 1998; Kapahi et al., 2000). The relationship between antioxidant capacity and species lifespan is, however, uncertain with reports of contrasting results. Some studies have shown that antioxidant capacity is positively correlated with species longevity (Tolmasoff et al., 1990; Ku and Sohal, 1993; Sohal et al., 1993). For example, a comparative study between white-footed mice (*Peromyscus leucopus*) and house mice (*Mus musculus*) found that the longer-lived white-footed mice (~8 years) had catalase and glutathione peroxidase activities that were twice those of house mice (~4 years) (Sohal et al., 1993). Conversely, others have demonstrated an inverse relationship between antioxidant activities and lifespan, with short-lived species (e.g., rat) having higher antioxidant capacities than long-lived species

(e.g., pigeon) (Perez-Campo et al., 1994). Based on these conflicting results it has been suggested that antioxidant activities are not the primary determinants of species longevity (Perez-Campo et al., 1998). Alternatively, variation in species longevity could exist due to differential levels of damage repair and not from differences in antioxidant capacities. Comparative studies have indeed shown a positive correlation between DNA damage repair and species lifespan (Grube and Burkle, 1992; Foksinski et al., 2004). While research exploring this relationship has been limited, greater damage repair may be responsible for elevated oxidative stress tolerance in long-living species, and thus contribute to their prolonged longevity.

1.3 Specific Aims

The central aim of my doctoral work was to test the Oxidative Stress Theory by determining if it can adequately explain the 9-fold difference in maximum lifespan between long-living naked mole-rats (28.3 years) and similarly-sized mice (~3.5 years). According to this theory, delayed rates of aging observed in NMRs, when compared to mice, are a consequence of species differences in the oxidative stress pathway that lead to lower damage accrual in this long-living rodent. Similarly, age-related changes in components of the oxidative stress pathway are likely to differ between NMRs and mice, leading to distinct profiles of damage accumulation during aging in these two species. I addressed these issues by comparing levels of various biochemical biomarkers in tissues and urine of physiologically age-matched mice and naked mole-rats at different stages of life. The specific aims of my project were the following:

a) To compare antioxidant defenses in mice and NMRs and therefore determine if the long-living NMRs possess a superior antioxidant suite than shorter-living mice.
b) To assess if NMRs have lower levels of oxidative stress than mice and if they are thus less likely to generate oxidative damage.
c) To compare oxidative damage repair in these two species and therefore ascertain if NMRs are better able to remove incurred damage.
d) To measure oxidative damage generation in NMRs and mice and thus establish if NMRs produce less damage.
e) To quantify levels of accrued oxidative damage to various biological molecules, and thus establish if damage content is lower in NMR tissues.
f) To compare age-related profiles of damage generation and accrual in NMRs and mice and therefore asses they differ in these two species during aging.

CHAPTER 2: ANTIOXIDANTS DO NOT EXPLAIN THE DISPARATE LONGEVITY BETWEEN MICE AND THE LONGEST-LIVING RODENT, THE NAKED MOLE-RAT.

(This work is currently published. Andziak B, O'Connor TP, Buffenstein R, 2005. Mechanisms of Ageing and Development. 126, 1206-1212). Reprinted with permission.

2.1 Abstract

The maximum lifespan of naked mole-rats (NMRs; *Heterocephalus glaber*) is greater than that of any other rodent. These hystricognaths survive in captivity >28 years, 8-times longer than similar-sized mice. The present study tested if NMRs posses superior antioxidant defenses compared to mice and if age-related interspecies changes in antioxidants were evident Activities of Cu/Zn superoxide dismutase (Cu/Zn SOD), Mn SOD, catalase and cellular glutathione peroxidase (cGPx) were measured in livers of physiologically equivalent age-matched NMRs (30, 75 and 130 months) and CB6F1 mice (4, 12 and 18 months). In mice, Mn SOD activity increased with age, while the activity of catalase and cGPx declined. None of the antioxidants changed with age in mole-rats. cGPx activity of NMRs was 70-times lower ($p<0.0001$) than in mice, and resembled that of cGPx knock-out animals. NMRs may partially compensate for the lower cGPx when compared to mice, by having moderately higher activities of the other antioxidants. It is nonetheless unlikely that antioxidant defenses are responsible for the 8-fold longevity difference between these two species. Maintenance of constant antioxidant defenses with age in NMRs concurs with previous physiological data, suggesting delayed aging in this species.

2.2 Introduction

Maximum lifespan potential (MLSP) is positively correlated with body size, such that large animals live considerably longer than smaller species, although the slope and intercepts of these allometric relationships vary among different phylogenetic groups (Prothero and Jurgens, 1987). Tremendous variability in MLSP exists around this descriptor, such that for their size humans, NMRs and bats are extremely long-lived, living ~5-10 times longer than predicted by mass (Prothero and Jurgens, 1987; Austad and Fischer, 1991), while opossums live ~0.5 times that expected by size (Austad and Fischer, 1991; Buffenstein and Jarvis, 2002).

NMRs (*Heterocephalus glaber*) are mouse-sized (~ 40 g) subterranean hystricognath rodents endemic to northeast Africa (Kingdon, 1974). These small mammals can survive in captivity for more than 28.3 years (Buffenstein, 2005) and even in the wild longevity reportedly exceeds 17 years (S. Braude, pers. comm.). This extraordinary MLSP is even greater than that reported for much larger porcupines, making NMRs the longest-living rodent species known (Buffenstein and Jarvis, 2002). NMRs live in large underground colonies that are protected from predation and climatic extremes, which likely lead to low levels of extrinsic mortality in this species. In addition, these small mammals are eusocial, exhibiting a division of labor in the colony that includes cooperative foraging and care of young (Jarvis, 1981). The social structure and underground existence of naked mole-rats would be predicted to result in stronger selection against the effects of deleterious genes expressed later in life, ultimately leading to their impressive longevity through the evolution of mechanisms that protect and maintain physiological function (Kirkwood and Austad, 2000).

Proximate mechanisms, that enable extended longevity of long-lived organisms and in particular of NMRs, remain to be elucidated. One commonly held proximate theory that provides a biochemical mechanism for aging is the Oxidative Damage theory (Harman, 1956; Beckman and Ames, 1998*a*). It proposes that aging is caused by oxidative damage to molecular and cellular components, and that differences in MLSP may be due to disparate rates of damage accrual among species. Damage should accumulate when there is an imbalance between the amount of endogenously generated reactive oxygen species (ROS) and the ability of organisms to prevent and/or repair ROS-induced modifications (Finkel and Holbrook, 2000). Oxidative damage may in turn lead to irreversible declines in physiological function - the characteristic feature of aging that makes older organisms more frail and susceptible to fatal pathological processes such as cancer, cardiovascular disease and neurodegeneration (Ames et al., 1993; Beal, 2002).

Antioxidants reduce damage by scavenging ROS and converting these to less reactive and destructive molecules. Principal enzymatic antioxidants include various superoxide dismutases (SOD), catalases and glutathione peroxidases, which "may function both in sequence and in parallel" (Beckman and Ames, 1998*a*). Specifically, SOD converts superoxide anions into hydrogen peroxide (McCord et al., 1971), which is then metabolized by either catalase (Fridovich, 1978) or glutathione peroxidase (Chiu et al., 1976) to oxygen and water. The effect of variation in antioxidant activity as a determinant of MLSP is controversial (Sohal and Weindruch, 1996; Barja 2002), with reports of positive, negative and/or no correlation between antioxidant activity and lifespan (Sohal et al., 1993; Perez-Campo et al., 1994; Brunet-Rossini, 2004). Similarly, the assertion that aging per se results in reduced antioxidant capacity and thus a

progressive decline in biochemical defenses in older organisms has been criticized (Rikans and Hornbrook, 1997). These equivocal findings have raised debate regarding the functional significance of age-related modifications in antioxidant activity, as well as the relative contribution of these biochemical defenses to determining MLSP (Beckman and Ames, 1998; Barja, 2002).

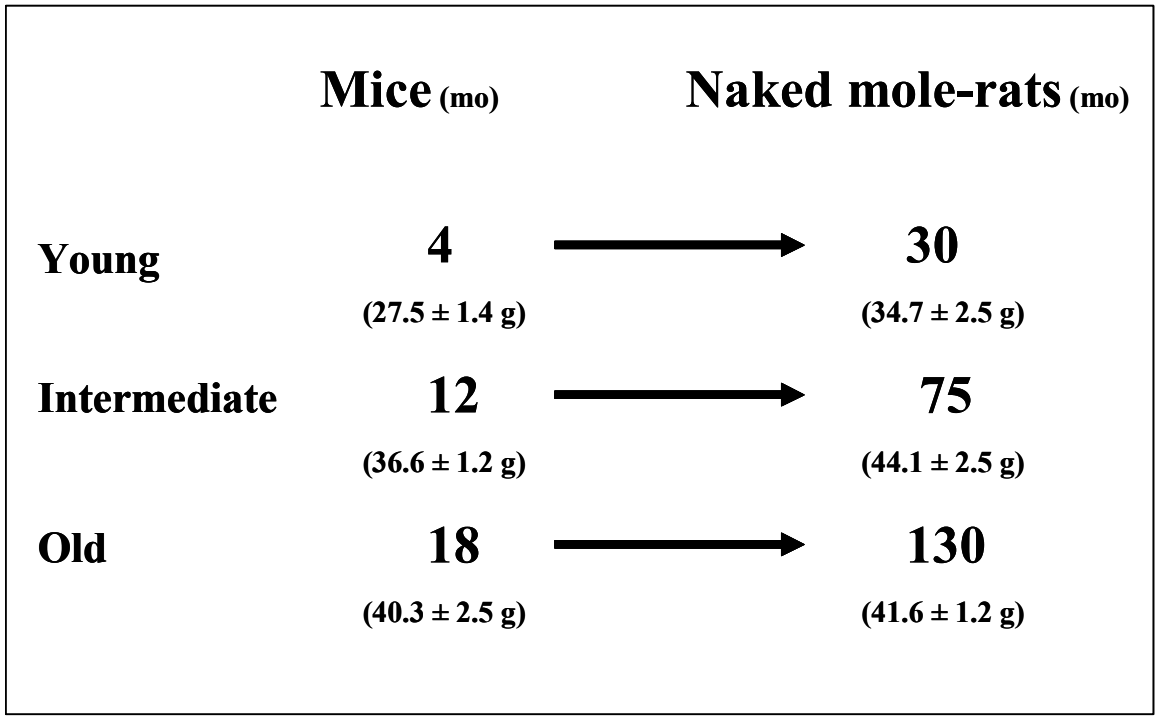
We addressed both these issues in a study comparing two similar-sized rodent species with disparate longevity, the naked mole-rat (MLSP >28.3 years) and CB6F1 mouse (MLSP ~3.5 years). Activities antioxidant enzymes (cellular glutathione peroxidase, catalase, Mn SOD and Cu/Zn SOD) were quantified in liver tissues of mice and NMRs at three physiologically equivalent ages, (young, middle aged and older adults: see methods). We hypothesized that given the role of antioxidants in neutralizing ROS, NMRs would have higher antioxidant activities than mice, and that any age-associated declines in antioxidants would be attenuated in mole-rats when compared to mice.

2.3 Materials and methods

Animals

Activities of antioxidant enzymes were compared in male mole-rats and CB6F1 mice (*Mus musculus*). Three age cohorts were specifically chosen in accordance with the guidelines outlined by Miller and Nadon (2000), to represent these two species at physiologically equivalent ages (see Figure 1). All animal handling procedures were reviewed and approved by the City College of New York (CCNY; New York, NY) IACUC.

Figure 1) Age cohorts of animals used in this study. The three cohorts were determined as proportions of their MLSP; such that the young animals are adults just beyond puberty and old cohorts were typical of older individuals whose survival was still over 90% (Miller and Nadon, 2000). The intermediate age cohorts represent animals during the middle stages of their adult life. Average cohort body weights are in parentheses ± SEM.



NMRs were born in captivity and maintained in colonies at CCNY. The parental stock originated from animals captured in Kenya in 1980. Animals were housed in simulated, multi-chambered burrow systems under constant climatic conditions that aimed to approximate their native habitat (30°C; 75% RH). NMRs were given an ad lib supply of fruit and vegetables (apples, butternut squash, corn, grapes and green beans, carrots, lettuce and yams), supplemented with a high protein and vitamin enriched cereal (Pronutro, South Africa).

Hybrid CB6F1 mice were purchased from Charles River Laboratories (Wilmington, MA) and from the National Institute of Aging (NIA; Bethesda, MD). The two younger age groups of mice were purchased at 7 weeks of age and housed at the CCNY until they reached the appropriate ages for this study. Animals were maintained in standard mouse cages (4 animals per cage) on a 12:12 hour light/dark cycle and on an ad lib diet of mouse chow (5001 Rodent Diet, PMI Nutrition International) and water. The oldest mice were obtained at ~18 months of age from the NIA.

Tissue collection and preparation

Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (60mg/kg). For each age cohort ten naked mole-rats, and six mice were killed and livers harvested. Tissues were dissected out within 5 minutes of injection and rinsed in ice-cold Ringer solution, prior to being sub-divided for different biochemical assays and flash frozen in liquid nitrogen for storage at -80°C.

The protocols for preparing samples were similar for all assays. Sample preparation was performed on ice and all centrifugation was done at 4°C. Liver sub-

samples were minced with a blade and then rinsed once in ice cold 0.9% NaCl with 0.16 mg/mL of heparin and once in their respective homogenizing buffer. Samples were homogenized manually on ice in a glass Duall mortar using a Teflon pestle.

For Cu/Zn SOD assays livers were homogenized in 10 mM potassium phosphate buffer (pH = 7.4 at 4°C) containing 5 μM EDTA and 30 mM KCl (1:10, w/v), followed by a 10 minute centrifugation at 8,500 g. The resulting supernatants were retained. Mn SOD samples were homogenized in the same buffer as above (1:4, w/v), however the homogenates were centrifuged first at 800 g for 10 minutes, with the resulting supernatants centrifuged again at 20,000 g for 10 minutes. Mitochondrial pellets were re-suspended in homogenization buffer. Liver sub-samples assayed for catalase activity were homogenized in 20 mM potassium phosphate buffer (pH = 7.4 at 4°C) containing 5 mM EDTA (1:5, w/v). Homogenates were centrifuged at 10,000 g for 12 minutes and resulting supernatants collected. For glutathione peroxidase assays, livers were homogenized in 50 mM Tris-HCl buffer (pH = 7.5 at 4°C) containing 5 mM EDTA and 1 mM 2-mercaptoethanol (1:6, w/v). These were centrifuged at 8,000 g for 12 minutes and the supernatant was retained. All homogenization products were kept frozen at - 80°C until time of assay. All chemicals were obtained from Sigma (St. Louis, MO).

Antioxidant assays

Antioxidant activities are expressed per mg of protein. Total protein content of the samples was determined using the Pierce BCA Protein Assay (Pierce, Rockford, IL).

Activity of both Cu/Zn and Mn superoxide dismutase was quantified using the BIOXYTECH SOD-525 (OxisResearch, Portland, OR) assay. Immediately prior to the

Cu/Zn SOD assay, all Mn SOD and Fe SOD present in the respective samples were inactivated by an ethanol/chloroform (62.5/37.5 (v/v)) extraction (manufacturer's instructions). Mn SOD samples were sonicated (Microson, Misonix, Farmingdale, NY) on ice (3 times for 10 seconds, with a 20 second pause between each sonication) prior to assay. The SOD assay is based upon change in absorbance (525 nm) that results from an increased rate of SOD-mediated autoxidation of 5,6,6a,11b-trihydroxybenzo[c]flourene under alkaline conditions. Both types of samples were pre-treated (1 minute) with 1,4,6-trimethyl-2-vinylpyridinium to remove potentially interfering mercaptans. The ratio of autoxidation of 5,6,6a,11b-trihydroxybenzo[c]flourene in the presence (sample; VS) and absence (water blank; VC) of SOD was used to determine SOD activity (U) .

Catalase activity was determined using the BIOXYTECH Catalase-520 (OxisResearch, Portland, OR) assay. Samples were initially incubated for 1 minute in the presence of hydrogen peroxide (H2O2, 10 mM), and the remaining H_2O_2 was established via a coupling reaction of 4-aminophenazone and 3,5-dichloro-2-hydroxybenzenesulfonic acid in a horseradish peroxidase catalyzed reaction. Absorbance of the resulting quinoneimine dye was measured at 520 nm. At the time of assay, values for a set of standards were recorded and used to generate a curve from which sample catalase activity (U) was calculated.

Activity of cellular glutathione peroxidase (cGPx) was quantified using the BIOXYTECH c-GPx-340 (OxisResearch, Portland, OR). This method measures the rate of cGPx catalyzed oxidation of reduced glutathione (GSH) in the presence of tert-butyl hydroperoxide. Other constituents of the reaction mixture, glutathione reductase and NADPH, maintain a constant GSH concentration through a glutathione reductase

catalyzed reaction of oxidized glutathione (GSSG) in the presence of NADPH. The activity of cGPx (mU) is proportional to a decline in absorbance at 340 nm of the reaction mixture that accompanies the oxidation of NADPH to $NADP^{+}$.

Statistical Analyses

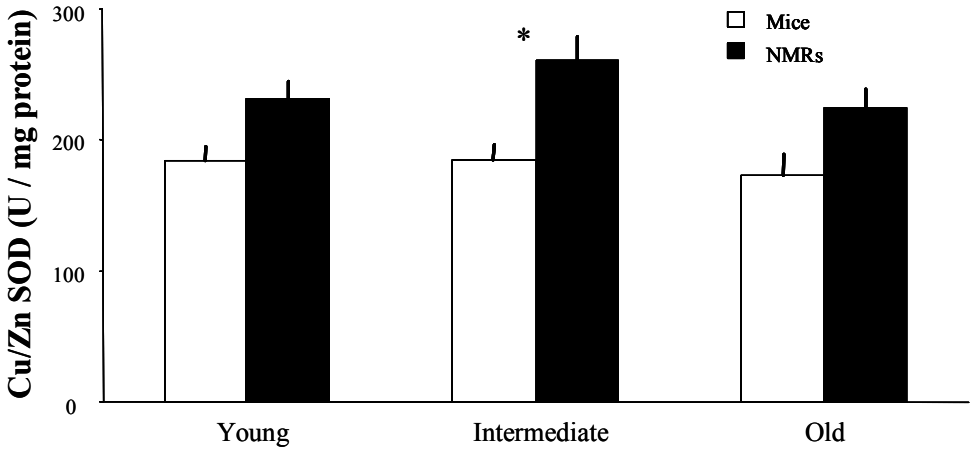
Statistical analyses were performed using NCSS Version 97 (Kaysville, UT). Results are presented as means ± standard error of means (SEM) for all values. The effects of age and inter-specific differences in antioxidant activities were tested for significance using a two-way analysis of variance (ANOVA), with age group and species as independent factors, followed by the Tukey-Kramer multiple-comparison test. Statistical significance for all analyses was $p<0.05$.

2.4 Results

Cu/Zn Superoxide dismutase

There was no significant effect of age, nor an interaction effect of age and species (Figure 2) for Cu/Zn SOD activity in mice and NMRs. However, a significant species effect ($p < 0.0001$) was found with an overall 1.35 fold higher Cu/Zn SOD activity in NMRs (241.4 ± 9.4 U/mg) than in mice (180.5 ± 7.2 U/mg protein).

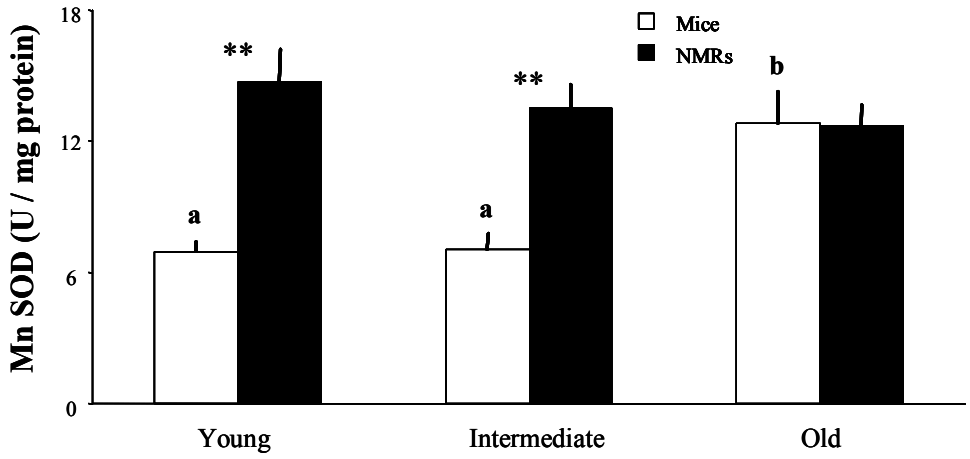
Figure 2) Activity of Cu/Zn superoxide dismutase (Cu/Zn SOD) in liver homogenates of mice and naked mole-rats at different ages. Cu/Zn SOD activity remained constant throughout adulthood in these two species, although its levels were greater in naked mole-rats than in mice. Results are means ± SEM. Significant differences between age-matched mice and naked mole-rats: * $p < 0.05$.



Mn Superoxide dismutase

The interaction of age group and species had a statistically significant effect ($p < 0.01$) on Mn SOD activity (Figure 3). In the young and intermediate age groups, Mn SOD activity was significantly greater in NMRs (14.7 ± 1.5 U/mg and 13.9 ± 1.2 U/mg, respectively, for young and intermediate), than in mice (young, 7.0 ± 0.5 U/mg and intermediate, 7.0 ± 0.8 U/mg). There was no difference in activity between the two species in the older cohort (NMRs, 12.6 ± 1.1 U/mg and mice, 12.9 ± 1.4 U/mg). The significant interaction effect results from the fact that there was an age-associated increase in Mn SOD activity in mice, but not in NMRs (Figure 3).

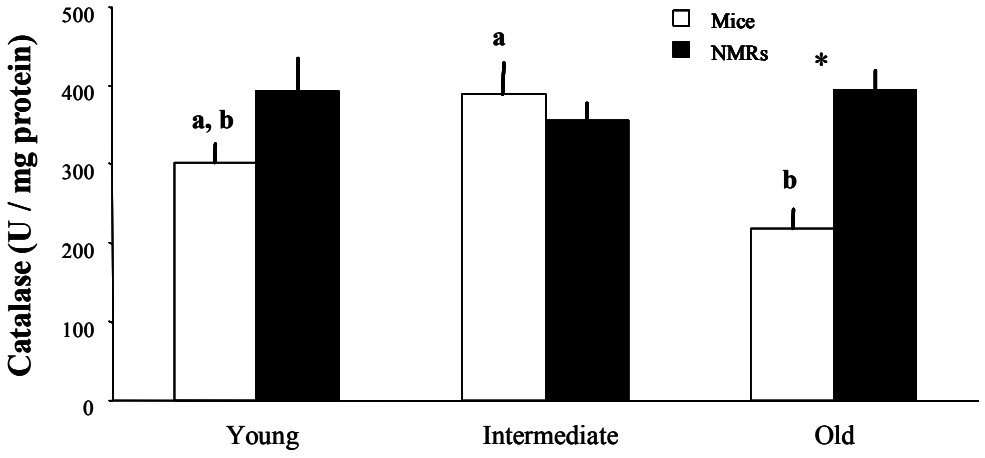
Figure 3) Activity of Mn superoxide dismutase (Mn SOD) in liver homogenates of mice and naked mole-rats of different ages. The age-related pattern of activity differed in naked mole-rats and mice. Mn SOD activity remained constant in naked mole-rats and increased in mice. Results are means ± SEM. Significant differences between age-matched mice and naked mole-rats: ** $p < 0.01$. "a" and "b": mouse group means that do not share a common superscript are significantly different. ($p < 0.05$).



Catalase

As observed for Mn SOD, the interaction effect of age group and species was statistically significant ($p < 0.05$) for catalase activity (Figure 4). This resulted from disparate values at the intermediate age group of both species. Catalase activity was highest in this age group for mice and lowest for NMRs (Figure 4). Furthermore, catalase activity of old mice (218.5 ± 25.0 U/mg) was significantly lower than that of the intermediate age group (390.0 ± 39.4 U/mg), while there were no significant differences in the three mole-rat cohorts. Catalase activity of old NMRs (395.0 ± 29.2 U/mg) was significantly higher than that of old mice.

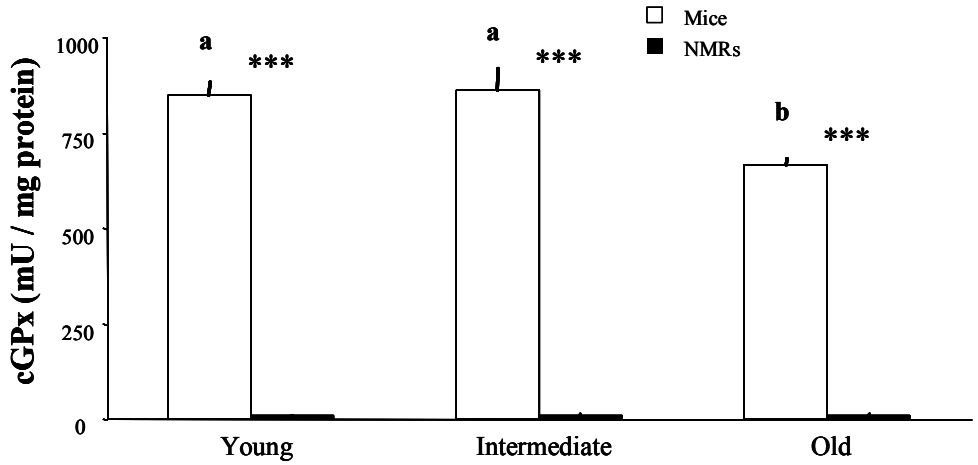
Figure 4) Activity of catalase in liver homogenates of mice and naked mole-rats of different ages. Mice and naked mole-rats displayed disparate age-associated patterns of catalase activity. While mole-rats maintained catalase activity constant, in mice it was reduced in the old cohort. Results are means ± SEM. Significant differences between age-matched mice and naked mole-rats:* $p < 0.05$. "a" and "b": mouse group means that do not share a common superscript are significantly different. ($p < 0.05$).



Cellular glutathione peroxidase

The age group by species interaction effect also had a statistically significant effect on cellular glutathione peroxidase activity ($p < 0.001$; Figure 5). The significant interaction effect is due to mice showing a statistically significant decline with age in cGPx activity (850.2 ± 34.1, 861.1 ± 59.5 and 666.5 ± 17.5 mU/mg in the young, intermediate and old cohorts, respectively) that was not found in NMRs. However, the result with the greatest biological significance is the observation that cGPx activity in NMRs (9.9 ± 1.3 mU/mg), which does not show any age-associated changes, is ~70 times less than that of mice across ages.

Figure 5) Activity of cellular glutathione peroxidase (cGPx) in liver homogenates of mice and naked mole-rats of different ages. Age-associated patterns of cGPx activity differed in these two species, such that in mole-rats cGPx remained constant and in mice it declined with age. More importantly, however, cGPx activity of naked mole-rats was near absent and far lower than that of mice across all ages. Results are means ± SEM. Significant differences between age-matched mice and naked mole-rats: *** $p < 0.001$. "a" and "b": mouse group means that do not share a common superscript are significantly different. ($p < 0.001$).



2.5 Discussion

Relative inter-species differences in antioxidant activities may contribute to observed differences in MLSP among species (Beckman and Ames, 1998). Our study tested this premise by comparing antioxidant defenses of NMRs and shorter-lived mice. In addition, we assessed if antioxidant defenses are maintained throughout life in both these species. Antioxidant activity differed between species: cGPx activity of NMRs was ~70 times lower than in mice, while the activities of the three other antioxidants were moderately greater in NMRs than in mice, although this was only true for certain age cohorts. Antioxidant activities of NMRs did not change with age, where as those of mice showed contrasting age-related patterns. Catalase and cGPX in mice liver homogenates declined with age, while Mn SOD was elevated in the oldest mouse cohort. These data support the premise that antioxidant enzymes are regulated independent of each other and that age-related patterns of activity are both enzyme and species specific (Rikans and Hornbrook, 1997).

Surprisingly cGPx activity in NMRs was 0.014x that of mice. Indeed, cGPx activity of NMRs was quantitatively similar to that of selenium (Se) deficient (Weitzel et al., 1990) and cGPx knockout (Ho et al., 1997) mice. Tissues of both Se deficient and cGPx knockout mice are more susceptible to oxidative stress when compared to wild-type animals (de Haan et al., 1998; Fu et al., 1999) and this appears to be the case for mole-rats. They exhibit significantly higher levels of oxidative damage than mice in all biomolecules (lipids, proteins and DNA) measured to date (Andziak et al., 2004; Buffenstein 2005). Although cGPx knockout mice reportedly do not compensate for their lack of cGPx by elevating catalase or SOD activities, they nonetheless show normal

survival under laboratory conditions and effectively cope with hyperoxia (Ho et al., 1997). Similarly, NMRs have evolved in a hypoxic underground habitat (Buffenstein, 2000) and do not appear to be harmed by their captive lifestyle under relatively hyperoxic laboratory conditions, given their extraordinary longevity.

Low thyroid hormone concentration is another physiological feature shared by NMRs and Se deficient mice (Arthur et al., 1990; Buffenstein et al., 2001). This may partially reduce ROS generation by lowering metabolic rate. It is, however, difficult to establish if these two common features between NMRs and Se deficient mice reflect a nutritional Se deficiency in captivity, or if it is an evolved trait of altered features in the biochemical cascade.

cGPx activity is responsive to environmental cues, especially during early development (Frank and Groseclose, 1984; Buzadzic et al., 1992). For instance, cGPx activities in squirrels that hibernate are elevated during winter. Increase in cGPx during hibernation is speculated to afford protection during the metabolically demanding periods of arousal. Similarly, when animals are in arrested states in response to dehydration and anoxic stress, cGPX is elevated, (Storey, 1996). In addition, cGPX is reportedly "dispensible in unstressed animals" (Brigelius-Flohe, 1999) and elevated in tissues of animals subjected to marked fluctuations in oxygen availability or usage (Selman et al., 2000; Hermes-Lima and Zenteno-Savin, 2002). Near-absent cGPx activity in NMRs may concur with this premise: NMRs live in a thermally stable warm milieu and expend very little energy on thermoregulation (Buffenstein and Yahav, 1991). These animals have extremely low basal metabolic rates, labile body temperatures and rely heavily upon ectothermic thermoregulatory mechanisms such as huddling in tight groups and basking

in warm areas of the burrow (Yahav and Buffenstein, 1991). Animals that are highly dependant upon endothermic heat generation have higher levels of glutathione peroxidase than ectotherms, with cGPx values for chickens and rats two- and seven- times greater respectively than that of lizards and frogs (Venditti et al., 1999).

Lack of cGPx activity in NMRs may be due to phylogeny, for cGPx activity of guinea pigs (*Cavia porcellus*), another hystricognath species, is also reduced when compared to mice (Sohal et al., 1990; Himeno et al., 1993). Low cGPx activity of guinea pigs is offset by catalase and superoxide dismutase activities that exceed those of mice 5-10 fold (Sohal, 1990; Himeno et al., 1993). Furthermore, guinea pig catalase has a more widespread cellular distribution than in most other mammals, and is abundantly present in both peroxisomes and cytosol (Himeno et al., 1993; Brigelius-Flohe, 1999). Guinea pigs have unimpaired H2O2 detoxification capacity and do not accrue inordinate amounts of oxidative damage (Barja and Herrero, 2000; Pamplona et al., 2000). While both NMRs and guinea pigs share a similar pattern of antioxidant activity when compared to mice, mole-rat antioxidant activities are one quarter those of guinea pigs. NMRs, nevertheless, live four times longer than their much larger relatives. This suggests that similarly low cGPx activity in these two hystricognaths reflects shared phylogeny and is not a trait associated with species longevity.

NMRs may partially compensate for the near-absence of cGPx activity by augmenting other antioxidant enzymes activities. In each of the three age cohorts, at least one of the other antioxidants had a higher activity in NMRs than in mice. These differences were moderate relative to the disparity in cGPx activity. Indeed, the most pronounced difference in activity for any enzyme was that of Mn SOD in young animals;

here NMRs had twice the activity of mice. In mutant mice that over-express catalase and Cu/Zn SOD, similar differences (1.4-fold) in these antioxidants are associated with a 40% lifespan extension (Brown-Borg and Rackoczy, 2000; Hauck and Bartke, 2000). It is highly unlikely that these modest species differences of both NMRs and mutant mice in catalase, Cu/Zn SOD and MnSOD activities offset the near-absence of cGPx, let alone reflect superior antioxidant defense. Although, we have only measured these antioxidants in liver and do not know if post mitotic tissues show different patterns in antioxidant activity, our data, like that of several other studies, (Sohal et al., 1990; Perez-Campo et al., 1994; Brunet-Rossinni, 2004), do not support the hypothesis that antioxidant activities correlate with MLSP. Clearly antioxidant activities are not limiting factors in aging and their effects are only noticed when animals are deficient in these ROS scavengers.

Age-related changes in antioxidant activity patterns differed markedly for the two rodent species examined in our study. Changes with age in antioxidant activities of mice were enzyme specific: In the oldest mouse cohort, Mn SOD increased while activity of both catalase and cGPx declined. These findings agree with previous studies that showed that age-associated changes do not follow a common trend but are contingent on the antioxidant, species, strain, gender and tissue assayed (Sohal et al., 1993; Rikans and Hornbrook, 1997; Sverko et al., 2004). Late-life changes in activities of mouse antioxidants may reflect a response in older animals to altered ROS generation rates (Palomero et al., 2001). Indeed, in gerbils (Sohal et al., 1995), rats (Hagen et al., 1997) and mice (Sohal et al., 1994), generation rates of superoxide and hydrogen peroxide have been shown to increase with age. In our study, Mn SOD up-regulation may represent a

response to augmented mitochondrial superoxide production. Because superoxide has been shown to inhibit cGPx and catalase, its presence at significantly higher concentrations in older individuals could lead to greater hydrogen peroxide generation (Kono and Fridovich, 1982; Blum and Fridovich, 1985). When compounded with possible age-associated losses in ROS neutralizing efficiency (Kasapogolu and Ozben, 2001), older mice should be more susceptible to ROS attack.

In stark contrast to our mouse data, all four antioxidants remained unchanged with age in NMRs. This pattern in antioxidants is consistent with previous findings from our laboratory of retarded physiological modifications in this species with aging (Buffenstein and Jarvis, 2002; O'Connor et al., 2002). Specifically, unlike other mammalian models of aging research, NMRs display no significant changes in bone mineral density, body composition, basal metabolism, and gut function between the ages of 2-20 years of age (O'Connor et al., 2002). Furthermore, NMRs continue to breed throughout their lives (Buffenstein, 2005). Lack of change in antioxidants may be yet another manifestation of attenuated aging process in NMRs. These results imply that the overall capacity to neutralize ROS remains unaltered throughout adult life of mole-rats. We are currently assessing if ROS generation is low compared to other species and/or if it changes with age.

In conclusion, our data refute the hypothesis that antioxidant enzyme activities are a key determinant of MLSP. The modest differences in three of the four key enzymes between NMRs and mice cannot account for the ~8-fold difference in longevity. Very low cGPx, activity levels combined with only moderate up-regulation of other antioxidant enzymes suggests that other mechanisms, such as reduced ROS generation or

greater tolerance of oxidative damage, may play a more important role in their prolonged longevity. Absence of age-related changes in antioxidant activity levels add further support to the premise that rates of aging are retarded in this extremely long-living small mammal.

Acknowledgements

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CHAPTER 3: HIGH RATES OF OXIDATIVE DAMAGE IN THE LONGEST-LIVING RODENT. (This work has been submitted to and peer reviewed in Science. Andziak B, O'Connor TP, Buffenstein R.)

3.1 Abstract

Oxidative stress is widely regarded to shape aging processes and influence longevity. We undertook a comparative study to test this theory as a determinant of species-specific lifespan in the longest-living rodent species known (naked mole-rats, maximum captive lifespan >28.3y), and shorter-living mice. We compared both tissue (liver, kidney, heart) and urinary markers of lipid peroxidation, protein oxidation and DNA modification. Surprisingly, naked mole-rats consistently had accrued more oxidative damage than physiologically age-matched (0.3y) mice. Even when old (2y) mice were compared with same-aged mole-rats, damage levels were equal to or higher in the longer-living species. Given that mole-rats live an order of magnitude longer than mice, despite higher levels of oxidative damage, our data challenge the oxidative stress theory of aging and suggest that other mechanisms serve as determinants of species longevity.

3.2 Main text

Considerable insights into mechanisms involved in aging may be gleaned by exploiting the large natural variation in maximum lifespan (MLS) among species. Similarly, the validity of current theories of aging can be tested in comparative studies between both similar-sized and phylogenetically-related species that show disparate longevity. Surprisingly, there are few comparative studies in aging research that are designed this way, or that include naturally long-living animals, even though these animals generally exhibit slow rates of age-related deterioration in physiological capacity, reproductive rate, and/or disease resistance (Finch et al., 1990; Austad, 2001). Indeed, species with extraordinary longevity could provide unique insights into mechanisms of aging and processes that promote longevity that are not readily available from more conventional short-lived species. We report the results of a comparative study testing a widely accepted paradigm of aging – the Oxidative Stress Theory- in two similar-sized (~35g) rodents (naked mole-rats and mice) that show almost an order of magnitude difference in longevity.

The naked mole-rat, *Heterocephalus glaber* (Bathyergidae, Figure 1) lives five times longer than predicted by allometry, with a MLS (>28.3y) that even exceeds that of all other long-living, much larger rodents. These subterranean hystricognaths live in sealed, thermally-buffered burrows that provide protection from both climatic extremes and predation. This lifestyle contributes to low extrinsic species mortality, and possibly has led to the evolution of physiological and biochemical mechanisms that promote somatic maintenance and extend fecundity in this species (Buffenstein, 2005).

Figure 1) The ecology of naked mole-rats. A) Naked mole-rats (NMRs) are eusocial, strictly subterranean hystricognaths that are endemic to north-east Africa. These mouse-sized rodents commonly survive in captivity into the third decade of life (maximum lifespan >28.3y). This longest-living rodent species shows attenuated age-related declines in physiological function and does not develop cancers or other pathologies commonly associated with aging. The only obvious visual sign of aging in NMRs is a lighter, parchment-like skin. The photograph shows older NMRs, (wild caught, and maintained in captivity for >25y, with tattoos) compared to younger animals (~5y; without tattoos). B) NMRs maintain reproductive capacity throughout their long lives, and the oldest breeding female in our population was wild caught and >26 years old when she died during parturition. The pregnant female shown here is >15 years old.

A

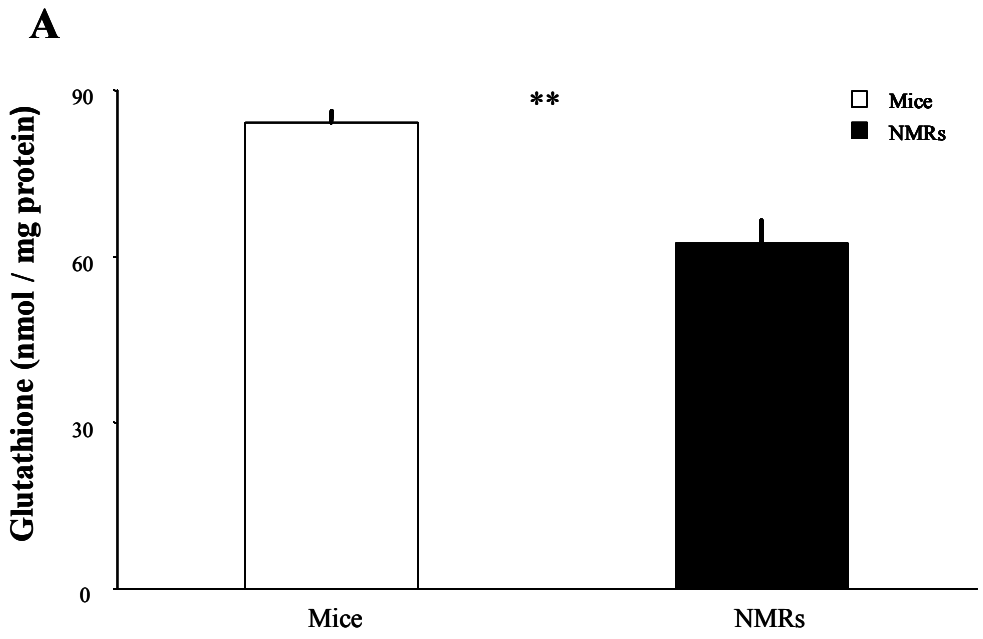
B

The Oxidative Stress Theory is currently the most widely accepted mechanistic theory of aging (Harman, 1956). It postulates that physiological declines characteristic of aging are due to accrued oxidative damage to cellular macromolecules, induced by endogenously generated reactive oxygen species (ROS). Interspecific variation in MLS therefore could result from different rates of oxidative damage accrual that in turn may affect the onset times and/or rates of aging (Sohal and Weindruch, 1996). Although antioxidants may prevent damage by neutralizing ROS, their influence on MLS is equivocal. Rather, species longevity correlates better with attenuated rates of ROS generation and/or concomitantly low amounts of oxidative damage (Barja, 2002). We quantified differences in antioxidant defenses, oxidative damage, and redox status in young naked mole-rats (2y) and compared these with both physiologically age-matched young (0.3y) and chronologically equivalent (2y) CB6F1 hybrid mice. We hypothesized that accrued oxidative damage would be lower in naked mole-rats than in mice (MLS ~3.5 y), thereby retarding the aging process in this long-living species.

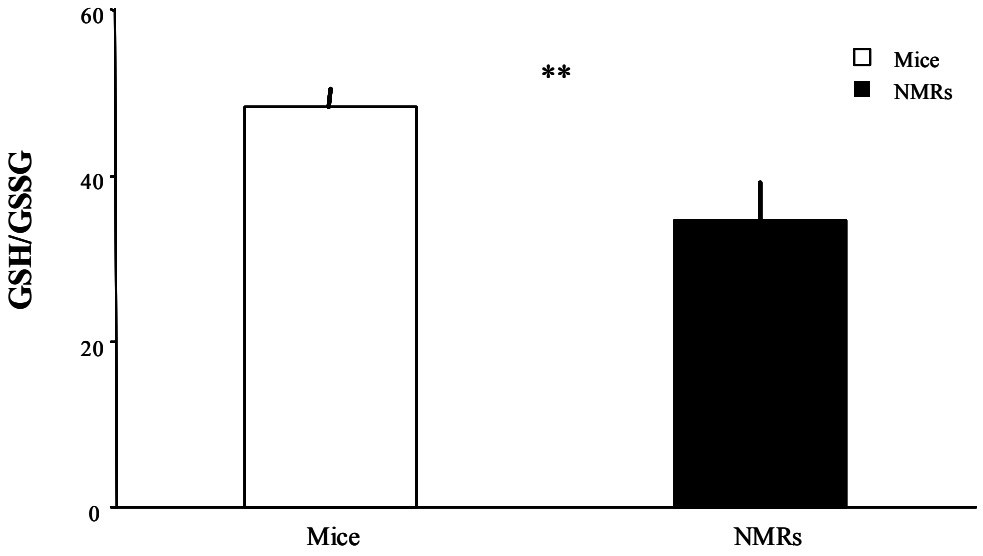
We assessed if enhanced antioxidant defenses contribute to naked mole-rat longevity by measuring levels of a ubiquitous non-enzymatic antioxidant - reduced glutathione (GSH) in liver of mice and naked mole-rats (Meister and Anderson, 1983). GSH levels were lower in naked mole-rats than in mice (Figure 2A). These observations, together with the near-absence of cellular glutathione peroxidase activity without compensatory up-regulation of other antioxidants (Andziak et al., 2005), imply that naked mole-rats do not have superior antioxidant defenses compared to mice. Antioxidant status is clearly not a consistent determinant of MLS.

To compare levels of oxidative stress, we measured ratios of the glutathione redox pair (GSH/GSSG, reduced:oxidized). Lower GSH/GSSG ratios in naked mole-rats than in mice (Figure 2B) are indicative of a more pro-oxidative cellular environment in this long-living species (Jones, 2002). This may reflect the relatively hyperoxic captive living conditions (21% O_2) compared to those (<15%O_2) in their natural subterranean habitat (Buffenstein, 2000). Hyperoxia, increases ROS generation and can rapidly induce considerable oxidative damage, even in long-living species (i.e., budgerigars; Jaensch et al., 2001) whose cells are reportedly more resistant to oxidative stress than short-living species (Ogburn et al., 1998). While laboratory-reared naked mole-rats are capable of thriving in this artificial environment, routinely surviving and breeding more than 26 years, these findings demonstrate greater levels of oxidative stress in this species.

Figure 2) Levels of glutathione and glutathione redox pair ratios in mice and NMRs. A) Levels of reduced glutathione (GSH) in liver homogenates of young mice (0.3y) are higher than in young (~2y) naked mole-rats (NMRs) (** $p < 0.01$). Lower NMR GSH levels suggest that antioxidants are not responsible for the impressive longevity of this species. B) The ratio of the glutathione redox pair (reduced/oxidized; GSH/GSSG) provides an estimate of cellular redox status. Lower liver GSH/GSSG ratios in naked mole-rats than in mice (** $p < 0.01$) imply higher levels of oxidative stress in this long-living species.



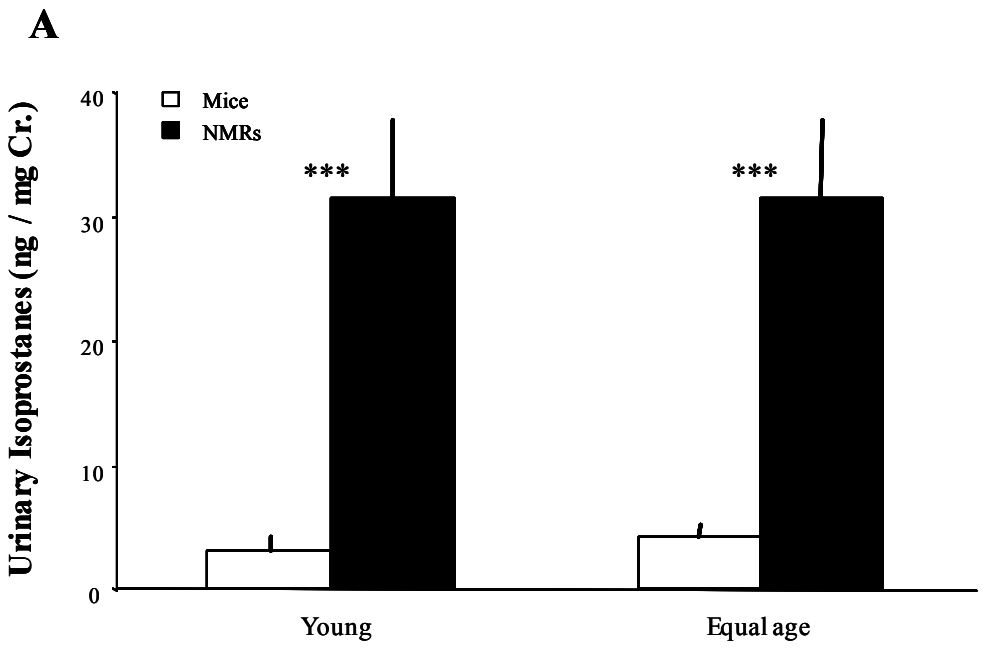
B



To assess rates of in vivo oxidative stress to specific biological molecules, we quantified urinary markers of lipid peroxidation (isoprostanes) and DNA oxidation (8-hydroxy-2'-deoxyguanosine, 8-OHdG). Isoprostanes are prostaglandin-like compounds formed via ROS-induced peroxidation of arachidonic acid (Morrow et al., 1990) and concentrations of these biologically active compounds are elevated in response to oxidative stress, as well as in various pathological states including Alzheimer's disease, atherosclerosis and diabetes (Roberts and Morrow, 2000). Urinary isoprostane levels were significantly greater in naked mole-rats than in both young and chronologically matched mice (Figure 3A), suggesting that these long-living rodents have greater rates of lipid peroxidation than mice.

8-OHdG is formed by the hydroxylation of deoxyguanosine at the C-8 position, and its urinary level may be indicative of whole animal oxidative DNA stress (Kasai et al., 1986). Naked mole-rats had lower 8-OhdG in urine than young mice (Figure 3B). While this result concurs with previous studies showing an inverse correlation between urinary 8-OHdG and MLS (Foksinski et al., 2004), an alternate interpretation of these data is also possible and may, indeed, be more meaningful. Urinary rates of 8-OHdG excretion are reportedly indicative of DNA repair (Cooke et al., 2005), which may decline with age in both rats and mice (Fraga et al., 1993, Figure 3B). Urinary 8-OHdG levels of young naked mole-rats were similar to that of old mice and lower than in physiologically age-matched, young mice. Our data would therefore imply less DNA repair in the longer-living species.

Figure 3) Urinary measures of lipid peroxidation (isoprostanes) and DNA (8-OHdG) oxidative damage reflect rates of in vivo damage generation and/or repair. We compared young naked mole-rats (NMRs ~2y) to young mice (~0.3y) and also to mice at similar chronological ages (~2y). Both biomarkers were assayed using previously published ELISA techniques (Stein and Leskiw, 2000; Kadiiska et al., 2005). (A) Isoprostane concentrations were ~10x higher in NMRs than in mice at both physiologically equivalent ($p < 0.001$) and chronologically similar ages ($p < 0.001$). (B) Urinary 8-OHdG levels were four times greater ($p < 0.001$) in young mice than in young NMRs, and similar at the same chronological age. NMRs may have higher levels of lipid peroxidation and less efficient DNA damage repair than mice.



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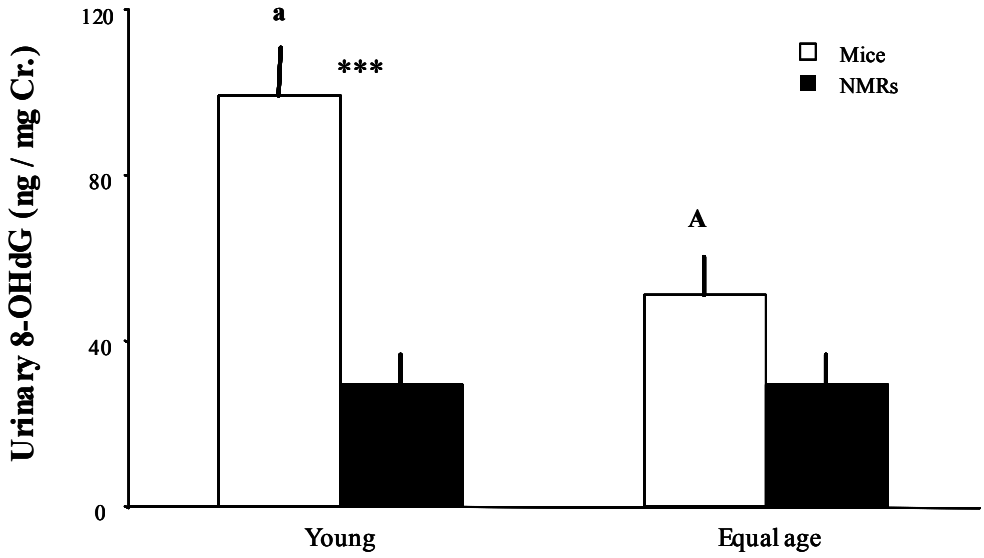
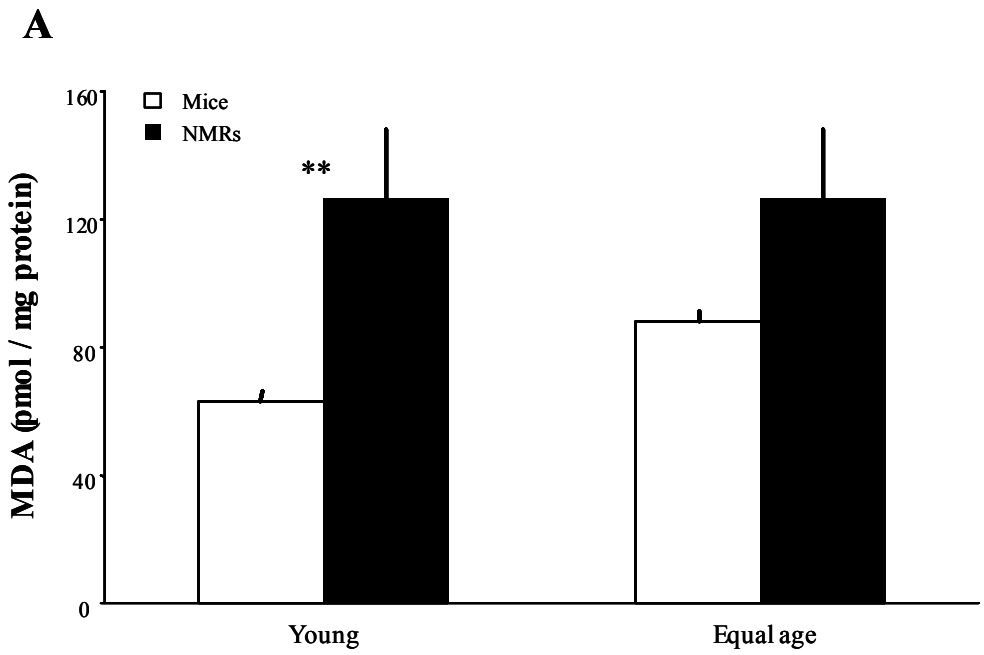
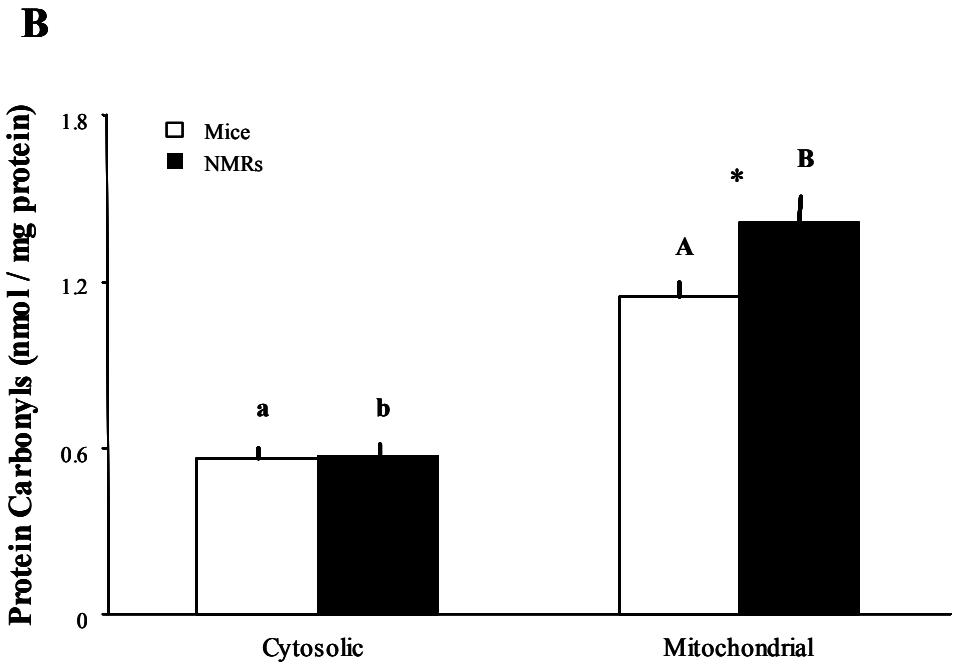


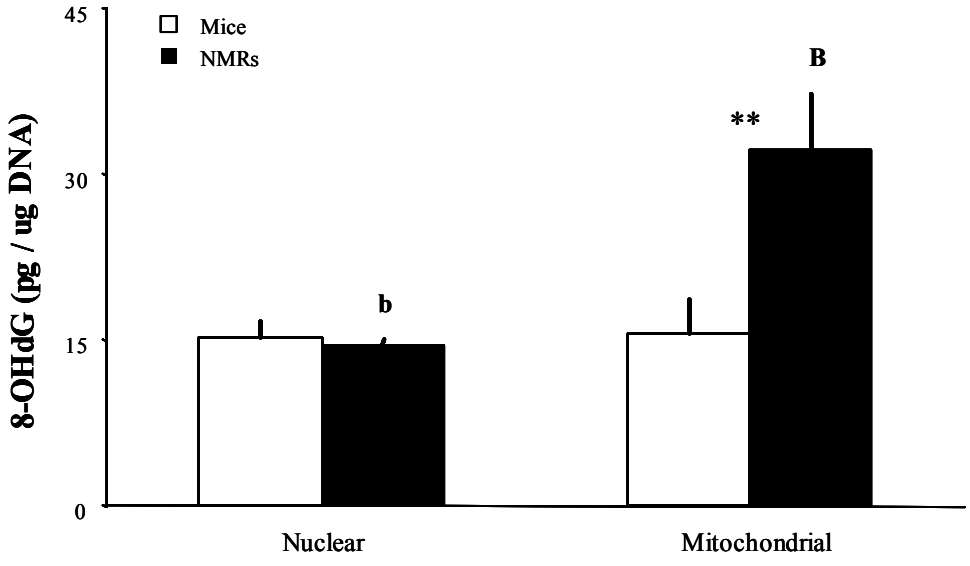
Markers of accrued oxidative damage to lipids (malondialdehyde, MDA; Esterbauer et al., 1991), proteins (protein carbonyls; Berlett and Stadtman, 1997) and DNA (8-OHdG), were surprisingly greater in tissues of naked mole-rats than in shorter-living mice. Species differences in accrued protein carbonyls and 8-OHdG were apparent in mitochondria but not in the other cell fractions tested (Figure 4). This is not unexpected since mitochondria are the primary sites of ROS generation during aerobic metabolism, and their macromolecules are especially susceptible to oxidative stress (Harman, 1972). These measures of MDA, protein carbonyls and 8-OHdG provide strong evidence that naked mole-rats accumulate higher levels of oxidative damage than mice at a physiologically equivalent age. Chronologically age-matched mice and mole-rats had similar amounts of MDA in liver tissue. Lipid damage over the first two years of life was therefore the same in both species (Figure 4), yet naked mole-rats have the potential to live 26 more years, while mice have less than 2 additional years of likely survival.

Figure 4) Naked mole-rat (NMR) tissues have higher levels of oxidative damage to lipids (malondialdehyde, MDA), proteins (protein carbonyls) and DNA (8-OHdG) than do mouse tissues. We compared these three biomarkers in young physiologically age-matched animals (~0.3y versus ~2y). In addition, MDA was compared at similar chronological ages (~2y). MDA was assayed spectrophotometrically, while both protein carbonyls and 8-OHdG were determined using previously published ELISA protocols (Kakimoto et al., 2002; Kujoth et al., 2005). (A) Liver MDA levels in young NMRs were twice ($p < 0.05$) that of young mice while MDA content in same aged animals was 1.3x greater in NMRs, but this difference did not reach significance. (B) Protein carbonyl content was 1.3x higher in NMR mitochondria than in mice ($p < 0.05$). For both mice ($p < 0.001$) and NMRs ($p < 0.001$) protein carbonyls were more abundant in mitochondrial fractions than in cytosolic fractions. Cytosolic protein carbonyl levels were similar in both species. (C) Kidney 8-OHdG levels in mitochondrial DNA of NMRs were double those of mice ($p < 0.01$). Mitochondrial DNA damage of NMRs was twice that of nuclear DNA damage ($p < 0.001$). Nuclear 8-OHdG levels were similar in both species.


A
Mice
NMRs
**
MDA (pmol / mg protein)
160
120
80
40
0
Young
Equal age


B
Mice
NMRs
Protein Carbonyls (nmol / mg protein)
1.8
1.2
0.6
0
a
b
A
*
B
Cytosolic
Mitochondrial

C



Collectively, our findings lead us to reject the proposed hypothesis that naked mole-rats accrue less oxidative damage than a shorter-lived, similar-sized, phylogenetically-related species. The consistency of our findings among various markers and tissues suggests that these results are robust even though we used immunoassays, instead of other commonly utilized techniques (e.g., HPLC or GC/Mass spectroscopy). Our data support previous findings of higher oxidative damage in long-living birds than in shorter-living rodents (Hamilton et al., 2001*b*), but they diverge from other results correlating reduced oxidative damage with MLS (Barja and Herrero, 2000), and are at variance with the most widely accepted paradigm of aging. Similarly, transgenic heterozygous mice, in which mitochondrial superoxide dismutase activity is compromised, show high levels of oxidative damage without any deleterious effect on lifespan (Van Remmen et al., 2003). Clearly factors other than oxidative damage accumulation influence longevity and may be more important in determining age-related declines in physiological function. Despite the high levels of accrued oxidative damage from an early age, naked mole-rats not only routinely survive into their third decade, but also do not exhibit age-related impairment to physiological (O'Connor et al., 2002) or reproductive function.

Using assessments of oxidative damage to key macromolecules (lipids, proteins and DNA) as well as GSH/GSSG ratios reflecting redox status, our data consistently refute the hypothesis that the prolonged longevity of naked mole-rats is due to reduced oxidative damage accrual and/ or attenuated oxidative stress. Naked mole-rats clearly tolerate high levels of oxidative damage, but their impressive lifespan appears to be unrelated to any of the oxidative stress parameters measured. While oxidative damage

may be a byproduct of aging, other mechanisms, such as specific cell and molecular properties (e.g., DNA and protein stability; or membrane phospholipid composition) and their concomitant influences on cell signaling pathways, seem more likely to serve as pacemakers of the aging process.

3.3 Materials and Methods

Experimental Design

We tested the Oxidative Stress Theory in two rodents of disparate longevity- mice (CB6F1 hybrid; *Mus musculus*, MLS ~ 3.5y) and longer-living naked mole-rats (*Heterocephalus glaber*, MLS >28.3y). We focused our study on both young, physiologically age-matched individuals (mice: ~0.3y and mole-rats: ~2y), at approximately 10% of their respective maximum lifespan potentials and also on chronologically age matched (2y old) individuals. It has been suggested (Austad, 2005) that young-adults of long-living species inherently posses mechanisms facilitating longevity and thus can be used to effectively study the underlying causes of aging. This enabled us to test if attenuated damage accrual correlates with species differences in longevity, or if damage accumulation occurs at similar rates in these two rodent species.

As an estimate of antioxidant defenses we measured a ubiquitous non-enzymatic antioxidant, reduced glutathione (GSH), in liver tissue. We obtained an estimate of cellular oxidative stress by calculating the ratios of the glutathione redox pair (reduced/oxidized, GSH/GSSG) in liver. To compare *in vivo* organismic oxidative stress we quantified urinary concentrations of isoprostanes (lipid peroxidation) and 8-hydroxy-2'-deoxyguanosine (8-OhdG, DNA oxidation/repair). We measured markers of oxidative

damage accrual to lipids (malondialdehyde; MDA) in liver, proteins (protein carbonyls) in heart, and DNA (8-OHdG) in kidney tissues. Protein carbonyls were assayed in both cytosolic and mitochondrial fractions, while 8-OhdG was quantified in nuclear and mitochondrial DNA. Unless otherwise stated, we compared levels of the various biomarkers in young naked mole-rats (n=7) with those in both physiologically (n=6) and chronologically (n=6) age-matched mice.

All measurements were made using commercially available colorimetric assays using either a spectrophotometer or a microplate reader (DU530, Beckman-Coulter, Fullerton, CA and SpectraMAX 340PC, Molecular Devices, Sunnyvale, CA). Values for both urinary markers are reported per mg of creatinine, while malondialdehyde, protein carbonyl and glutathione data are expressed per mg of protein. Kidney 8-OhdG values are expressed per pg of DNA, which we calculated from spectrophotometric OD260 values.

Animals

Naked mole-rats were born in captivity and originated from parental stock caught in Kenya in 1980. We housed naked mole-rats in colonies at the City College of New York (New York, NY) in simulated multi-chambered burrow systems, under constant climatic conditions that approximated natural conditions encountered by this species (30°C; 75% RH). Naked mole-rats were provided with an ad libitum supply of fruit and vegetables, supplemented with a high protein and vitamin enriched cereal (Pronutro, South Africa).

CB6F1 mice were purchased from the National Institute of Aging (Bethesda, MD) and Charles River Laboratories (Wilmington, MA). Animals were kept in standard mouse

cages (4 per cage) at 25ºC on a 12/12 hour light/dark cycle, and were given an ad libitum supply of mouse chow (5001 Rodent Diet, PMI Nutrition International) and water.

Urine and tissue collection

We collected a 6 hour urine sample from mice and naked mole-rats during 12:00 - 18:00. Mice and naked mole-rats were housed individually in plastic collection chambers (Sterilite, Townsend, MA) lined with parafilm (American National Can, Menasha, WI). A wire platform was mounted inside the chamber to prevent animals from physically dispersing or contaminating excreted urine. Animals were constantly monitored and upon detection of micturition, all urine voided was collected and transferred on ice until stored at -80°C. Prior to urinary 8-OHdG and isoprostane assays, samples were centrifuged at 500 g for 3 minutes (4°C) to remove any debris (e.g., skin or fur) that may have been accidentally collected.

Animals were euthanized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg). Tissues were dissected out within 10 minutes of injection, rinsed in ice-cold Ringer's solution and flash frozen in liquid nitrogen for storage at -80°C.

We used similar tissue preparation protocols for all our assays. Tissues were minced and manually homogenized on ice in their respective homogenizing buffers using a Duall glass homogenizer. Homogenates were centrifuged, as per specific assay protocol (4°C), and the resulting samples were stored at -80°C until assayed.

Glutathione

Levels of both reduced (GSH) and oxidized (GSSG) glutathione were determined in livers of young mice (n=5) and naked mole-rats (n=5) using the BIOXYTECH GSH/GSSG-412 kit (OxisResearch, Portland,OR). We assessed the cellular redox status, as an estimate of oxidative stress, in these two species by comparing the ratios of GSH to GSSG (GSH/GSSG).

We homogenized livers in a buffer consisting of 0.25 M sucrose, 3 mM EDTA and 10 mM Tris-HCl (pH 7.4, 4°C). Homogenates were centrifuged at 3000 g for 10 minutes and supernatants collected. We adapted the manufacturer's protocol to a micro-plate by proportionally reducing the volumes of samples and reagents by 75%, and confirmed that this did not alter the efficacy of this technique using calibration standards.

Lipid Peroxidation

We quantified urinary isoprostane content using EA 85 ELISA kits (Oxford Biomedical Research, Oxford, MI), specific for 15-isoprostane F2t. ELISA kits have frequently been used to measure urinary isoprostane content (e.g., Stein and Leskiew, 2000; Mathur et al., 2002; Chen et al., 2003; Dobrian et al., 2003), although this approach is not as common as Gas Chromatography/Mass Spectrometry. Total liver MDA was measured using the BIOXYTECH MDA-586 assays (OxisResearch, Portland, OR).

Liver samples were washed with 0.9% NaCl containing 0.16 mg/mL of heparin and 2.5 mM butylated hydroxytoluene (BHT) in acetonitrile and homogenized in 20 mM potassium phosphate with 2.5 mM BHT (pH 7.4, 4°C). Homogenates were centrifuged at 3,000 g for 10 minutes and supernatants collected.

Absorbance readings were made across a range of wavelengths (650 nm – 450 nm, 0.5 nm interval, DU530, Beckman-Coulter, Fullerton, CA), spanning the expected peak absorbance (586 nm) of the resulting assay reaction product (carbocyanine dye). We isolated distinct MDA peaks using 3rd derivative spectroscopy.

Protein Carbonyls

We measured protein carbonyl levels in cytosolic and mitochondrial heart fractions of young naked mole-rats (n=7) and mice (n=7) using the Zentech PC ELISA tests (Zentech, Dunedin, NZ). This protocol has numerous advantages over the classic colorimetric method (Dalle-Donna et al., 2003), and has, indeed, been used in a number of studies on aging (e.g., Davies et al., 2001; Kujoth et al., 2005).

Tissue samples were homogenized on ice in 210 mM mannitol, 70 mM sucrose, 10 mM Tris-HCl, 1 mM diethylenetriaminepentaacetic acid (DTPA), 1 mM desferal, (pH 7.4 at 4°C), supplemented with a protease inhibitor cocktail (MiniComplete, Roche Applied Sciences, Indianapolis, IN). Large fragments were removed by slow centrifugation at 1000 g for 3 minutes, and the supernatant was centrifuged again at 10,000 g for 10 minutes and both cytosolic (aqueous) and mitochondrial (solid) fractions were collected (Davies et al, 2001). Mitochondrial pellets were washed twice, re-suspended in homogenizing buffer and disrupted using a Fisher Scientific F60 sonic dismembrator (Misonix, Farmingdale, NY).

Prior to analyses, we adjusted protein content of our samples to 40 mg/mL. We concentrated our samples using a Savant DNA110 SpeedVac (Savant, Farmingdale, NY), after adding both ribonuclease A (cytosolic, 20 μg/mL and mitochondrial, 100 μg/mL)

and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) in methanol (final conc., 25 mM). RNase A was added to prevent spurious carbonyl readings (Buss et al., 1997), whereas trolox, a vitamin E mimetic, prevented sample oxidation during this process.

DNA Damage

Content of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in both urine, and kidney nuclear and mitochondrial DNA extracts was assayed using BIOXYTECH 8-OHdG-EIA kits (OxisResearch, Portland, OR). This protocol uses a monoclonal antibody (N45.1) specific for 8-OHdG, and while it is not as popular as commonly used HPLC-EC technique, numerous studies have been published using this methodology (e.g., Kang et al., 1998; Radak et al., 1999; Kakimoto et al., 2002; Kadiiska et al., 2005). Furthermore, the values obtained by ELISA kits have been shown to correlate well with those found using HPLC-EC, for both urine (Toyokuni et al., 1997) and extracted tissue DNA (Evans et al., 1999). We compared levels of urinary 8-OHdG in young naked mole-rats (n=6) with those in both physiologically and chronologically age-matched mice (n=6 per cohort), whereas tissue DNA 8-OHdG concentrations was compared only in physiologically age-matched animals (n=6 per cohort).

Kidney nuclear DNA was extracted using DNA Extractor WB kits (Wako BioProducts, Richmond, VA), while mitochondrial DNA was isolated using mtDNA Extractor CT kits (Wako BioProducts, Richmond, VA). Kidneys were homogenized in manufacturer-supplied buffers to which deferoxamine mesylate (Desferal; 0.1 mM final conc.) was added, to prevent sample oxidation (Helbock et al., 1998). RNAse A (20

μg/mL final conc.) was used during extraction to remove RNA from nuclear samples while for mitochondrial samples this was done during DNA hydrolysis (RNAse A final conc. 100 μg/mL).

We combined mitochondrial DNA from two same-age con-specifics, whereas individual samples were used for nuclear DNA analyses. DNA hydrolysis (Helbock et al., 1999) involved enzymatic digestion with nuclease P1 (1 U/μl, USBiological, Swampscott, MA) and calf intestine alkaline phosphatase (1 U/μl, Roche Applied Sciences, Indianapolis, IN). Digested DNA samples were centrifuged at 5,000 g for 40 minutes (4°C) in 10,000-Da micro-filtration tubes (UltraFree-MC, Millipore, Bedford, MA). The resulting filtrates were stored on ice and assayed immediately thereafter. We lowered the incubation temperature from 37°C to 25°C, and compensated for this by doubling the incubation time to two hours thereby removing micro-plate edge effects.

Creatinine and Protein Content

Both creatinine and protein were measured using commercially available assays (creatinine:- CR 01; Oxford Biomedical Research, Oxford, MI and protein:- Pierce BCA Protein Assay, Pierce, Rockford, IL).

Statistical Analyses

Results are presented as means ± SEM. Differences between naked mole-rats and mice biomarkers of oxidative stress were determined using Student's t-test (NCSS Version 97; Kaysville, UT). Prior to statistical analyses data were transformed to their

natural logarithm to meet assumptions of the statistical tests that data be normally distributed. Statistical significance for all analyses was set at $p < 0.05$.

Acknowledgements

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CHAPTER 4: DISPARATE LIPID DAMAGE PROFILES DURING AGING IN MICE AND IN THE LONGEST-LIVING RODENTS, NAKED MOLE-RATS.

(This work is currently in preparation for submission to the journal Aging Cell. Andziak B, Buffenstein R.)

4.1 Abstract

Differences in oxidative damage generation and accrual may underlie the natural variation in species longevity. We compared age-related profiles of whole-organism lipid peroxidation (urinary isoprostanes), accrued liver lipid damage (malondialdehyde), and liver non-heme iron in long-living naked mole-rats (NMRs>28.3y) and shorter-living CB6F1 hybrid mice (~3.5y). Surprisingly, even at a young age, both markers of lipid peroxidation, as well as iron were at least 2-fold higher (p<0.005) in NMRs. Age-related profiles of these parameters were species-specific. Accrued damage doubled in mice, reflecting the maintenance of steady levels of whole-organism lipid peroxidation and iron during adulthood. In NMRs, however, whole organism lipid peroxidation declined by half with age (p<0.001), despite increases in tissue iron (p<0.05). Lipid damage levels did not change with age in NMRs, contrary to the predictions of the Oxidative Stress theory. Our results thus imply that the extended longevity of NMRs may be independent of oxidative stress parameters.

4.2 Introduction

The Oxidative Stress Theory asserts that declines in organismal function, characteristic of the aging process, result from a progressive accrual of endogenously induced modifications to cellular constituents (Harman, 1956). Damage is caused by a broad spectrum of reactive oxygen species (ROS) that may either be direct or indirect by-products of normal aerobic respiration: ROS-induced products of oxidative processes are often highly reactive themselves inducing damage to other biological molecules. Accumulation of damage during aging thus reflects a chronic imbalance between the rate of ROS generation and both the ability to neutralize ROS and/or repair any ROS-induced damage (Beckman and Ames, 1998*a*). Organismal antioxidant defenses scavenge and neutralize ROS, while repair mechanisms remove damaged biological macromolecules preventing their accrual in tissues (Finkel and Holbrook, 2000). Within this context, natural variation in species longevity is thought to reflect disparate rates of aging caused by unequal oxidative damage accumulation (Sohal and Weindruch, 1996).

Membrane lipids, especially those in the mitochondria, are particularly susceptible to oxidative stress, since they are located in close proximity to sites of ROS generation. In addition, damage to lipids occurs through a sequence of self-propagating peroxidative reactions (Esterbauer et al., 1991). Lipids are important ubiquitous components of biological membranes. Their modification by oxidative processes may lead to altered membrane fluidity and permeability properties with concomitant widespread aging effects (Davies, 2000). Susceptibility of membranes to lipid peroxidation also depends upon membrane fatty acid composition; membranes higher in

polyunsaturated fatty acids (PUFAs) and in particular (n-3 PUFAs, such as docosahexanoic acid) are particularly vulnerable to oxidative stress (Hulbert, 2005).

Lipid peroxidation and subsequent damage accrual during aging can be affected by the cellular environment. Intracellular concentrations of transition metals, especially iron, initiate Fenton type reactions with hydrogen peroxide to form highly reactive hydroxyl radicals (Marzabadi et al., 1988), or with lipid hydroperoxides to produce alkoxy radicals (Spiteller, 2001). Elevated iron levels in tissues should thus result in increased oxidative damage (Sohal et al., 1999). This is indeed the case such that reported increases in tissue iron during aging correlate with age-related changes in oxidative damage levels (Massie et al., 1983; Cook and Yu, 1998).

Studies on age-associated changes in accrued lipid damage have been equivocal, with results being contingent on the species, tissues and gender of animals assayed (Palomero et al., 2001; Sverko et al., 2002; Oxenkrug and Requintina, 2003). The lack of consensus in the literature has led some authors to conclude that progressive accrual of oxidative lipid damage is not an integral component of organismal aging (Rikans and Hornbrook, 1997). While data on age-related changes in oxidative lipid damage have been inconsistent, a number of comparative studies have reported an inverse relationship between lipid damage and longevity, such that longer living birds accrue less damage than similar sized mammals (Pamplona et al., 1999). These patterns have been related to a lower peroxidation index associated with lower levels of n-3 PUFAs in long-living species (Pamplona et al., 1998; Hulbert, 2005). We asked if lipid peroxidation of the longest-living rodent (the naked mole-rat) may provide useful insights into this controversy.

Naked mole-rats (*Heterocephalus glaber*, NMRs) are mouse-sized subterranean hystricognath rodents that in captivity survive to approximately 28 years (Buffenstein and Jarvis, 2002). This longevity is not only exceptional for a 35g mammal, exceeding five- to ten-fold that predicted by body mass (Prothero and Jurgens, 1987; Austad and Fischer, 1991), but also holds the record for longevity among rodents (Buffenstein, 2005). This longevity quotient (ratio of actual lifespan to that predicted based on body mass) is similar to that observed in humans and exceeded only by that reported for bats (Austad and Fischer, 1991). Not only do NMRs live an extremely long time, but they also do not exhibit age-associated physiological modifications commonly observed in other mammalian models of aging research (O'Connor et al., 2002), and continue to reproduce successfully into the third decade of life (Buffenstein and Jarvis, 2002). Collectively, these findings suggest that aging is delayed in this long-living species and that NMRs may be a useful animal model with which to assess mechanisms involved in resisting aging.

NMRs, despite maintaining organismal function well into their third decade of life, do not posses superior antioxidant defenses than shorter-living mice (Andziak et al., 2005). Surprisingly, these rodents have reportedly very low activity of an important mammalian antioxidant enzyme, cellular glutathione peroxidase, without significant compensatory up-regulation of other enzymatic antioxidants. While antioxidant defenses are not considered significant species longevity determinants (Barja, 2002), the near-absence of this important H_2O_2 neutralizing enzyme (Chiu et al., 1976), raises the possibility that NMRs may be particularly susceptible to high rates of oxidative stress and

is indeed reflected by high levels of oxidative damage to several macromolecules in this long-living species (Andziak et al., unpublished data).

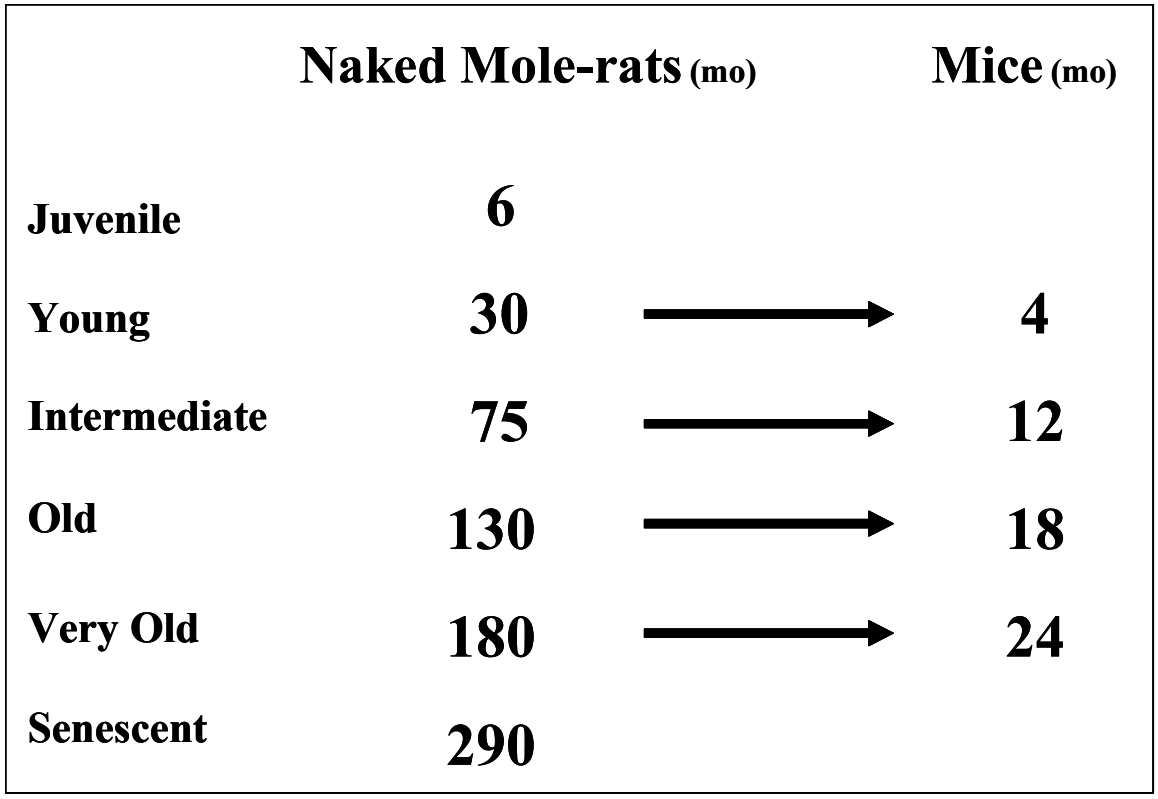
In the present study we compared damage generation and accrual during aging by assessing age-related changes of two markers of lipid peroxidation (isoprostanes and malondialdehyde) in two similar-sized rodent species with disparate maximum longevity; NMRs (>28.3 years) and CB6F1 hybrid mice (~3.5 years). We also compared levels of non-heme iron during aging to establish if species differences in the intracellular environment correlate with the observed patterns of oxidative damage generation and accrual. We hypothesized that long-living NMRs would have lower levels of lipid damage generation than similar-sized, yet shorter-lived mice, and that damage would accrue at a slower rate in the longer living species. In addition, we hypothesize that intracellular non-heme iron would be lower NMRs than in mice, reflecting a cellular environment less conducive to oxidative stress.

4.3 Materials and methods

Animals

Lipid damage and non-heme iron levels were determined in male NMRs and hybrid CB6F1 mice (*Mus musculus*) at different ages. Age cohorts were specifically selected to facilitate comparaisons among these two species at physiologically equivalent ages (Figure 1). Animal handling procedures were reviewed and approved by the City College of New York (CCNY; New York, NY) IACUC (#0414 and #0415).

Figure 1) Age cohorts of animals used in this study. For comparisons between mice and NMRs, age-cohorts were specifically chosen as proportions of their respective longevities. In addition, "Juvenile" and "Senescent" NMRs cohorts were used when comparing urinary isoprostane excretion at different stages of life in this species.



All NMRs, with the exception of the "Senescent" cohort (290 months), were born in captivity. Senescent animals were captured in Kenya in 1980, as juveniles, and comprised the parental stock from which all the other experimental NMRs originated. Animals were maintained in colonies at CCNY and housed in simulated, multi-chambered burrow systems under constant climatic conditions (30°C; 75% RH), that resemble those encountered in the wild. NMRs were fed *ad libitum* a diverse diet of fruits and vegetables, which was supplemented with a high protein and vitamin enriched cereal (Pronutro, South Africa).

Mice were obtained from Charles River Laboratories (Wilmington, MA) and from the National Institute of Aging (NIA; Bethesda, MD). A number of the animals in the two younger age groups were purchased at 7 weeks of age and housed at CCNY until they reached the appropriate ages for this study. Animals were maintained in standard mouse cages (4 animals per cage) on a 12:12 hour light/dark cycle and on an *ad libitum* diet of mouse chow (5001 Rodent Diet, PMI Nutrition International) and water.

Urine Collection

A six-hour urine (12:00 - 18:00) sample was collected from mice and NMRs housed individually in plastic collection chambers (Sterilite, Townsend, MA). Chambers were lined with parafilm (American National Can, Menasha, WI), on top of which we mounted a wire platform preventing animals from physically dispersing or contaminating excreted urine. Animals were constantly monitored and upon detection of micturition, all urine was collected and transferred on ice for storage at -80°C. Prior to the isoprostane

assays, urine samples were centrifuged at 500 *g* for 3 minutes (4°C) to remove any debris (e.g., skin or fur) that may have been accidentally collected.

Liver Collection

Animals were euthanized with an intraperitoneal injection of sodium pentobarbital (60mg/kg). Liver was dissected out within 5 minutes of injection, rinsed in ice-cold Ringer solution and flash frozen in liquid nitrogen and stored at -80°C until later analyses.

Urinary Isoprostanes

Urinary isoprostane content was quantified with a competitive enzyme-linked immunoassay kit (EA 85, Oxford Biomedical Research, Oxford, MI), which uses an anti-body specific for 15-isoprostane F_{2t}. Given the potential for cross-reactivity with other isomers present in urine (Proudfoot et al., 1999), values obtained are considered indicative of overall isoprostane status. Urinary isoprostanes are reported *per* mg of creatinine.

Malondialdehyde (MDA)

Total liver MDA content was determined using the BIOXYTECH MDA-586 assay (OxisResearch, Portland, OR). Liver samples were minced on ice and washed with 0.9% NaCl containing 0.16 mg/mL of heparin and 2.5 mM butylated hydroxytoluene (BHT) in acetonitrile. We manually homogenized the tissues in 20 mM potassium phosphate containing 2.5 mM BHT (pH 7.4 at 4°C) using a glass Duall homogenizer

(Kontes, Vineland, NJ). Homogenates were centrifuged at 3,000 *g* for 10 minutes at 4°C and the resulting supernatants stored at -80°C until time of assay.

Absorbance readings were made across a range of wavelengths (650 nm – 450 nm, 0.5 nm interval, DU530, Beckman-Coulter, Fullerton, CA), spanning the expected peak absorbance (586 nm) of the resulting carbocyanine dye. Distinct MDA peaks were isolated using 3$^{rd.}$ derivative spectroscopy. Liver MDA data are expressed *per* mg of protein.

Non-Heme Iron

Liver non-heme iron content was determined using the modified method of Rebouche et al. (2004). Liver samples were homogenized in high purity water using a mechanical homogenizer. Homogenates were combined with an equal volume of protein precipitation solution (1 N HCl and 10 % trichloroacetic acid), mixed and incubated at 95°C for 120 minutes. Following the incubation, the solutions were mixed and centrifuged at 8200 *g* for 10 minutes (20°C). The resulting supernatants were stored on ice until assay later that same day.

Equal volumes of sample and chromogen (0.508 mM ferrozine, 1.5 M sodium acetate and 0.1% (v/v) thioglycolic acid) were reacted in micro-plates. The mixtures were incubated in the dark for 30 minutes (20°C), after which absorbance readings were made at 562 nm. Sample concentrations were determined using a calibration curve based on a set of iron standards (High Purity Standards, Charleston, SC), which were treated in the same manner as liver homogenates. Non-heme iron data are expressed *per* mg of protein

Creatinine and Protein Content

Creatinine was determined in urine using the CR 01 kits (Oxford Biomedical Research, Oxford, MI). Creatinine levels in urine provide an estimate of glomerular filtration rate and are thus also useful indicators of kidney function. Protein concentration was determined in sample homogenates with a BCA Protein Assay (Pierce, Rockford, IL).

Statistical Analyses

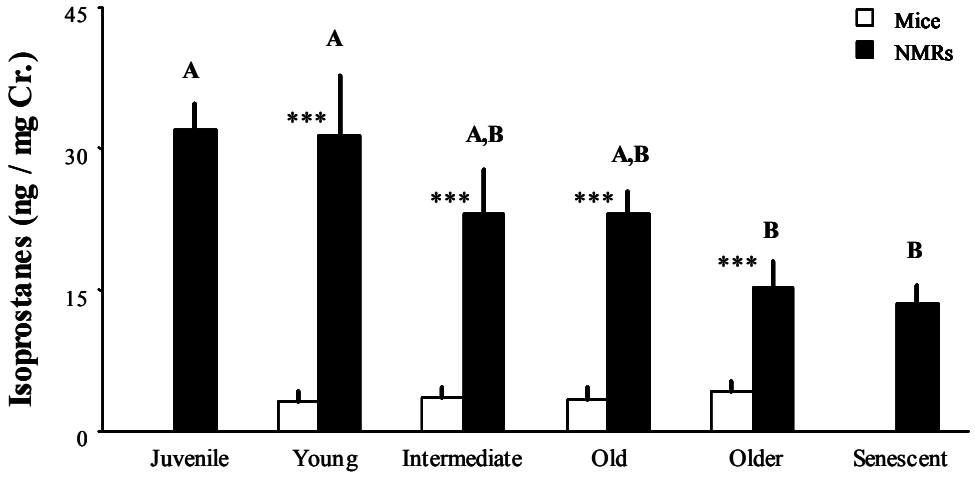
Statistical analyses were performed using NCSS Version 97 (Kaysville, UT). Prior to statistical analyses data were transformed to their natural logarithm. The effects of age and species differences on urinary isoprostanes, MDA and non-heme iron in liver were tested for significance by a two-way analysis of variance (ANOVA) using a general linear model. In addition, we tested the effect of age of isoprostanes in the six NMR age cohorts using a one-way ANOVA. All age-cohort contrasts were made using the Bonferroni multiple-comparison test. Results are presented as untransformed means ± SEM. Statistical significance for all analyses was set at $p < 0.05$.

4.4 Results

Urinary Isoprostanes

Urinary isoprostane levels of mice and NMRs showed neither a significant effect of age, nor an interaction effect of age and species (Figure 2). However, there was a significant and pronounced species effect ($p < 0.001$), such that NMRs exhibited 5- to 10-fold higher isoprostane levels (23.30 ± 2.16 ng/mg) than did mice (3.64 ± 0.51 ng/mg), in the four comparative age cohorts assessed ($p < 0.005$). When we examined a larger age-range of NMR data, including 6 month-old and 290 month-old animals, we found a significant ($p < 0.001$) effect of age on urinary isoprostane levels, such that this marker of whole-body lipid peroxidation declined with age (Figure 2). The two youngest (6 mo, 31.98 ± 2.87 ng/mg; 30 mo, 31.47 ± 6.39 ng/mg) mole-rat cohorts had significantly higher isoprostane concentrations than both 180 month-old (15.29 ± 2.81 ng/mg; $p < 0.05$) and 290 month-old (13.67 ± 1.95 ng/mg; $p < 0.01$) animals.

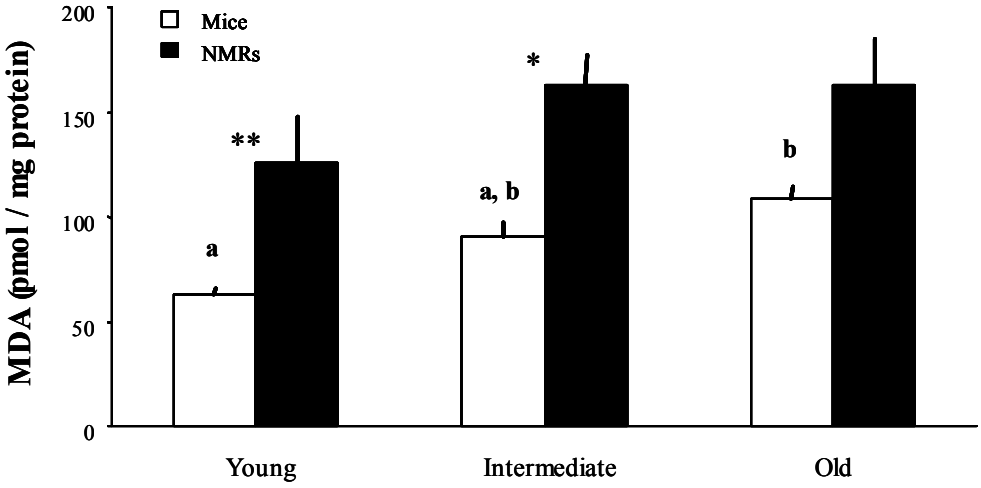
Figure 2) Urinary isoprostanes levels in mice and NMRs at different ages. While urinary isoprostane excretion remained constant with age in the four mouse cohorts, there was an age-related decline across the six in NMRs age groups. NMRs had higher isoprostane content than mice for all four physiologically-equivalent age groups. Results are means ± SEM. Significant differences between age-matched mice and NMRs: *** – $p < 0.001$. A and B: NMR age-cohort means that do not share a common superscript are significantly different.



Malondialdehyde (MDA)

Age-related changes in liver lipid damage (MDA) showed a similar pattern in both species (Figure 3) resulting in no significant interaction effect of age and species among mice and NMRs. Both 30 month-old (125.8 ± 21.9 pmol/mg) and 75 month-old (161.2 ± 13.9 pmol/mg) NMRs had significantly more MDA than age-matched mouse cohorts (4 mo, 63.0 ± 3.3 pmol/mg, $p < 0.01$; and 12 mo, 90.3 ± 7.4 pmol/mg, $p < 0.05$) resulting in a significant species effect ($p<0.001$). While all three mole-rat cohorts had similar MDA values, lipid damage of 18 month-old mice (108.9 ± 5.8 pmol/mg) was nearly twice that of the 4 month-old animals ($p < 0.05$) resulting in a significant age effect on accrued lipid damage ($p < 0.01$).

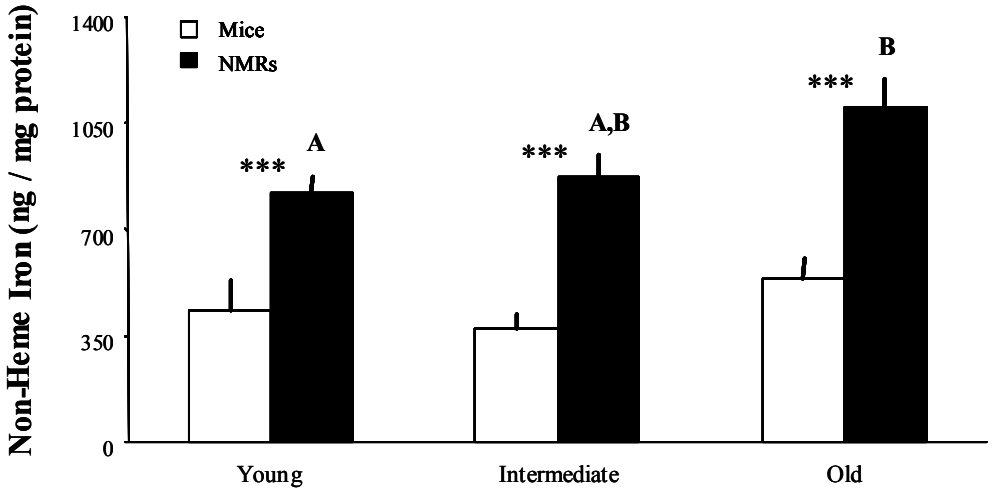
Figure 3) Accrued liver malondialdehyde (MDA) levels in mice and NMRs at physiologically equivalent ages. MDA content increased with age in mice, yet remained constant in NMRs. NMRs had higher MDA levels than mice in the two younger age cohorts. Results are means ± SEM. Significant differences between age-matched mice and NMRs: * * – $p < 0.01$, * – $p < 0.05$. a and b: mouse cohort means that do not share a common superscript are significantly different.



Non-heme Iron

For all the age groups examined, NMRs (30 mo, 821 ± 64 ng/mg; 75 mo, 877 ± 72 ng/mg; 180 mo, 1109 ± 86 ng/mg) had higher iron levels than age-matched mice (4 mo, 435 ± 98 ng/mg, $p < 0.001$; 12 mo, 378 ± 40 ng/mg, $p < 0.001$; 18 mo, 536 ± 64 ng/mg, $p < 0.001$), resulting in a significant effect of both species ($p < 0.001$) and age ($p < 0.05$), although the interaction effect of age and species was not significant (Figure 4). Although there was a significant effect of age on iron levels and 180 month-old NMRs seemed to have higher non-heme iron than the younger age groups, none of within-species pair-wise comparisons was statistically significant. When we analyzed the NMR iron data on its own, we found an age-associated increase, with levels in 180 month-old animals being higher than in 30 month-old animals ($p < 0.05$).

Figure 4) Liver non-heme iron levels in mice and NMRs of different ages. Iron levels did not change with age in mice, but increased in NMRs. NMRs had higher liver levels of non-heme iron than mice for al three age-cohorts assayed. Results are means ± SEM. Significant differences between age-matched mice and NMRs: * * * – $p < 0.001$. A and B: NMR age-cohort means that do not share a common superscript are significantly different.



4.5 Discussion

This study set out to assess if variation in longevity among species may be explained by differences in lipid peroxidation and oxidative damage accrual. Specifically, we asked if the longest-living rodent and shorter-living mice accrue similar amounts of oxidative damage to lipids at physiologically equivalent ages, and if amounts of lipid peroxidation can be correlated with non-heme iron levels. The results were contrary to predictions based upon the Oxidative Stress theory. Our comparative data did not correspond to differences in species longevity, as young NMRs had significantly higher urinary isoprostane levels, more accrued oxidative damage (MDA) in liver, as well as greater amounts of non-heme iron than mice. In addition, very different species responses were evident during aging. Although there was an age-related increase in non-heme iron in naked mole-rats, urinary isoprostane levels declined by half with age (6-290 months) in this species, while liver MDA content remained constant. In mice, while there were no age-associated changes in non-heme iron and urinary isoprostane concentrations, accrued liver damage doubled with age (4-18 months).Oxidative damage generation and accumulation thus follow different species patterns that do not reflect observed species longevity differences.

Young animals

Urinary isoprostanes are key markers for arachidonic acid peroxidation and are indicative of organismal oxidative damage generation (Morrow et al., 1990; Esterbauer et al., 1991). High levels of urinary isoprostanes may reflect the higher proportion of arachidonic acid and other n-6 PUFAs, and lower amounts of n-3 PUFAs in naked mole-

rat membranes when compared to mice (Hulbert et al., in press). It is therefore possible that assays of peroxidation markers representative of docosahexanoic acid, an n-3 PUFA with a higher susceptibility to peroxidation, may reveal species differences that are divergent from those reported in the present study. Our findings may also stem from high levels of non-heme iron in NMR tissues, since iron can initiate peroxidation of lipids (Clothier et al., 2000; Gerhard et al., 2002). Interspecific differences in membrane composition and non-heme iron concentrations may also explain higher MDA content in NMRs, even at a young age. Previously, we reported that young NMRs also have more DNA and protein damage than age-matched mice (Andziak et al., in press). This suggests that high levels of lipid damage in NMRs can not be simply attributed to a greater proportion of arachidonic acid in their membranes, and simply reflect more substrate for peroxidative reactions. The 10-fold higher isoprostane levels and 2-fold greater MDA content in young NMRs more likely reflect elevated rates of oxidative damage generation by this species.

Greater rates of damage generation in NMRs may be due to relatively higher oxygen concentrations experienced by laboratory-reared animals, when compared to those in their natural burrow conditions (Buffenstein, 2000). Indeed, increased isoprostane levels are reportedly associated with hyperoxia-induced oxidative stress in both clinical (Delanty et al., 1997) and experimental (Jaensch et al., 2001) settings. Newborn and young NMRs may be especially susceptible to rapid oxidative damage accrual resulting from laboratory-induced hyperoxia, since their antioxidant defenses are not likely to be fully developed (Utsami et al., 1977; Fantel and Person, 2002) and unable to cope with this oxidative stressor. Pronounced lipid damage generation in NMRs during

early stages of life is consistent with previous studies in mice, which reported highest lipid peroxidation rates during prenatal and neonatal development (Utsumi et al., 1977; Salminen et al., 1988). In addition, greater damage accrual during early stages of life could reflect augmented ROS generation due to high cell proliferation and metabolic rates (Fraga et al., 1993; Fantel and Person, 2002). The 10-fold difference in levels of urinary isoprostanes, which reflects oxidative damage generation, does not lead to a similar differential in damage accumulation and NMRs have only twice the accrued damage as mice. This implies that NMRs may have efficient lipid damage repair mechanisms that restrict its accumulation in tissues. High levels of accrued oxidative damage at an early age do not induce long term deleterious effects in NMRs, since captive animals show no evidence of declines in physiological function until well into their third decade of life.

Non-heme iron levels of NMRs were greater than in mice, suggesting that the cellular milieu in this long-living species is more favorable to oxidative damage generation and accrual than it is in shorter-living mice. Indeed, in certain mouse strains non-heme iron levels are reportedly positively correlated with hepatic lipid peroxidation (Clothier et al., 2000; Gerhard et al., 2002) and feeding treatments that overload iron in the diet can lead to significant increases in oxidative damage (Stimson and Fischer, 1997; Valerio and Petersen, 1998). Elevated non-heme iron can potentially contribute to higher rates of oxidative damage observed in NMRs. High non-heme iron content in NMR tissues may be a trait associated with a subterranean existence in lateritic soils, rich in iron oxides and iron hydroxides (Brett, 1991). The elevated steady-state tissue iron levels that have evolved in NMRs in response to their natural habitat are still probably

expressed in our captive-reared animals. It is therefore highly likely that although the internal environment of NMRs may be more conducive to lipid peroxidation than it is in mice, non-heme iron levels are unrelated to the mechanisms involved in longevity determination.

Given that aging is assumed to be a continuous process, it is generally believed that levels of key biochemical markers of aging should be lower in young individuals of long-living species than those found in age-matched shorter-living species. The higher levels of lipid peroxidation in young NMRs than much shorter living mice, suggest that lipid peroxidation may not be an integral mechanistic components of longevity determination. Therefore, interspecific variation in oxidative damage of young animals may not be good indicators of comparative differences in maximum lifespan, and age-related changes in lipid damage generation and accrual may be more insightful.

Age-related changes

Isoprostane and MDA levels correlate well with each other in biological samples (Roberts and Morrow, 2000), and age-related changes in tissue MDA levels commonly mirror age-associated profiles of urinary isoprostanes. Mice and NMRs displayed disparate age-related profiles of isoprostane excretion and MDA accrual (Figure 2 and 3). In mice, urinary isoprostane excretion remained constant during adulthood (4 – 24 months), suggesting maintenance of steady rates of damage generation. Constant rates of damage generation in mice nevertheless resulted in progressive increases in liver MDA, such that levels of accrued damage doubled between 4 and 18 month-old cohorts. Rates of lipid damage generation declined with age in NMRs, such that isoprostane excretion

by 180 and 290 month-old NMRs was half those observed in 6 and 30 month-old cohorts. Liver MDA levels were moderately higher (~28%) in 75 month-old NMRs than they were in 30 month-old individuals. However, the two older age NMR cohorts had quantitatively equivalent accrued MDA levels, implying that rates of oxidative damage generation were matched by antioxidant defense and damage repair during that period.

Elevated isoprostane excretion is reportedly associated with certain pathological conditions including, Alzheimer's disease (Pratico et al., 1998; Montine et al., 2002) and atherosclerosis (Pratico et al., 1997; Pratico et al., 2001), and is similarly noted in transgenic mouse models of these diseases. Interestingly, age-associated increases in isoprostanes are found in individuals afflicted with one of these diseases and not age-matched controls (Pratico et al., 2001; Montine et al., 2002). This suggests that age-related increases in isoprostanes may be linked to pathological states and not the aging process itself. The absence age-associated increases in isoprostanes of either mice or NMRs supports this premise in that both mice and NMRs used in this study were healthy and devoid of any apparent pathology. Indeed in the case of NMRs isoprostanes declined with age, as presumably animals acclimated to the relatively high oxygen content in the laboratory.

Cellular iron can initiate peroxidative reactions of lipids and changes in its levels during aging may affect species-specific patterns of damage generation and accrual (Massie et al., 1983). Age-associated increases in liver iron have been shown to accompany age-related increases in accrued lipid damage in both rats (Cook and Yu, 1998) and C57BL/6J mice (Massie et al., 1983). In our CB6F1 mice non-heme-iron levels remained constant, possibly reflecting the strain-specific nature of this response

(Gerhard et al., 2002). Given that our mice had progressively more oxidative damage with increasing age, this suggests that the relationship between non-heme iron and lipid damage may be weakly correlative and not causative. In NMRs, even in young animals, non-heme iron was high and increased with age. Elevated iron levels were not associated with greater oxidative damage, but rather with a reduction in both, rates of lipid damage generation and its accrual in liver. This pattern parallels that of energetically restricted mice, which also display attenuated oxidative damage accrual during aging, despite age-related increases in non-heme iron (Lass et al., 1998; Sohal et al., 1999). Our results from both mice and NMRs thus do not support the hypothesis that changes in iron during aging correlate with rates of oxidative damage accrual.

The disparate patterns of damage generation and accrual in mice and NMRs during aging may also result from age-associated changes in the amount of substrate available for peroxidative reactions. Lipid peroxidation rates are reportedly affected by substrate availability (Cutler, 1985), and thus age-associated changes in membrane content of arachidonic acid may have led to the observed patterns of isoprostane excretion and MDA accrual. Phospholipids membrane composition of NMRs, unlike that of mice (Imre et al., 2000), does not appear to change with age (Hulbert et al., unpublished data). This thus suggests that age-related declines in lipid peroxidation of NMRs do not reflect a reduction in arachidonic acid levels.

Differences in antioxidants between NMRs and shorter-living mice may provide an alternative explanation for higher lipid damage levels in the long-living species. Glutathione levels of NMRs are lower than in mice (Andziak et al., unpublished data) and the hepatic activity of cellular glutathione peroxidase (cGPx) is reportedly 0.014-times

that reported in mice, without a concomitant up-regulation of other antioxidant enzymes (Andziak et al., 2005). This suggests that NMRs may have a compromised ROS-neutralizing capacity, rendering these rodents susceptible to oxidative stressors. Indeed, preliminary studies in our lab suggest that NMRs, unlike other long-living species (Ogburn et al., 1998; Kapahi et al., 1999), are less tolerant of oxidative stressors and show lower survival than mice following treatment with diquat (Andziak et al., unpublished data). Parallel findings have been reported for cGPx knock-out mice that are more vulnerable than wild-type animals to diquat and paraquat (de Haan et al., 1998; Fu et al., 1999), yet their development and survival are not affected by hyperoxia (Ho et al., 1997). Similarly, longevity of mice that are deficient in Mn superoxide dismutase does not differ from that of normal animals, even though these mutants show higher levels of accrued oxidative damage (Van Remmen et al., 2003).

Regardless of putative causes for the observed differences in oxidative damage between mice and NMRs, our findings of considerably higher levels of lipid peroxidation in the longer living naked mole-rat are in conflict with the widely accepted Oxidative Stress theory. Urinary isoprostane levels of NMRs were 5 to 10-fold greater than in mice for all age-cohort comparisons, suggesting higher rates of lipid damage generation in this long-living species. Furthermore, accrued MDA levels in 30 and 75 month-old NMRs were approximately 2-fold higher than in physiologically age-matched mice. Our findings are divergent from previously published studies showing inverse correlations between susceptibility to peroxidation (Cutler, 1985) and/or lipid damage accrual (Pamplona et al., 1998) and species longevity. NMRs generate more damage than mice and accrue it in greater quantities early in life, yet nonetheless they are able to live with

this damage, routinely surviving and breeding for more than 25 years while 2y old mice have less than 2 more years of potential lifespan.

Conclusions

Our findings of high levels of oxidative damage generation and accrual, as well as high non-heme iron levels in tissues of the longest living rodent species, even at a young age do not support the key tenet of the Oxidative Stress theory that sustained low levels of oxidative damage are an important component of longevity. Rather other factors such as enhanced DNA and/or protein stability may be important indicators of species maximum lifespan potential. While age-associated increases in non-heme iron were evident, there was no concomitant age-related increase in markers of oxidative damage generation and accrual over at least a 10 year period, suggesting that these animals were better able to maintain steady states of accrued oxidative damage than mice are. Indeed the dramatic age-associated decline in urinary isoprostane excretion to less than one half that of young animals suggests that unlike most other mammals, as naked mole-rats age, rates of oxidative damage generation decline and steady state levels of damage are maintained. Although the mechanisms behind this are as yet unknown, this may be of considerable importance in retarding the aging process in this rodent species.

4.6 Acknowledgements

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CHAPTER 4: SYNOPSIS

Aging is a complex, yet universal process that is thought to result in progressive declines in physiological and reproductive function with increasing age. Extensive research on aging has led to the development of numerous ultimate and proximate theories explaining this process. In this doctoral project I tested a widely held proximate mechanism of aging, namely the Oxidative Stress Theory by comparing various biochemical markers associated with responses to oxidative stress in long-living naked mole-rats (*Heterocephalus glaber*, 28.3 years) and much shorter-living CB6F1 hybrid mice (*Mus musculus*, ~3.5 years). A central tenet of the Oxidative Stress Theory is that oxidative damage accumulates progressively with increasing age, leading to physiological declines that make older organisms increasingly susceptible to pathology and death. Factors that either facilitate or delay damage accumulation are thought to affect rates of aging and may thus account for the observed natural variation in species longevity.

This study examined if the mechanism proposed by the Oxidative Stress Theory can explain the 9-fold difference in longevity between similarly-sized mice and naked mole-rats. Using a comparative approach I tested six specific hypotheses to assess both inter-specific differences in oxidative damage and the putative mechanisms employed by the longer-living species in response to oxidative stress: Antioxidant levels during aging were compared to establish if naked mole-rats are better able to neutralize ROS and thereby prevent oxidative damage generation. Components of the cellular environment of naked mole-rats were analyzed to determine if it is less pro-oxidative than in mice and thereby establish if these long-living rodents experience less oxidative stress. Oxidative

DNA damage repair was quantified to assess if naked mole-rats are better able to remove incurred damage than mice. *In vivo* levels of oxidative damage during aging were compared to establish if naked mole-rats generate less oxidative damage than mice, as well as to determine if there are species differences in age-related profiles of damage generation. Levels of accrued oxidative damage to various biological molecules were quantified to establish if naked mole-rats indeed accumulate less damage than mice. In addition, age-associated patterns of damage accumulation were assessed. None of my comparative results supported predictions based on the Oxidative Stress theory. Collectively these data suggest that this widely held mechanistic theory of aging may not explain the pronounced difference in longevity between mice and naked mole-rats.

Antioxidants are a diverse group of enzymatic and non-enzymatic compounds that neutralize ROS by converting them into less reactive forms, and their levels would thus be predicted to be higher in long-living naked mole-rats than in mice. To test this I compared the activities of four antioxidant enzymes, as well as levels of a non-enzymatic antioxidant (reduced glutathione). Naked mole-rats do not possess a superior antioxidant suite when compared to mice, suggesting that their impressive longevity is unrelated to antioxidant defenses. While poor antioxidant defenses have been previously reported in long-living species and are thought to reflect low ROS generation rates (Barja, 2002), the near-absence of cGPx activity in naked mole-rats, combined with only moderate up-regulation of other antioxidant enzymes suggests that this long-living species may have poor ROS neutralizing capacity. The age-related antioxidant profiles differed in these two species; there were enzyme-specific activity changes in mice, while in naked mole-rats

activities of all antioxidants remained constant. This implies that age-related changes in antioxidants may not play a central role in the aging process of naked mole-rats.

Oxidative stress results from an imbalance between ROS generation and their removal by antioxidant defenses. The status of the cellular environment may affect levels of oxidative stress by making conditions either more or less favorable to ROS generation. I assessed if the cellular environment of naked mole-rats is less conducive to ROS generation than it is in mice, and followed this by comparing specific levels of oxidative stress in these two species. The finding that naked mole-rats had significantly more non-heme iron in liver tissue than mice, suggests that the cellular milieu of this long-living species is more favorable for pro-oxidative reactions and affords little protection from oxidative stressors. This finding was confirmed by higher levels of oxidative stress in naked mole-rats, as indicated by their lower glutathione redox pair (GSH:GSSG) in naked mole-rats. While these findings may reflect specific environmental differences between captive housing and their natural habitat, they nonetheless imply higher ROS generation rates in captive naked mole-rats. Despite this, naked mole-rats are able to survive well into their third decade in captivity.

Oxidative damage repair mechanisms remove oxidation products preventing their accrual in tissues. These mechanisms would be predicted to be especially efficient in naked mole-rats, thus limiting damage accumulation during their long-lives. This study compared DNA repair (urinary 8-hydroxy-2'-deoxyguanosine, 8-OH-dG) in these two rodent species. Urinary 8-OHdG content was lower in naked mole-rats than in mice at equal physiological ages, indicating less DNA repair in the long-living rodent. Various factors, including metabolic rate can affect urinary 8-OHdG excretion (Foksinski et al.,

2004). Metabolic rate reportedly declines with age in commonly studied rodent models of aging, and this may explain the disparate urinary 8-OHdG values in 4 and 24 month-old mice. Differences in metabolic rate can not alone explain the 3-fold difference in urinary 8-OHdG content between these two species at equal physiological ages, since murine metabolic rate is only 1.3-times greater than that of naked mole-rats. It is also possible that lower urinary 8-OHdG in naked mole-rats reflect attenuated oxidative DNA damage generation. However, given that oxidative stress levels are greater in naked mole-rats than in mice, this long-living species likely generates considerable amounts of oxidative damage. Low 8-OHdG excretion by naked mole-rats is thus likely due to poor damage repair and not low rates of damage generation. These findings are the opposite of what one would predict for an exceptionally long-living rodent.

The Oxidative Stress Theory predicts that long living species should have low rates of oxidative damage generation. This was assessed through a comparison between naked mole-rats and mice of age-related changes in a marker of *in vivo* lipid peroxidation (urinary isoprostanes). Naked mole-rat data for *in vivo* lipid peroxidation show that young animals incur considerably more oxidative damage than do similar aged mice. While this order of magnitude difference in isoprostane levels may reflect species differences in phospholipid membrane composition (Hulbert et al., in press), these findings demonstrate that naked mole-rats generate extensive amounts of oxidative damage. Urinary isoprostane levels remained constant in mice during aging, while in naked mole-rats they exhibited significant age-associated declines. Given these disparate age-related damage generation profiles, species patterns of damage accrual may therefore also differ between these two species.

Age-related declines in physiological function characteristic of the aging process may result from progressive accrual of oxidative damage. Long-living species are thought to exhibit slow rates of damage accrual that lead to attenuated rates of aging. To test this key tenet of the Oxidative Stress Theory levels of markers of DNA (8-OHdG), protein (protein carbonyls) and lipid (malondialdehyde) oxidation were compared in mice and naked mole-rats. Oxidative damage content of naked mole-rats was greater than that of mice for all damage markers assayed, suggesting that quantitative differences in this oxidative stress parameter do not explain the 9-fold difference in longevity between these two species. Since accumulated oxidative damage levels reflect a variety of factors upstream along the oxidative stress pathway, thus while not supporting the Oxidative Stress Theory, my results are perhaps not surprising. Specifically, when compared to mice, naked mole-rats have a similar antioxidant suite, higher levels of oxidative stress, poorer damage repair and elevated damage generation rates, which together would be expected to result in greater damage accumulation in this long-living rodent. High levels of oxidative damage, even at a young age, clearly do not affect physiological performance of naked mole-rats, since they are able to successfully function and reproduce into the third decade of life.

The age-related patterns of damage accrual were different in these two rodents. Rates of oxidative damage accumulation remained constant in mice, potentially leading to progressively greater damage levels in tissues. In naked mole-rats, however, damage accrual rates declined with age resulting in similar damage levels in both 75 and 130 month-old cohorts. This long-living species is thus able to attenuate damage accrual during life, maintaining it at steady levels over long-periods of time. This age-related

accrual profile may reflect a reduction in oxidation damage generation. Our findings therefore illustrate that progressive accrual of tissue damage with increasing age, as predicted by the Oxidative Stress theory, is not an inevitable component of organismal aging.

The current findings are in direct contrast to the plethora of previously published papers, suggesting that the Oxidative Stress Theory *per se* is not the mechanism responsible for lifespan differences among rodents. While this indeed may be the case, an extensive body of literature exists that show elevated oxidative damage in various animal models, as a consequence of age-related pathology (Mezzetti et al., 2000; Beal, 2002;), as well as in response to different experimental treatments that induce oxidative stress (Delanty et al., 1997; Kadiiska et al., 2005). Based on this literature it is most likely that oxidative stress parameters are, at least partially, involved in the aging process and an outright dismissal of this theory is premature. Instead, our results can provide insights in to the mechanisms, within the framework of this theory, that result in both greater oxidative damage content in these long-living rodents and the observed age-related patterns of damage accumulation in these two species.

The delayed rates of aging and extended longevity of naked mole-rats may reflect a number of evolved physiological adjustments to high levels of oxidative damage (Buffenstein, 2005). One of these adjustments may involve a delay in reaching a universal "threshold" associated with a certain amount of oxidative damage, beyond which organismal function rapidly deteriorates and leads to the development of pathology and/or a failure of a critical physiological systems (e.g., cardiovascular). To achieve this, naked mole-rats may have attenuated lifetime damage generation and/or greater damage

repair, when compared to shorter-living mice. Alternatively, oxidative damage may have lesser physiological effects on naked mole-rats than it does on mice. This could be due either to an elevated damage "threshold" or an increased physiological tolerance of damage in these long-living rodents. Consequently, the disparate longevity of these two species may be unrelated to differences in absolute oxidative damage levels, even though damage accumulation can still affect their physiological function and be a component of their respective aging processes.

Oxidative damage levels present at any given instant in an organism's life reflect a difference between the amount of damage generated up to that point, and the proportion of it, which has been efficiently repaired. Rates of damage generation may differ during life due to age- and/or environment-specific variation in oxidative stress, as well as age-related changes in organismal repair mechanisms (Martinez-Vicente et al., 2005). It is thus possible that rates of oxidative damage accrual vary throughout life, resulting in non-linear age-associated profiles of damage accumulation. Although the details of age-related damage profiles may differ between species, the common pattern should be universal and correspond to three principal stages; a) development, b) adulthood, and c) old age.

The developmental period between birth and adulthood is very likely characterized by significant oxidative damage accumulation. At birth, organisms are introduced in to greater ambient oxygen concentrations than those encountered *in utero*, which when coupled with the absence of fully functional antioxidant defenses and damage repair mechanisms can lead to high ROS generation and oxidative damage accrual. Oxidative stress experienced by laboratory-reared naked mole-rats may be

especially great given the disparity in oxygen concentrations between the laboratory and their natural subterranean habitat. Indeed, highest levels of damage generation in our study were in the two youngest age cohorts, likely resulting in these animals having twice the levels of accrued damage as age-matched mice. Additionally, elevated rates of oxidative damage generation during development may be a consequence of elevated metabolic rates necessary for normal growth. The fact that oxidative lipid damage accumulated by our mice and naked mole-rats, presumably, during the first 10% of their lifespan was greater than that accrued in the subsequent 40% of their natural longevity, supports the importance of early-life events on oxidative damage levels.

Adulthood is a period of life associated with reproduction. Therefore, it is likely that oxidative damage levels are maintained below the "threshold" associated with declines in organismal function, which if reached, may restrict reproductive output. To do this organisms rely on fully functional defense mechanisms that not only allows them to cope with steady-state rates of ROS and damage generation, but also provides them with a reserve to endure periods of increased oxidative stress brought on by stochastic events. This premise may also hold true for naked mole-rats, even though most colony members never breed, since individuals maintain a capacity throughout adult life to become breeders. Specifically, in colonies with a distinct age structure the large females that compete for breeding status, when the previous breeding female is removed, are usually the original daughters of the founder pair. In naked mole-rat males, the frequency of disperser morph individuals that are readily accepted in to a novel colony and likely to become breeders is proportionately greater among older adults (7 to 15 years). Rates of damage accrual during adulthood should thus be moderate. Indeed, damage to DNA and

proteins for which damage repair mechanisms have been well established in literature, increase only moderately during adulthood (Hamilton et al., 2001*b*; Levine and Stadtman, 2001). However, because of the self-propagating nature of peroxidative reactions and the apparent absence of repair mechanisms, lipid damage is more likely to increase with age. This may explain our observed age-related damage profiles in mice and suggests age-related declines in function of this species may result from an excessive accrual of lipid damage (Hulbert, 2005).

In commonly studied models of aging research, old age is characterized by attenuated physiological and reproductive function, increased frequency of pathology, as well as accelerated rates of oxidative damage accrual. While increased damage accumulation during old age may be due to augmented ROS generation, declines in damage repair are also likely to be involved in this phenomenon. The effect of age-related declines in oxidative damage repair can be two-fold. A direct consequence of declining repair is an increasingly skewed imbalance between damage generation and its removal that may lead to increased damage levels. As products of oxidative damage accumulate, those that themselves are biologically active could initiate novel oxidative reactions. In addition, oxidation products can reportedly impair repair mechanisms (Grune et al., 2004). These products would further accelerate damage accumulation. Our present data on oxidative damage accrual unfortunately do not contain any animal cohorts that are older than two-thirds of their species longevity, a point beyond which these rapid increases in damage levels reportedly occur. It is thus presently difficult to definitively assess if naked mole-rats adhere to this proposed mechanism as they get older, warranting further studies addressing this important issue.

Although we did not have accrued oxidative damage data, the lipid damage generation (i.e., urinary isoprostanes) component of our study included a senescent naked mole-rat cohort that had lived to 85% of its species' longevity. Surprisingly, this very old cohort had quantitatively lower isoprostane levels than those observed in young and adult animals. The observed declines in damage generation may be a response by naked mole-rats to high damage content incurred early-life and suggests that during life this species maintains damage at steady-state levels, and below the "threshold" that results in impaired function. The oldest individuals on record were breeders implying that even though they were chronologically very old, they were not necessarily "physiologically" old. What is classically defined as "old age" likely occurs beyond what is presently considered of as their maximum life span and strongly suggests that the longevity of these mouse-sized rodents extends into the fourth decade.

In conclusion, the results from our study testing the Oxidative Stress Theory strongly suggest that this widely held mechanism may not explain the 9-fold difference in longevity between these two species. While the Oxidative Stress Theory is still likely to be involved in aging, its exact effects on longevity are probably species specific. Consequently, future comparative studies should consider species differences in age-related patterns of damage accrual and appreciate the likely contribution of other mechanisms to this process. Our results demonstrate that naked mole-rats are a formidable model for testing the various extant theories of aging, with a potential to challenge prevailing intellectual frameworks, generate novel ideas, and stimulate debate, which when resolved should result in a greater understanding of the aging process.

CHAPTER 6: FUTURE STUDIES

Our present results suggest that the Oxidative Stress Theory may not explain the 9-fold difference in maximum longevity between mice and naked mole-rats. Given the obvious significance of our data for the field of the biology of aging, our exploratory study should supplemented by future, more detailed experiments comparing oxidative stress parameters in naked mole-rats and shorter-living mice. Only then will it be possible to assert whether or not naked mole-rats are indeed an exception to the Oxidative Stress Theory, or if rather our present data reflect adjustments in various components of this mechanism that delay the aging process in these rodents.

The Oxidative Stress Theory predicts that oxidative damage progressively accrues with increasing age, although it is likely that rates of damage accumulation may differ during different stages of life. While our study focused on adult animals, future experiments could compare oxidative stress parameters in developing and senescent individuals that may not posses fully functional antioxidant defenses and damage repair mechanisms. Together with our current data, results of such studies would illustrate if damage accumulates progressively throughout life, and if its age-related profile differ between long-living naked mole-rats and mice.

The findings of the current project are inconsistent with both the Oxidative Stress theory, as well as previous comparative studies relating longevity to specific rates of ROS generation (Barja et al., 1994; Brunet-Rossinni, 2004). Although our data imply that long-living naked mole-rats generate more ROS than mice, the evidence for this assertion is indirect (i.e., similar antioxidant suite, yet lower GSH/GSSG ratios). Future experiments could directly compare ROS generation in these two species through assays

of either superoxide and/or hydrogen peroxide production in different tissue preparations (i.e., whole and mitochondrial). If indeed naked mole-rats generate higher and/or equal amounts of ROS as shorter-living mice, then this would corroborate our present results and strongly suggest that these long-living rodents are an exception to the Oxidative Stress Theory. However, if naked mole-rats have lower ROS-generation rates than mice then such an outcome, while not detracting from our findings that differences oxidative damage do not explain the disparate longevity in these two species, would warrant further studies aimed at elucidating the causes for higher levels oxidative damage naked mole-rats.

Our results imply that naked mole-rats can tolerate considerable oxidative damage, effectively surviving for nearly three decades with high levels of damage accrued early in life. Furthermore, our GSH/GSSG data also suggests that naked mole-rats can tolerate high levels of oxidative stress. However, cGPx activity of these rodents parallels that reported for cGPx knock-out mice, which are also extremely vulnerable to various oxidative stressors (de Haan et al., 1998). Treatment of mice and naked mole-rats with an oxidative stressor (e.g., diquat), followed by measurements of oxidative stress (i.e., GSH/GSSG ratio), as well as of oxidative damage (i.e., isoprostanes, MDA, mt protein carbonyls and mt 8-OHdG) may establish if their cGPx deficiency have a functional consequence.

The population of naked mole-rats used in this study was reared in the laboratory under hyperoxic conditions, likely augmenting their ROS generation rates when compared to animals living in their natural subterranean habitat. A comparison of both oxidative stress and oxidative damage markers in tissues from captive-reared and wild-

caught naked mole-rats would address if the high levels of oxidative damage observed in our study population resulted from life in the laboratory.

Antioxidants can affect levels of organismal oxidative stress by neutralizing ROS and/or converting them to more benign forms. Very low glutathione peroxidase activity in naked mole-rats, without a concomitant up-regulation of other antioxidant enzymes, as well as comparatively low glutathione levels, suggest a depressed glutathione cycle in this long-living species. Future studies could explore this further, by comparing other components of the glutathione cycle in naked mole-rats and mice. Such studies could include comparisons of glutathione reductase activity, reflecting the ability to re-cycle glutathione into its active reduced state, as well as measurements of phospholipid hydroperoxide glutathione peroxidase (GPx4) activity, which is the enzyme responsible for the removal of hydroperoxides out of phospholipids *in situ*. Furthermore, the current experimental design may be extended to include comparisons between naked mole-rats and guinea pigs, which are another Hystricognath species with reportedly low cGPx activity. This may thus establish if observed differences in the glutathione cycle between mice and naked mole-rats are specific to naked mole-rats or if instead they are linked to Hystricognath phylogeny.

Organismal oxidative damage repair mechanisms restrict damage accrual by removing oxidation products, and therefore species differences in their efficacy can result in disparate rates of damage accumulation. Lower urinary 8-OHdG excretion in naked mole-rats than mice implies that this long-living species has poor DNA damage repair. Our present findings could be extended by a series of more detailed experiments assessing not only specific DNA repair mechanisms, but also those involved in removing

other types of oxidative damage. Although the exact mechanisms of lipid damage repair still remain to be elucidated, there are a number of known pathways responsible for removal of DNA damage (Bohr, 2002; Cooke et al., 2005) and oxidized proteins (Martinez-Vicente et al., 2005). Species comparisons of DNA glycosase activities (e.g., oxoguanine DNA glycolase 1 or its mitochondrial isoform mitochondrial Oxidative Damage Endonuclease) may establish if the observed differences in 8-OHdG between naked mole-rats and mice, result from lower Base Excision Repair (BER) in the long-living rodent. Meanwhile, comparisons of either the autophagic and/or the ubiquitin-proteosome repair systems in these two species may ascertain if protein damage repair of naked mole-rats is also lower than it is in mice. Together these findings can explain if disparate DNA and protein damage repair between two species can explain our observed difference in oxidative damage accrual.

Peroxidative reactions of arachidonic acid result in the formation of both isoprostanes and malondialdehyde, and thus it is possible that disparate levels of these oxidative markers in naked mole-rats and mice reflect differences in arachidonic acid content of their phospholipid membranes. Substrate availability affects the kinetics of peroxidative reactions of lipids (Cutler, 1985), and reportedly naked mole-rat membranes have relatively higher proportions of n-6 PUFAs (e.g., arachidonic acid) than mice (Hulbert et al., in press). Future studies, may thus focus on comparing lipid peroxidation products of n-3 PUFAs (e.g, docosahexanoic acid), which more abundant in mice (Hulbert et al., in press). Neuroprostanes are a peroxidation product of docosahexanoic acid and comparisons of its levels in mice and naked mole-rats could establish if our results indeed reflect species differences in membrane composition or if in fact lipid

damage generation and accrual are greater in naked mole-rats regardless of the marker assayed.

These suggestions for future work are specific to further exploring and testing the oxidative theory of aging. Obviously, this exceptionally long-lived rodent may prove to be a useful animal model with which to test all the current and future theories of aging, for it certain that animals with exceptional aging can and will provide useful insights into how and why we age.

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