Intracellular antioxidant and DNA repair enzymes as correlates of stress resistance and longevity in vertebrates

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Abstract

In animals, both stress resistance and longevity appear to be influenced by the insulin/insulin-like growth factor-1 signaling (IIS) pathway, the basic organization of which is highly conserved from invertebrates to vertebrates. Reduced IIS or genetic disruption of the IIS pathway leads to the activation of forkhead box transcription factors, which is thought to upregulate the expression of genes involved in enhancing stress resistance, including perhaps key antioxidant enzymes as well as DNA repair enzymes. Enhanced antioxidant and DNA repair capacities may underlie the enhanced cellular stress resistance observed in long-lived animals, however little data is available that directly supports this idea. I used three experimental approaches to test the association of intracellular antioxidant and DNA base excision repair (BER) capacities with stress resistance and longevity: (1) a comparison of multiple vertebrate endotherm species of varying body masses and longevities; (2) a comparison of long-lived Snell dwarf mice and their normal littermates; and (3) a comparison of hypometabolic animals undergoing hibernation or estivation with their active counterparts. The activities of the five major intracellular antioxidant enzymes as well as the two rate-limiting enzymes in the BER pathway, apurininc/apyrimidinic (AP) endonuclease and polymerase β, were measured. These measurements were performed in one or more of the following: (1) cultured dermal fibroblasts; (2) brain tissue; (3) heart tissue; (4) liver tissue. My results indicate that antioxidant enzymes are not universally upregulated in association with enhanced stress resistance and longevity. I also did not find that BER enzyme activity was positively correlated with longevity, in an inter-species context, though there was evidence for enhanced BER in long-lived Snell dwarf mice. Thus, while there were
instances in which enhanced antioxidant and BER enzyme activities were associated with increased stress resistance and/or longevity, this was not universally the case, indicating that other mechanisms must be involved. These results suggest the need to re-examine existing 'oxidative stress' hypotheses of longevity and probe further into the molecular physiology of longevity to discover its mechanistic basis.
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Table of Contents

Chapter 1. Literature Review.................................................................................................................. 13
  1.1 Introduction ..................................................................................................................................... 13
  1.2 Inter-species comparisons ............................................................................................................... 16
  1.3 Intra-species comparisons ............................................................................................................ 18
  1.4 Stress resistance as a key characteristic of longevity ................................................................. 21
  1.5 Stress resistance, longevity and IIS ............................................................................................... 22
  1.6 Snell dwarf mice – an intra-species model of stress resistance and longevity ......................... 24
  1.7 Quiescence and longevity ............................................................................................................... 26
  1.8 Putative molecular mechanisms of longevity ............................................................................... 27
  1.9 Oxidative stress and damage ......................................................................................................... 28
  1.10 Intracellular antioxidant enzymes ............................................................................................. 30
    1.10.1 MnSOD ........................................................................................................................................ 32
    1.10.2 CuZnSOD ................................................................................................................................. 32
    1.10.3 GPx and GR ............................................................................................................................ 33
    1.10.4 CAT .......................................................................................................................................... 34
  1.11 DNA damage and repair .............................................................................................................. 34
    1.12 BER .............................................................................................................................................. 35
    1.12.1 8-oxoguanine DNA glycosylase ............................................................................................ 36
    1.12.2 APE ......................................................................................................................................... 38
    1.12.3 Po1β .......................................................................................................................................... 38
    1.12.4 DNA ligase III ......................................................................................................................... 39
  1.13 Hypotheses and Approach .......................................................................................................... 39

Chapter 2. Plasma IGF-1 is negatively correlated with body mass in a comparison of 36 mammalian species ........................................................................................................................................................................... 41
  Hypothesis ............................................................................................................................................... 41
  Objective ................................................................................................................................................ 41
  Publications of results .......................................................................................................................... 41
  Contributions ......................................................................................................................................... 41
  2.1 Introduction ..................................................................................................................................... 42
  2.2 Experimental Procedures .............................................................................................................. 43
  2.3 Results ............................................................................................................................................ 44
  2.4 Discussion ...................................................................................................................................... 54
Chapter 3. Antioxidant enzyme activities are not broadly correlated with longevity in 14 vertebrate endotherm species

Hypothesis
Objective
Publications of results
Contributions
3.1 Introduction
3.2 Experimental Procedures
  3.2.1 Materials
  3.2.2 Animals
  3.2.3 Tissue collection
  3.2.4 Tissue homogenization
  3.2.5 Immunodetection of MnSOD and CuZnSOD in Snell mouse tissues
  3.2.6 In-gel superoxide dismutase assay
  3.2.7 Other enzyme assays
  3.2.8 Statistical analyses
3.3 Results
3.4 Discussion
3.6 Supplemental Data

Chapter 4. DNA base excision repair in mammals and birds is negatively correlated with body mass, but has no relationship to species maximum lifespan

Hypothesis
Objective
Contributions
4.1 Introduction
4.2 Experimental Procedures
  4.2.1 Materials
  4.2.2 Animals
  4.2.3 Tissue collection
  4.2.4 Isolation of nuclear fractions
  4.2.5 Base Excision Repair Assays
  4.2.6 Statistical analyses
4.3 Results
4.4 Discussion
Chapter 5. Mechanisms of stress resistance in Snell dwarf mouse fibroblasts:

Hypothesis ................................................................. 114
Objective ................................................................. 114
Publications of results ............................................. 114
Contributions ........................................................... 114

5.1 Introduction ....................................................... 115
5.2 Experimental Procedures .................................... 117
  5.2.1 Materials ......................................................... 117
  5.2.2 Preparation of Snell Mice Dermal Fibroblasts .... 118
  5.2.3 Preparation of Cell Lysates .............................. 119
  5.2.4 Western Blots ............................................... 119
  5.2.5 Antioxidant and Metabolic Enzyme Assays ....... 119
  5.2.6 Base Excision Repair Assays ........................... 120
  5.2.7 Assessment of mitochondrial abundance and membrane potential ... 120
  5.2.8 Statistical Analysis ........................................ 121
5.3 Results ............................................................... 121
  5.3.1 Antioxidant and BER enzymes in normal and dwarf mouse fibroblasts in complete and serum-free media ......................................................... 121
  5.3.2 Effects of exogenous oxidants on antioxidant and BER enzymes .......... 126
  5.3.3 Mitochondrial and fermentative metabolism in normal and dwarf mouse fibroblasts ................................................................. 128
5.4 Discussion ........................................................ 131
5.6 Supplemental Data .............................................. 139

Chapter 6. Contribution of intracellular antioxidant enzymes to stress resistance during hibernation ................................................................. 140
Hypothesis ................................................................. 140
Objective ................................................................. 140
Publications of results ............................................. 140
Contributions ........................................................... 140
6.1 Introduction ....................................................... 141
6.2 Experimental Procedures .................................... 144
  6.2.1 Materials ......................................................... 144
  6.2.2 Animals ......................................................... 144
  6.2.3 Tissue Homogenization .................................... 145
Chapter 7. Contribution of intracellular antioxidant enzymes to stress resistance during estivation

Hypothesis

Objective

Publications of results

Contributions

7.1 Introduction

7.2 Experimental Procedures

7.2.1 Materials

7.2.2 Animals

7.2.3 Tissue collection and homogenization

7.2.4 Western blots

7.2.5 In-gel superoxide dismutase assay

7.2.6 Other enzyme assays

7.2.7 Protein Carbonyl assay

7.2.8 Statistical analysis

7.3 Results

7.4 Discussion

Chapter 8. General discussion

IIS and antioxidant enzymes in stress resistance and longevity

DNA repair in stress resistance and longevity

Are all cells considered equal?

Molecular mechanisms of stress resistance in the Snell dwarf mouse

Molecular mechanisms of stress resistance in long-lived vertebrate species

Molecular mechanisms of stress resistance in hibernation and estivation

Conclusion

References
List of Tables

Chapter 2
Table 2.1: Plasma IGF-1 values used in this analysis.......................................................... 47
Table 2.2: Adult body mass, maximum lifespan (MLSP) and mean plasma total .......... 51

Chapter 3
Table 3.1: Sex, age, mass and maximum lifespan (MLSP) of the mammalian and avian
species. Species MLSP data are from AnAge (de Magalhaes et al., 2005)..................... 67
Table 3.2: Statistical analysis of linear regressions, enzyme activity as a function of
lifespan.................................................................................................................................. 78
Table S3.1: Statistical analysis of linear regressions, enzyme activity as a function of
mass...................................................................................................................................... 89
Table S3.2: Statistical analysis of linear regressions, CS activity as a function of MLSP.
........................................................................................................................................... 89
Table S3.3: Statistical analysis of linear regressions, CS activity as a function of body
mass...................................................................................................................................... 89

Chapter 4
Table 4.1: Sex, age, mass and maximum lifespan (MLSP) of the mammalian and avian
species.................................................................................................................................. 100
Table 4.2: Oligonucleotides used to measure BER enzyme activity.............................. 101
Table 4.3: Results of correlational analysis of enzyme activities as a function of lifespan.
............................................................................................................................................. 106
Table 4.4: Results of correlational analysis of enzyme activities as a function of body
mass........................................................................................................................................ 106

Chapter 7
Table 7.1: Changes in antioxidant expression and/or activity during estivation.......... 175
List of Figures

Chapter 1

Fig. 1. 1: Insulin/IGF-1 signalling within *C. elegans, D. melanogaster* and *M. musculus.* .................................................................................................................................................. 24

Fig. 1. 2: GPx uses reduced glutathione (GSH) as a source of electrons to reduce H$_2$O$_2$ to water .................................................................................................................................. 31

Fig. 1. 3: 8-oxodG, which is the most common form of oxidative damage, is removed from DNA by OGG1. ........................................................................................................ 37

Chapter 2

Fig. 2. 1: Phylogeny of 36 mammalian species included in the data set. ........................................... 45

Fig. 2. 2: Plasma total IGF-1 concentrations are negatively correlated with species’ adult body mass .................................................................................................................................. 52

Fig. 2. 3: Re-analysis of data set using Felsenstein’s phylogenetically independent contrasts (FIC) .................................................................................................................................. 53

Fig. 2. 4: Correlation of MLSP with plasma total IGF-1 concentration ........................................... 55

Fig. 3. 1: Phylogeny of the 14 species used in this study. ................................................................. 64

Fig. 3. 2: CuZnSOD does not correlate with lifespan ........................................................................... 74

Fig. 3. 3: Correlation between MnSOD activity and animal MLSP .................................................. 77

Fig. 3. 4: Correlation between CAT activity and animal MLSP ...................................................... 79

Fig. 3. 5: GPx activity does not correlate with animal MLSP .......................................................... 80

Fig. 3. 6: GR activity does not correlate with animal MLSP .......................................................... 81

Chapter 3 Supplemental

Fig. S3. 1: Correlations between MnSOD activity and species MLSP ............................................. 90

Fig. S3. 2: The activity of brain and heart CAT ............................................................................... 91

Fig. S3. 3: Citrate synthase activity in the human is relatively moderate compared to the other species in this data set ......................................................... 91
Chapter 4

Fig. 4.1: Phylogeny of the 15 species used in this study. .............................................. 96

Fig. 4.2: APE activity does not correlate with MLSP....................................................... 102

Fig. 4.3: Polβ nucleotide incorporation activity negatively correlates with MLSP..... 104

Fig. 4.4: Incorporation activity negatively correlates with species body mass. .......... 106

Fig. 4.5: Incorporation plus ligation activity negatively correlates with species MLSP and body mass........................................................... 107

Fig. 4.6: Significant negative correlation between 8-oxodG levels within heart nuclear DNA and species body mass........................................ 110

Chapter 5

Fig. 5.1: Antioxidant protein levels and activities in normal and Snell dwarf mouse skin derived fibroblasts cultured at physiological (3%) and standard (20%) oxygen in complete and serum-free media..................................................... 123

Fig. 5.2: Base excision repair activities of normal and Snell dwarf mouse skin derived fibroblasts cultured at physiological (3%) and standard (20%) oxygen conditions in complete and serum-free media..................................................... 125

Fig. 5.3: Effect of paraquat and hydrogen peroxide on antioxidant protein levels and activities of normal and Snell dwarf mouse skin derived fibroblasts cultured at standard (20%) oxygen conditions in serum-free media. ..................................................... 127

Fig. 5.4: Effect of paraquat and hydrogen peroxide on base excision repair activities of normal and Snell dwarf mouse skin derived fibroblasts cultured at standard (20%) oxygen conditions in serum-free media..................................................... 128

Fig. 5.5: Mitochondrial content and membrane potential in normal and Snell dwarf mouse skin derived fibroblasts cultured in complete and metabolically stressful conditions................................................................. 130

Chapter 5 Supplemental

Fig. S5.1: Genotype does not affect MnSOD protein levels that have been corrected for mitochondrial abundance (COX activity).......................................................... 139

Chapter 6

Fig. 6.1: Immunodetection of MnSOD and CuZnSOD squirrel tissues. ...................... 147
Fig. 6. 2: MnSOD and CuZnSOD protein levels in squirrel tissues are unaltered during hibernation. ................................................................. 148

Fig. 6. 3: MnSOD and CuZnSOD activities are unaltered during hibernation. .......... 149

Fig. 6. 4: Glutathione peroxidase, glutathione reductase and catalase activities are unaltered during hibernation. ........................................ 150

Chapter 7

Fig. 7. 1: Representative Western blot showing MnSOD and CuZnSOD levels in brain tissue from control, fasted and estivating lungfish. ................................................................. 164

Fig. 7. 2: MnSOD and CuZnSOD protein levels in heart and brain tissue of lungfish. .. 165

Fig. 7. 3: MnSOD protein activities in heart and brain of lungfish. ......................... 166

Fig. 7. 4: Catalase, glutathione peroxidase and glutathione reductase protein activities measured in heart and brain of lungfish. ................................................................. 168

Fig. 7. 5: Levels of oxidative damage in heart and brain tissues of control, estivating and fasted lungfish. ................................................................. 169
Chapter 1. Literature Review

1.1 Introduction

In the wild, lifespan is highly dependent on the prevalence of disease, adequate nutrition and predation. Smaller animals, which are particularly susceptible to predation, have generally evolved to mature and reproduce relatively quickly and this is associated with relatively shorter lifespans. In contrast, larger animals, which tend to be less vulnerable to predation, have slower rates of maturation, take longer to reach reproductive maturity and have longer lifespans. In protected environments, differences in species’ lifespans are equally striking, and persist even under relatively sterile conditions, when disease load is minimal, nutrition is excellent and predation is absent. It is often under the latter conditions that maximum lifespan (MLSP) is determined. Although MLSP measurements refer to a single time point in a single animal, these measurements are increasingly used within comparative studies. To improve the reliability of MLSP measurements, the free database AnAge (de Magalhães et al., 2005), which is the main resource of MLSP data has recently included estimates of the samples sizes in which MLSP measurements have been derived (de Magalhães and Costa, 2009). This includes sample sizes for captive and when possible wild animals. MLSP is considered to reflect the intrinsic rate of aging in the absence of detrimental extrinsic forces. In comparison, mean lifespan for a species varies with nutritional status, disease load and predation. Although species mean and maximum lifespans are readily determined, and we have some insight into their ecological explanations, we know surprisingly little about how increased longevity has been achieved in animal species in
which it has evolved. Similarly, our mechanistic understanding of longevity within a species, such as humans, is in an early stage.

Numerous hypotheses have been advanced over nearly a century of research to explain the mechanistic basis of longevity in animals. Rubner (1908) noted an inverse relationship between energy metabolism and body mass, and that larger animals lived longer. Upon multiplying the mass-specific rate of energy expenditure by species maximum lifespan (MLSP) he concluded that a gram of body tissue expends approximately the same amount of energy during the lifespan regardless of the size of the animal, and inferred that energy expenditure per lifespan may be a fixed quantity. This idea suggests that the quicker energy is used up the faster death will arrive, which Pearl (1928) proposed as his ‘rate-of-living’ theory. Over time, evidence has weakened this theory: for example, mass-specific metabolic rates within bird and bat species are relatively high despite their long lifespans (see Speakman, 2005a; Austad, 2010, for reviews). Similarly, caloric restriction, which is the most widely recognized lifespan extending intervention, does so without reducing mass-specific metabolic rates in fruit flies, mice and rats (reviewed by Hulbert et al., 2007). In fact, in some organisms mass-specific metabolic rate is increased by caloric restriction (Masoro et al., 1982; Houthoofd et al., 2002; Lin et al., 2002).

Even with the above limitations, metabolism is still believed by some to influence longevity (reviewed by de Magalhães et al., 2007). For instance, evidence suggests a relationship between mass-specific metabolic rate and the level of polyunsaturated fatty acids within cellular membranes (Gudbjarnason et al. 1978; reviewed by Hulbert et al., 2007), of which the latter negatively correlates with lifespan. These relationships sparked
the membrane pacemaker theory of aging (reviewed by Pamplona et al., 2002; Hulbert, 2003). Unfortunately, the pacemaker hypothesis of aging has recently come under criticism due to the failure to remove the confounding effects of species body mass and phylogeny (see below; Speakman, 2005b; Valencak and Ruf, 2007).

Another mechanism believed to correlate with animal longevity includes replicative cellular senescence. Röhme (1981) observed a positive correlation between fibroblast population doublings and MLSP of eight mammalian species; however this study is weakened with the use of a small sample size (see below). Recently a correlative study with eleven mammalian species revealed a positive relationship between replicative senescence and body mass (Lorenzini et al., 2005), suggesting that the original correlation between replicative senescence and MLSP reported by Röhme was primarily a function of body mass. A mechanism by which replicative senescence is limited is the erosion of telomeres; however telomere shortening can be avoided via increased activity of telomerase. Interestingly, Seluanov et al. (2007) found that telomerase activity negatively correlates with body mass in 15 rodent species. Together, the data do not support one another, if indeed telomerase activity is believed to be a preventative mechanism of replicative senescence. Furthermore, due to the nature of these experiments, very little is known about how this relates to in vivo conditions. Cells are routinely cultured at atmospheric oxygen levels (20%) which have been shown to induce senescence in mouse embryonic fibroblasts (MEFs); in contrast, MEFs do not senesce when cultured at more physiological oxygen levels (3%) (Parrinello et al., 2003).

Longevity and lifespan are influenced by processes that are multifactorial and complex, and likely the above mechanisms among other factors are responsible for
influencing lifespan. Genetic modifications have revealed several putative ‘longevity
genes’ that confer increased lifespan within some model organisms. To date, such
longevity genes include those whose protein products are involved in cellular
maintenance, protection and/or repair (reviewed by Kuningas et al., 2008).

Inter-specific and intra-specific comparisons are two widely used approaches to
test the above mechanistic basis of animal species longevity. The inter-specific approach
relies on comparing the longevity trait of interest between different animal species. In
contrast, the intra-specific approach often relies on genetic manipulation; however
includes comparing the effects of caloric restriction. These two approaches address
different longevity related questions; whereas inter-specific comparisons primarily
examine mechanisms that have co-evolved with longevity, genetic modifications
addresses which mechanisms can increase or decrease animal longevity.

1.2 Inter-species comparisons

Inter-species comparisons are necessary to address the basic question of how
longevity has evolved in animals. Applied correctly, the comparative approach can
identify individual molecular character traits that have co-evolved with longevity and
therefore may have played a permissive role. This approach is certainly not novel.
Comparative studies of putative longevity genes have been done for decades (e.g. Hart
and Setlow, 1974). However, many such comparisons have included only two or three
species (Ku and Sohal, 1993; Herrero and Barja, 1998; Brunet-Rossini 2004; Pérez et
al., 2009a; Salmon et al., 2009), an approach with little or no statistical validity (Garland
and Adolph, 1994). It is highly unlikely that all selective pressures will be based around
longevity; rather, many environmental factors will contribute to evolved differences in
molecular traits. Thus, it is not possible to conclude that any trait differing between two species must be related to differences in longevity. Nonetheless, two species comparisons continue to appear in the literature, often focusing on species with extreme longevity for their size, such as bats and naked mole rats (Brunet-Rossini 2004; Pérez et al., 2009a; Salmon et al., 2009).

One way to overcome the limitations inherent in two species comparisons is to simply include more species and test for correlations. Correlations between longevity and specific character traits require at least three data points because the degrees of freedom are N-2 (a statistical correlation between two species cannot be calculated because the final degrees of freedom would equal zero; reviewed by Garland and Adolph, 1994). However, studies with a low number of species will be negatively affected by the increased probability of occasional outlying data points biasing the results and potentially leading to the acceptance of a hypothesis that should be rejected.

Comparative studies using multiple species have been used to examine relationships between lifespan and; 1) mitochondrial ROS production (Labinskyy et al. 2006; Lambert et al. 2007), 2) cellular resistance to a variety of exogenous stressors (Kapahi et al. 1999; Salmon et al. 2008), 3) antioxidant enzyme activities (Tolmasoff et al. 1980; Cutler, 1991; Sohal et al. 1990; Brown and Stuart 2007), 4) DNA nucleotide excision repair (Hart and Setlow, 1974; Francis et al. 1981; Hall et al. 1984; Licastro and Walford, 1985), and 5) mitochondrial cysteine content (Moosmann and Behl, 2008). Unfortunately, limitations associated with some of these multi-species comparisons have prevented their confident interpretation. For example, López-Torres et al. (1993) suggested a negative correlation between liver glutathione peroxidase (GPx) activity and
MLSP; however, three of the eight species used in this study were ectotherms and the measured enzyme activities were not corrected for the relatively low body temperature of those species relative to the mammals included in the study, temperature has been shown to influence the rate of GPx activity (Doucet-Beaupré et al., 2010). Two other major limitations of comparative studies of longevity done to date have been the failure to account for the confounding effects of body mass and the non-independence of data points representing individual species, due to their phylogenetic relatedness (see Speakman 2005b for review). Many biological traits correlate with body mass, and body mass in turn correlates with species MLSP (Speakman, 2005b). Therefore MLSP will correlate with traits that are also related to body mass. This problem can be addressed by correlating the residuals calculated from the regression line of MLSP and body mass with the residuals calculated from the regression line of MLSP and the trait of interest (Speakman, 2005b). All extant animals share their evolutionary history with distinct common ancestors, and therefore individual animal species are only approximations of independent data points, which violates a key assumption of the correlative approach. An appropriate approach, which has been used in some comparative studies of longevity (de Magalhães et al., 2007; Lambert et al., 2007; Moosmann and Behl, 2008) involves the transformation of raw data into Felsenstein’s phylogenetic independent contrasts (FIC; Felsenstein, 1985) to account for phylogenetic relatedness. Despite the limitations outlined above, which can be overcome by simple modifications to study design and data analysis, the comparative approach has tremendous value as a means of identifying molecular traits that have co-evolved with longevity.

1.3 Intra-species comparisons
Much of the interest in aging research lies in discovering strategies for successful and healthy lifespan extension in humans. This type of study typically focuses on a single 'representative' species to test putative longevity traits. Intra-specific comparisons benefit from greatly reduced genetic variability (particularly compared to inter-specific studies) and the ability to strictly control dietary and environmental conditions. Much of the literature describing intra-species comparisons describes the use of genetic manipulation to increase or decrease the expression of putative longevity genes. These studies are invaluable and can offer insight into how specific genes can influence lifespan within a particular species (see below). However, the results generated using this approach need to be interpreted with caution due to several weaknesses associated with this methodology.

One concern with the majority of intra-specific studies is their use of highly inbred laboratory model organisms, such as *Caenorhabditis elegans*, *Drosophila melanogaster* and *Mus musculus*. Laboratory mice, for example, are routinely selected for early reproductive maturity and large litter size. This selective pressure appears to inversely correlate with lifespan; in fact wild caught mice reach sexual maturation more slowly and live longer than laboratory reared mice (Miller et al., 2002). Inbreeding may therefore remove from the mouse genome alleles that function to slow the rate of aging, and modifications that appear to lengthen lifespan may simply be the restoration of a compromised genetic background. In addition, the reliance on highly inbred model organisms has generated inter-laboratory variability, which has often been due to genetic drift in genetically isolated strains (Harrison et al., 2009). For example, copper zinc superoxide dismutase (CuZnSOD) overexpression in *D. melanogaster* appears to increase
maximum lifespan only when the strain used is relatively short-lived (e.g. *w^{118}*; Sun and Tower, 1999), suggesting the restoration of something lacking in short-lived strains (reviewed by Orr and Sohal, 2003). Similarly, Hu et al. (2007) reported that the overexpression of manganese SOD (MnSOD) in mice increased maximum lifespan, a finding which conflicts with data presented by several authors (Jang et al., 2009; Pérez et al., 2009b; Pérez et al., 2009c), who found no extension in lifespan. The latter authors propose that the discrepancy might be related to the fact that the mice in the former study were particularly short lived, with controls having mean lifespans 15% shorter than controls mice used in studies by Richardson’s group. Interestingly, the maximum lifespan extension of MnSOD transgenic mice used by Hu et al. (2007) was also ~15%. These results suggest that MnSOD overexpression in the shorter-lived mice merely repairs the lifespan to ‘normal’ for the strain.

Even if modified expression of a particular gene alters lifespan in all available strains of a particular species, it may have no effect on another species. For example, genetic manipulations of multiple intracellular antioxidant enzymes in *D. melanogaster* suggest correlations between enzyme activities and lifespan. However, the same genetic manipulations within mice do not support this relationship, which cautions against extrapolating results from one species into ‘rules for longevity in animals’ (reviewed by Muller et al., 2007).

Typically, genetic manipulations occur at a global level which can obscure the importance of cell- and/or tissue-specificity. For example, MnSOD homozygous null mice die shortly after birth with reported severe dilated cardiomyopathy, metabolic acidosis and fatty liver (Li et al., 1995; Lebovitz et al., 1996). In comparison, liver-
specific MnSOD knockout mice show no signs of liver gross abnormalities, suggesting that the lipid accumulation within the livers of the MnSOD-deficient mice is not due to the loss of MnSOD activity within the liver but as a systemic effect from the loss of MnSOD in other tissues (Ikegami et al., 2002). In addition, genetic modifications are often coordinated during development which can interfere with interpretation as to whether these enzymes are indeed ‘longevity genes’. MnSOD overexpression extends maximum lifespan in *D. melanogaster*, however this is only observed under adult onset conditions (Mockett et al., 1999; Sun et al., 2002), suggesting that MnSOD overexpression negatively impacts normal *D. melanogaster* development.

Although there are limitations associated with genetic modifications, this approach can offer valuable insights into how genes may influence lifespan in different animal species, or perhaps within animals generally. However, it is necessary to verify these results using experimental models in which longevity has either evolved or been achieved by dietary manipulation or specific mutations. That is, if MnSOD, for example, is a ‘longevity gene’ its expression should be elevated in long-lived species or strains of species.

### 1.4 Stress resistance as a key characteristic of longevity

Species survival relies on the accurate transmission of the germ line to the next generation. In his disposable soma theory of aging, Kirkwood (1977) states that organisms need to balance available resources (i.e. energy) between reproduction and the maintenance of somatic cells. However, due to the investment of resources into reproduction, mutations and other forms of cellular damage accumulate within the soma.
The differences in resource allocation between reproduction and somatic maintenance can explain the discrepancy in lifespans between different organisms.

Species that are heavily predated, invest more energy into rapid growth and early reproductive maturity rather than somatic maintenance because these individuals are unlikely to survive long. When individuals of species are removed from their environmental niches and therefore from natural selection, difference in somatic maintenance can be observed within inter-specific contexts. Indeed positive correlations appear to exist between cellular maintenance, measured by stress resistance and species lifespan within an inter-specific (Kapahi et al., 1999) and intra-specific context (Salmon et al. 2005; Labinskyy et al. 2006; Harper et al. 2007). Furthermore, selective breeding of stress resistance corresponds to lifespan extension in *D. melanogaster* (Rose et al., 1992). Interestingly, extended longevity does not appear to result simply from enhanced resistance to one form of stress but rather to several forms. 'Multiplex' stress resistance has been observed for several organisms (Miller, 2009) suggesting that cells from longer-lived species and/or strains have enhanced protection due to the maintenance of cellular homeostasis.

1.5 Stress resistance, longevity and IIS

Stress resistance and animal species lifespan appear to be influenced by the insulin/insulin-like growth factor-1 (IGF-1) signalling (IIS) pathway (reviewed by Longo and Fabrizio, 2002). This pathway is highly conserved between *C. elegans*, *D. melanogaster*, and *M. musculus* (Fig. 1.1). For simplicity, the pathway within *C. elegans* will be detailed due primarily to the innovative experiments conducted on this organism. Disruption of the IIS pathway in *C. elegans* extends lifespan and increases stress
resistance through the activity of daf-16 which encodes a FOXO (forkhead box) transcription factor. Normally, insulin/IGF-1 binds to the daf-2 receptor which initiates a phosphorylation cascade via phosphoinositide 3-kinase and AKT/protein kinase B (PKB), resulting in the phosphorylation of daf-16 which is sequestered to the cytosol. Conversely, the loss of insulin/IGF-1 binding to daf-2 allows for the translocation of daf-16 into the nucleus where it regulates the expression of several genes whose protein products are associated with increased stress resistance including intracellular antioxidant enzymes and DNA damage response genes (reviewed by Carter and Brunet, 2007).

FOXO3a activity within mammals has been associated with increased expression of the intracellular antioxidant enzymes MnSOD and CAT (Kops et al., 2002; Nemoto and Finkel, 2002) as well as the regulation of the DNA damage response protein GADD45 (growth arrest and DNA damage-inducible protein 45; Tran et al., 2002).

Manipulations within the IIS pathway result in lifespan extension and increased oxidative stress resistance within several mutant mice models, including heterozygous insulin receptor (IR), heterozygous IGF-1 receptor (IGF1R), insulin receptor 1 knockout (Irs1-/-), growth hormone receptor knockout (GHRKO) and klotho transgenic mice (Coshigano et al., 2003; Holzenberger et al., 2003; Baba et al., 2005; Salmon et al., 2005; Kurosu et al., 2005; Yamamoto et al., 2005; Selman et al., 2008). Ames and Snell dwarf mice are also long-lived mutant mice with disrupted IIS (Brown-Borg et al., 1996; Miller, 1999; Flurkey et al., 2002) both of which have been reported to possess enhanced cellular and whole animal stress resistance and live longer than their normal sized littermates (Salmon et al., 2005; Bokov et al., 2009).
Fig. 1.1: Insulin/IGF-1 signalling within *C. elegans*, *D. melanogaster* and *M. musculus*. Insulin/IGF-1 binds the Daf-2 receptor (for example within *C. elegans*), initiating a phosphorylation cascade. Phosphorylation activates Age-1, which phosphorylates and activates AKT/PKB, which phosphorylates the transcription factor Daf-16. Daf-16 phosphorylation inhibits translocation into the nucleus and therefore Daf-16 remains in the cytosol. Reduced or inhibited insulin/IGF-1 signalling allows for unphosphorylated Daf-16 to translocate to the nucleus and function as a transcription factor. Arrow heads represent positive regulation of the enzymes, whereas blocked arrows represent negative regulation of the enzymes. Daf; dauer formation; dInR, Drosophila insulin receptor; IR, insulin receptor, IGF1R, IGF-1 receptor; PI3K, phosphatidylinositol 3-kinase; Age-1 is the *C. elegans* homologues of AKT/PKB; PBK, protein kinase B; Daf-16 is the *C. elegans* homologue of FOXO; dFOXO; Drosophila forkhead transcription factors (Kenyon, 2005).

1.6 Snell dwarf mice – an intra-species model of stress resistance and longevity

Snell dwarf mice have an inherited mutation within the *Pit-1* gene which encodes a transcription factor essential for the proper development of the anterior pituitary. As a
result, these mice have deficiencies in growth hormone (GH), thyroid stimulating hormone (TSH), prolactin (PRL) and also a secondary deficiency in circulating IGF-1 levels (Li et al., 1990; Camper et al., 1990; Hsieh et al., 2002). Neonatal development is normal in Snell dwarf mice; however by weaning age they are approximately half the weight of their normal-sized littermates and only reach one-third of normal weight as adults (reviewed by Flurkey et al., 2002). Initially, Snell dwarf mice were believed to be a model of accelerated senescence, however this was found to be due to poor husbandry. Snell dwarf mice, when caged with normal male littermates, do not compete well for food, are susceptible to cannibalism, and cannot successfully thermoregulate, due to increased surface area to volume ratios and reduced levels of TSH (reviewed by Flurkey et al., 2002). Optimal housing conditions, which include housing Snell dwarfs throughout their lifespan with normal-sized female “warmer” mice, revealed that Snell dwarf mice are actually a model of longevity, living up to 50% longer than their normal littermates (Miller, 1999; Flurkey et al., 2002).

Snell dwarf cellular stress resistance has been well characterized by Miller and colleagues. Dermal fibroblasts isolated from Snell dwarf mice are more resistant to oxygen-induced cellular senescence that typically occurs under 21% oxygen levels (Maynard and Miller, 2006), exposure to UV, heat, cadmium, hydrogen peroxide (H$_2$O$_2$), and paraquat (PQ) (Murakami et al., 2003) and the DNA alkylating agent, methyl methanosulfonate (Salmon et al., 2005). The multiplex stress resistance phenotype of Snell dwarf fibroblasts suggests the involvement of various protective mechanisms, including ROS neutralization and the repair of oxidative damage to DNA. Surprisingly,
limited studies have focused on the role the above mechanisms have in conferring the stress resistance phenotype of Ames and Snell dwarf mice (see chapter 2 and chapter 4).

1.7 Quiescence and longevity

Downregulation of the IIS pathway is also associated with periods of quiescence. Reduced IIS (Kenyon et al., 1993) leads *C. elegans* to enter dauer formation, which normally occurs when *C. elegans* face food scarcity. As dauers, *C. elegans* live four to eight times longer than the normal two week lifespan. However, the active lifespan (time not spent in dauer) of these nematodes remains at two weeks (Klass and Hirsch, 1976). In addition to increased lifespans, dauers have reduced metabolism (reviewed by Burnell et al., 2005) and increased resistance to oxidative stress, which may be the result of enhanced SOD and catalase activities (Larsen, 1993; Vanfleteren, 1993; Vanfleteren and De Vreese, 1995; Honda and Honda, 1999).

Other forms of animal quiescence appear similarly to be regulated by IIS. Reduced plasma insulin and IGF-1 levels have been measured in hibernating *Scotophilus heathi* bats and golden-mantled ground squirrels (*Spermophilus lateralis*) (Krishna et al., 1998; Schmidt and Kelley, 2001). Phosphorylation levels of Akt/PKB are reduced in several tissues during torpor in the little brown bat (*Myotis lucifugus*) compared to active bats (Eddy and Storey, 2003). Similarly, phosphorylation levels of Akt/PKB are reduced in brain, skeletal muscle and liver of hibernating ground squirrels (Cai et al., 2004, Abnous et al., 2008). Downregulation of Akt/PBK phosphorylation should increase FOXO translocation into the nucleus consequently increasing expression of genes associated with enhanced stress resistance. Reduced IIS signalling during hibernation may confer increased tolerance to hypoxia and ischemia in 13-lined and arctic ground
squirrels (Frerichs and Hallenbeck, 1998; Dave et al., 2006). Longevity is also positively correlated with hibernation in bat species (Wilkinson and South, 2002) and the Turkish hamster (Lyman et al., 1981). *C. elegans* dauer and mammalian hibernation share many similarities with respect to downregulated IIS, increased longevity and enhanced stress resistance. Dauer formation has been associated with enhanced antioxidant enzyme activity, yet surprisingly there is limited data focusing on the potential role of intracellular antioxidant enzymes in stress resistance and lifespan in higher forms of quiescence, including hibernation and estivation.

**1.8 Putative molecular mechanisms of longevity**

Thus far, mechanisms that are believed to influence longevity include genes encoding enzymes involved in cellular maintenance, protection and/or repair (reviewed by Kuningas et al., 2008). In support of this idea, enzymes that are involved in reactive oxygen species (ROS) neutralization, DNA repair and protein repair comprise the core stress proteome which is conserved from bacteria to humans. In fact, nine of the 12 most highly conserved stress response proteins function in one of the above protective roles (Kultz, 2003). Enhanced activity of the core stress proteome thus could account for the stress resistant phenotype observed in long-lived species. Interestingly, the IIS pathway transcription factor FOXO is thought to regulate the expression of enzymes involved in ROS neutralization and repair of oxidative damage (Kops et al., 2002; Tran et al., 2002). Enzymes involved in these protective and repair pathways are of considerable interest due to the increasing evidence that the accumulation of oxidative damage to proteins, lipids and DNA ultimately influences lifespan (Hamilton et al., 2001; see Levine, 2002; Stadtman, 2004 for reviews; Ward et al., 2005).
1.9 Oxidative stress and damage

Animals are continuously exposed to endogenously generated oxidative stress. Mitochondria are the main source of cellular ROS and reactive nitrogen species (RNS; for simplicity both will be referred to as ROS) generated as normal by-products of aerobic respiration (Tahara et al., 2009). It is often reported that 1-2% of oxygen consumed by mitochondria form superoxide anions (O$_2^-$), however recent estimates suggest that this value is likely less than 1% (St-Pierre et al., 2002; reviewed in Murphy, 2009). Several factors have been proposed to account for this discrepancy, including 1) the \textit{in vitro} use of a single mitochondrial substrate at saturating concentrations 2) \textit{in vitro} measurements are carried out at 21% atmospheric oxygen rather than physiological oxygen levels which are closer to 3%, and 3) that mitochondria \textit{in vivo} would be continuously synthesizing ATP which lowers protonmotive force and decreases O$_2^-$ production (reviewed by Murphy, 2009).

Regardless of the percentage, the majority of mitochondrial generated ROS originate from the electron transfer chain (ETC). Electrons leak from complex I and III, reducing oxygen to O$_2^-$ (St-Pierre et al., 2002). Although O$_2^-$ has a low reactivity with most intracellular macromolecules (reviewed by Benov, 2001), it undergoes further reactions to generate more damaging ROS. H$_2$O$_2$, which is produced via the dismutation of O$_2^-$, undergoes Fenton chemistry in the presence of iron (Fe$^{2+}$) or copper (Cu$^{1+}$) to generate the highly reactive hydroxyl radical (·OH). In addition, O$_2^-$ will react with nitric oxide (NO) to form peroxynitrite (ONOO$^-$), a strong oxidant and nitrating molecule (Pryor and Squadrito, 1995).
ROS are often thought of as negative entities especially in relation to aging and longevity; however ROS are involved in numerous protective roles. For example $O_2\cdot^-$ generated from NADPH enzymes within neutrophils are involved in the innate immune response aiding in microbial phagocytosis (Fialkow et al., 2007). ROS also function in cell signalling, playing an important role in cell cycle regulation (reviewed by Menon and Goswami, 2007). It is only when ROS generation surpasses the capacity for proper neutralization that oxidative damage begins to accumulate in proteins, lipids and DNA. The accumulation of oxidative damage within the cell has been postulated by Harman (1956, 1972) to result in aging and eventual death of an organism. Oxidation of proteins interferes with proper tertiary folding and can result in reduced activity or loss of function (Berlett and Stadtman, 1997). Oxidized proteins are removed from the cellular environment through proteasomal degradation, however heavily oxidized proteins often overwhelm the proteasomal machinery and instead of proper degradation lead to cell death (reviewed by Davies, 2001). Lipid peroxidation similarly disrupts cellular homeostasis by altering membrane fluidity and causing protein-modifications both of which negatively impact mitochondrial oxidative phosphorylation efficiency, once again increasing the risk of cellular death (Chen and Yu, 1994). Finally, the accumulation of oxidative damage to DNA is of utmost importance because cellular maintenance, protection and repair rely on proper DNA replication and protein transcription. Oxidized bases, abasic sites and DNA strand breaks are common oxidative DNA lesions that have been measured within both nuclear and mitochondrial DNA. Elevated levels of damage are reported within mitochondrial DNA, which likely arise due to the close proximity to ROS generation (Yakes and Van Houten, 1997). Oxidative DNA lesions can impact
cellular homeostasis in several ways. Blocked DNA replication and/or DNA replication of oxidized lesions can increase genomic instability, resulting in elevated incidences of cancer (Charames and Bapat, 2003; Zhivotovsky and Kroemer, 2004). DNA lesions can likewise result in blocked or improper gene transcription; therefore disrupting protein synthesis (Kathe et al., 2004). Finally, oxidative DNA lesions can trigger apoptotic cell death (Oka et al., 2008). All aerobic eukaryotic cells possess mechanisms that manage oxidative stress. ROS neutralization via intracellular antioxidant enzymes acts as the first line of defence against oxidative stress. Furthermore, protein and DNA repair enzymes repair damage that may arise from ROS that escaped neutralization. DNA repair however, may be the most vital as this mechanism functions to ensure that replication and transcription of the genome are not compromised and therefore cellular function maintained.

1.10 Intracellular antioxidant enzymes

All aerobic organisms possess intracellular antioxidant enzymes which have the capacity to neutralize $O_2^-$ and $H_2O_2$. SOD enzymes convert two $O_2^-$ to $H_2O_2$ (Equation 1.1; Okado-Matsumoto and Fridovich, 2001). MnSOD catalyzes this reaction within mitochondrial matrices. In contrast, CuZnSOD functions primarily in the cytosol; however evidence suggests the presence of CuZnSOD within the intermembrane space of mitochondria (Okado-Matsumoto and Fridovich, 2001).

Equation 1.1: 

$$M^{ox+}\text{SOD} + O_2^- \rightarrow M^*\text{SOD} + O_2$$

$$M^*\text{SOD} + O_2 + 2H^+ \rightarrow M^{ox+}\text{SOD} + H_2O_2$$

$M$ represents the transition metal
H$_2$O$_2$ is further neutralized to water (H$_2$O) within the mitochondria and cytosol by GPx and glutathione reductase (GR) (Fig. 1.2).

\[
\begin{align*}
\text{H}_2\text{O}_2 & \quad 2 \text{GSH} & \quad \text{NADP}^+ \\
\text{GPx} & \quad \text{GSSG} & \quad \text{NADPH} + \text{H}^+
\end{align*}
\]

**Fig. 1. 2:** GPx uses reduced glutathione (GSH) as a source of electrons to reduce H$_2$O$_2$ to water. Oxidized glutathione (GSSG) is reduced back to GSH via GR, which requires NADPH as the electron donor.

Although there are numerous GPx isozymes (reviewed by Margis et al., 2008), the most abundant is GPx1, the activity of which the majority of the literature is focused. Two molecules of H$_2$O$_2$ can also be converted to oxygen (O$_2$) and H$_2$O by catalase (CAT), which is localized to peroxisomes (Equation 1.2).

\[
2 \text{H}_2\text{O}_2 \xrightarrow{\text{CAT}} 2 \text{H}_2\text{O} + \text{O}_2
\]

Intracellular antioxidant enzymes neutralize ROS; therefore if the accumulation of oxidative damage influences aging and lifespan then the activity of antioxidant enzymes should slow the process of aging and correlate with animal species lifespan. This idea has been thoroughly tested using genetically modified mice (reviewed by Pérez et al., 2009b; 2009c), which will be discussed below.
1.10.1 MnSOD

MnSOD deficiency in mice causes neonatal lethality between 10-18 days depending on the genetic background (Li et al., 1995; Lebovitz et al., 1996), suggesting the importance of MnSOD in normal murine development. Surprisingly, a 50% reduction in MnSOD does not reduce lifespan in mice (Van Remmen et al., 2003), however these mice are more susceptible to oxidative stress (Tsan et al., 1998; Kim et al., 2002), and have elevated levels of DNA oxidative damage accompanied by increased incidences of cancer (Van Remmen et al., 2003) in comparison to wildtype mice. MnSOD heterozygosity also disrupts the integrity of the ETC and leads to increased rates of apoptosis (Kokoszka et al., 2000; Van Remmen et al., 2003). MnSOD transgenic mice have lower ROS levels than WT mice (Shen et al., 2006) which may result in the enhanced cellular and whole animal stress resistant phenotype reported in these mice (Jang et al., 2009). Unfortunately, lifespan studies on MnSOD transgenic mice are conflicting (Hu et al., 2007; Jang et al., 2009; Pérez et al., 2009c), however as mentioned above the lifespan extension reported by Hu et al. (2007) appears to be due to the recovery of a short lived mouse strain. The results from the above genetic modifications fail to definitively show a correlation between MnSOD activity and murine lifespan.

1.10.2 CuZnSOD

The ablation of CuZnSOD in mice reduces lifespan (Elruchi et al., 2005; Pérez et al., 2009c) and elevates levels of oxidative stress (Muller et al., 2006) in comparison to wildtype mice. Although CuZnSOD heterozygous (+/-) mice appear to have a reduced lifespan compared to wildtype, the difference is not significant, which may be attributed to the small sample size (Elchuri et al., 2005; Muller et al., 2006). Recently, increased oxidative DNA damage has been measured in liver tissue of CuZnSOD +/- mice (Bhusari
et al., 2010) Overexpression of CuZnSOD also fails to extend lifespan in mice (Huang et al., 2000; Pérez et al., 2009c), however CuZnSOD transgenic mice are more resistant to paraquat injection (Pérez et al., 2009c). Thus, similar to MnSOD, genetic modification of CuZnSOD does not appear to influence lifespan in mice, however the reliance on highly inbred strains of mice may also affect these studies (as mentioned above).

1.10.3 GPx and GR

Loss of the most abundant GPx isozyme, GPx1, does not affect murine lifespan (Ho et al., 1997), however these mice do show increased susceptibility to oxidative stress (de Haan et al., 1998; Fu et al., 1999; Esposito et al., 2000; Han et al., 2008). Conversely, ablation of GPx4 (phospholipid hydroperoxidase) results in embryonic lethality (Ran et al., 2007), suggesting the importance of GPx4 in development. Somewhat unexpectedly, GPx4 +/- mice have longer lifespans compared to wildtype mice (Ran et al., 2007) despite higher levels of apoptotic cell death and increased sensitivity to oxidative stress (Yant et al., 2003). Overexpression of GPx1 or GPx4 attenuates myocardial ischemia/reperfusion injury (Yoshida et al., 1996; Dabkowski et al., 2008) which may be due to increased protection from apoptotic cell death (Ran et al., 2004). However, GPx4 overexpression does not alter murine lifespan (Ran et al., 2004). Surprisingly, lifespan studies have not been reported for GPx1 transgenic mice. Data focusing on genetic manipulation of GR is very limited; and GR deficient mice do not appear any less tolerant to oxidative stress than wildtype mice (Rogers et al., 2006). Furthermore, lifespan studies have not been conducted on GR mutant mice.
There is currently no lifespan data for CAT deficient mice. However, normal development does not appear to be disrupted in these mice which are healthy at one year of age and show no signs of increased vulnerability to oxidative damage compared to wildtype littermates (Ho et al., 2004). CAT transgenic mice do not have extended lifespans compared to wildtype mice (Pérez et al., 2009c), however isolated hepatocytes and fibroblasts from CAT transgenic mice have enhanced oxidative stress resistance compared to cells isolated from wildtype mice (Chen et al., 2004). On the other hand, the overexpression of CAT that is also targeted to mitochondria extends lifespan by approximately 20% and reduces levels of oxidative damage to DNA in mice (Schriner et al., 2005).

Overall, the above genetic modifications fail to reveal a positive correlation between intracellular antioxidant enzyme activity and maximum lifespan in mice; however the results do reveal an inverse relationship between maximal enzyme activity and oxidative damage. This indicates that although intracellular antioxidants neutralize ROS and enhance stress resistance the activity of these enzymes does not confer longevity. Another potential protective mechanism that may correlate with animal species lifespan is the repair of oxidative DNA damage. The amount of oxidative damage within liver nuclear and mitochondrial DNA is negatively correlated with animal species lifespan (Barja and Herrero, 2000) suggesting that longer-lived animals may have enhanced DNA repair activities, however Speakman (2005b) shows that this relationship is driven by the correlation between damage and species body mass.

1.11 DNA damage and repair
DNA damage resulting from endogenous processes accounts for approximately 10,000 DNA lesions per cell per day (Lindahl, 1993). Replication errors, oxidation, alkylation and hydrolysis are all types of endogenously-induced DNA damage (Lindahl, 1993). Several repair pathways function to repair the specific types of damage that accumulates within DNA. Enzymes involved in nucleotide excision repair (NER) remove the bulky DNA adducts typically caused by exogenous radiation. Enzymes involved in mismatch repair (MMR) correct DNA damage that arises during replication. Finally, oxidation, alkylation and the hydrolysis of DNA is repaired via enzymes of the base excision repair (BER) pathway (reviewed by Barnes and Lindahl, 2004). The enzymes involved in BER are important in maintaining cellular homeostasis against oxidative stress and genetic modifications of these enzymes have been well characterized in mice.

1.12 BER

Two BER pathways, long- and short-patch, are responsible for the repair of nuclear oxidative DNA damage. However, evidence suggests that the majority of oxidative DNA lesions are repaired via short-patch (Pascucci et al., 2002), which is initiated by the recognition and removal of the oxidative lesion by specific DNA glycosylases that catalyze the hydrolysis of the N-glycosidic bond between the damaged base and the sugar moiety (reviewed by Bohr, 2002). The removal of the lesion leaves an abasic (AP) site which may also arise spontaneously or as the result of radiation and chemical toxicity. AP lyase activity, possessed by several DNA glycosylases and AP endonuclease (APE1) cleave the backbone at the AP site. Cleavage of the AP site produces a DNA strand break 5' to the baseless sugar leaving a 3' hydroxyl group and a
deoxyribose phosphate (dRP) group at the 5' terminus. DNA polymerase β (polβ) catalyzes β-elimination of the dRP group and fills in the one nucleotide gap. Finally, the nicked DNA is sealed by DNA ligase III (Fig. 1.3; Bohr, 2002).

The efficiency of BER activity is believed to be influenced by two rate-limiting steps. The 3'-phosphoesterase activity of APE1 has also been reported to act as a rate-limiting step specifically during *in vitro* repair of oxidative lesions (Izumi et al., 2000). Further studies have suggested that this activity is also rate-limiting *in vivo*, as the majority of endogenous AP sites in normal rat and human liver tissues is cleaved 5' to AP sites (Nakamura and Swenberg, 1999). Srivastava et al. (1998) demonstrated that the removal of the 5'dRP group by polβ acted as the only rate-limiting activity *in vitro* and evidence did not suggest a role for APE1 in the overall influence of BER. However, due to the potential of two rate-limiting steps within BER, it is of interest to measure the separate activity of both APE1 and polβ.

### 1.12.1 8-oxoguanine DNA glycosylase

8-oxodG is estimated to represent approximately 5% of all oxidative lesions (Dizdaroglu et al., 2002). Adenine can mispair with 8-oxodG leading to G·C→T·A transversions. Together three enzymes prevent mutagenesis due to 8-oxoG lesions; 8-oxoguanine DNA glycosylase (OGG1) removes 8-oxoG that is paired to cytosine, MYH (muty homolog; named for the bacterial Mut Y DNA glycosylase) removes adenines mispaired with 8-oxoG (Fig. 3), and MTH1 (mut T homolog) removes 8-oxodGMP from the nucleotide pool, preventing its incorporation into DNA (reviewed by David et al., 2007).
Fig. 1. 3: 8-oxodG, which is the most common form of oxidative damage, is removed from DNA by OGG1. The removal of the base generates an AP site which is further processed by APE1. Polβ incorporates the correct nucleotide and the DNA backbone is sealed by DNA ligase. MYH repairs the misincorporation of adenine across from 8-oxodG. (Modified from Stuart and Page, 2008).

The overlap between these three enzymes is identified within Ogg1 null mice which possess only a moderate spontaneous mutation rate (Klungland et al., 1999) and similarly within Myh/Ogg1 double knock out mice which have increased rates of tumorigenesis (Xie et al., 2004; Russo et al., 2004). Interestingly, MTH1 deficiency alone also increases the rate of tumorigenesis in mice compared to wildtype mice (Tsuzukis et al., 2001). The existence of three enzymes to prevent the accumulation of 8-oxoG emphasizes the importance of removal of this mutagenic lesion. Over-expression of
human OGG1 in Chinese hamster ovary cells increase the level of 8-oxoG repair compared to non-transfected cells, however this is only reported when the cells are exposed to potassium bromate (Hollenback et al., 1999), suggesting that Ogg1 expression is induced by oxidative stress (Mo et al., 2005). In contrast, OGG1 protein levels are reduced in Chinese hamster lung fibroblasts exposed to H2O2 (Kang et al., 2009). These differences may arise due to the type of oxidative stressor.

1.12.2 APE

Deletion of the Apex gene, which encodes APE, is embryonic lethal in mice (Xanthoudakis et al., 1996), suggesting the requirement for APE activity during normal development. Interestingly, APE, also known as ref-1, acts as a redox activator of several transcription factors (Xanthoudakis and Curran, 1992). Surprisingly, a 50% reduction in APE activity does not affect the lifespan of mice, although these mice are highly susceptible to oxidative stress (Meira et al., 2001) and have increased spontaneous levels of mutagenesis (Huamani et al., 2004). Like other BER enzymes, APE activity appears to be induced by oxidative stress, activating both DNA repair and redox activities (Tell et al., 2005).

1.12.3 Polβ

Similar to APE, Polβ is essential for normal murine development. Polβ null mice succumb to respiratory failure with increased apoptotic cell death, dying shortly after birth (Sugo et al., 2000). Lifespan is not reduced in Polβ +/- mice, (Cabelof et al., 2006), however increased levels of DNA strand breaks are reported in these mice compared to wildtype mice (Cabelof et al., 2002). As mentioned above, oxidative stress induces the expression of Polβ (Cabelof et al., 2002).
1.12.4 DNA ligase III

DNA ligase III is another BER enzyme that is essential during normal murine development; however heterozygous mice developed normally and appeared healthy at 20 months of age (Puebla-Osorio et al., 2006). The loss of APE and DNA ligase III is embryonic lethal in mice. In addition, Polβ null mice die shortly after birth. Together, these results reveal the importance of these enzymes and the BER pathway in early normal mouse development. These results may imply that DNA lesions, including AP sites and strand breaks, occur frequently during early embryonic development (Puebla-Osorio et al., 2006).

1.13 Hypotheses and Approach

Within an intra-species context plasma IGF-1 levels correlate with decreased body mass and increased lifespan. Surprisingly, these associations have not been examined within an inter-species context. However, due to the relationship between IGF-1 and stress resistance it would seem plausible that IGF-1 levels are reduced in longer lived species. Based on our knowledge of IIS regulation of cell stress resistance, this would suggest that longer lived species should also have increased levels of proteins that maintain cellular homeostasis.

The most proximal mechanism for dealing with oxidative stress due to excessive intracellular ROS production is direct and immediate detoxification. To determine if intracellular antioxidant enzymes play a role in enhancing cellular stress resistance concomitant with increasing longevity, the activity of the five major intracellular antioxidant enzymes were measured in the following; 1) brain, heart and liver tissue of 12 mammalian and 2 avian species (an inter-species model of longevity; Chapter 3); 2) brain
and heart tissue, and dermal fibroblasts isolated from Snell dwarf and normal mice (an intra-species model of longevity; Chapter 3 & 5); and 3) brain, heart and liver tissues of 13-lined ground squirrel (Spermophilus tridecemlineatus) and African slender lungfish (Protopterus dolloi) models of hibernation and estivation (and increased stress resistance), respectively (Chapter 6 & 7).

ROS can escape neutralization causing DNA damage; therefore enzymes involved in the repair of oxidative DNA damage may be upregulated in association with increases in lifespan. APE and Polβ activities were measured in 1) brain and liver tissues of 14 mammalian and 2 avian species (Chapter 4) and 2) brain and heart tissue, and isolated dermal fibroblasts, from Snell dwarf and normal mice (Chapter 4 & 5) to establish if these enzyme activities are upregulated concomitantly with increases in stress resistance and longevity.

If intracellular antioxidant and BER enzymes confer stress resistance and longevity the activities of these enzymes should be correlated with longevity in both inter- and intra-species models. This relationship is expected to support the increased stress resistant phenotype in long-lived animals.
Chapter 2. Plasma IGF-1 is negatively correlated with body mass in a comparison of 36 mammalian species

Hypothesis

Cellular stress resistance positively correlates with species maximum lifespan, in addition reduced IGF-1 levels have been associated with increased cellular stress resistance therefore plasma IGF-1 levels should correlate negatively with longevity in an inter-species context.

Objective

The primary objective of this project was to determine if plasma IGF-1 levels correlated with species maximum lifespan or body mass.

Publications of results


Contributions

I performed all phylogenetic and statistical analyses, and contributed to writing the methods and results sections of the chapter as well as editing throughout.
2.1 Introduction

In mammals, insulin-like growth factor (IGF-1) is produced in many tissues, and has endocrine, paracrine and autocrine functions during development and in the physiology of adults. Plasma IGF-1, however, is thought to originate primarily from the liver (see Stratikopoulos et al., 2008 and references therein). Both local and plasma IGF-1 contribute approximately equally to organismal growth and adult body mass of mice (Stratikopoulos et al., 2008). Intra-specific comparisons of plasma IGF-1 and adult body mass within dogs (Spichiger et al., 2006) or primates (Bernstein et al., 2007) illustrate significant positive correlations between the two variables in both species. Within a species, deficiencies in plasma IGF-1 are associated with dwarfism, and elevated IGF-1 with gigantism (Yakar et al., 2002). Thus, plasma IGF-1 is an important positive regulator of growth and development in mammals.

Plasma IGF-1 (Yuan et al., 2009) and growth per se (Rollo, 2002) are, however, also negatively correlated with longevity in mice and other animals (Kenyon, 2010). In a study of 31 laboratory strains, adult mice showed a significant negative correlation between plasma IGF-1 and median lifespan (Yuan et al., 2009). Presumably, this is related to the known physiological activities of this hormone. While its mitogenic properties stimulate growth and regenerative tissue repair (e.g. Philippou et al., 2007), higher IGF-1 levels are also permissive for cancer growth (Longo and Fontana, 2006). Indeed, much of the lifespan extension arising from reduced plasma IGF-1 in rodents may be due to the concomitantly reduced susceptibility to cancer (see Sonntag et al., 2006 and references therein). Reduced IGF-1 signalling is also associated with increased organismal and cellular stress resistance via the activation of FOXO transcription factors.
under these conditions (summarized in Robb et al., 2009). For example, mice deficient in IGF-1 signalling due to hemizygosity for the IGF-1 receptor are stress resistant (Holzenberger et al., 2003). In contrast, in a murine stroke model, high plasma IGF-1 is associated with greater brain damage, while reduced IGF-1 ameliorates brain damage (Endres et al., 2007).

Although intra-specific data is relatively robust in indicating associations between plasma IGF-1, body mass and longevity, there is no similar insight into the possible role(s) of IGF-1 in an inter-specific context. Mammalian species' body masses and maximum lifespans (MLSPs) range over six and two orders of magnitude, respectively, which lends itself to such an analysis. In addition, interspecies comparisons are facilitated by the fact that the IGF-1 amino acid sequence is highly conserved amongst mammals. For example, the sequence is 100% conserved between humans, pigs, dogs, cows, rabbits, and guinea pigs and 95% conserved between humans and rats, mice and hamsters. Here, I have analyzed published values for plasma total IGF-1 levels from young adults of 36 mammalian species with respect to body mass and MLSP. Cellular stress resistance positively correlates with species maximum lifespan, in addition reduced IGF-1 levels have been associated with increased cellular stress resistance therefore plasma IGF-1 levels should correlate negatively with longevity in an interspecies context. This analysis reveals a strong negative correlation between body mass and plasma IGF-1, which is opposite that observed within a species. Plasma IGF-1 levels did not correlate with MLSP which also differs from observations made within species.

2.2 Experimental Procedures
PubMed and Web of Science database searches were performed to find published values for plasma total IGF-1 in as many species and orders of the vertebrate class Mammalia as could be found.

A phylogeny for the 36 species used in this study (Fig. 2.1) was estimated by amalgamating mammalian phylogenies from Chatterjee et al. (2004), Delisle and Strobeck (2005), Lindqvist et al. (2009), Matthee and Davis (2001), Pitra et al. (2004), Springer and Murphy (2007), Yang and Yoder (2003).

Raw data were natural log-transformed prior to correlation analyses. Residuals were calculated from simple linear regression of the dependent variable of interest on body mass to remove the confounding effect of body mass. Significance of the coefficient of correlation was used to determine if the line was different from horizontal, and significance was based on t-values for one-tailed tests. A p-value of 0.05 was considered significant. Felsenstein's phylogenetically independent contrasts (FIC; Felsenstein 1985) were calculated using PDAP (Garland et al., 1992; Garland et al., 1993). FIC analysis employed the branch length estimates from the phylogenetic tree (Fig. 2.1) and all branch lengths set to one, as in the speciation model of character change (Martins and Garland, 1991; Price et al., 1997).

### 2.3 Results

Plasma total IGF-1 values from 36 mammalian species (Fig. 2.1) were obtained from published studies (Table 2.1). As IGF-1 tends to increase markedly during adolescence and then decrease in old age (Rosen and Conover, 1997), data was used from physically mature young adults, unless otherwise indicated. In some species, there are significant differences in plasma IGF-1 between the sexes. Therefore, wherever possible,
data from both males and females of a given species was used, calculating a species mean for the pooled data. Plasma IGF-1 is also affected by nutritional status, so data obtained from calorie restricted or starving animals was avoided. In the case of wild animals, wherever possible data from publications sampling what appeared to be well nourished individuals were used. Pregnancy and lactation also influence IGF-1, and so data from pregnant and lactating individuals were avoided as well, though in a few instances this was not possible (as indicated in Table 2.1).

Fig. 2.1: Phylogeny of 36 mammalian species included in the data set. Common names shown; see Table 2.1 for species names.

The data in Table 2.1 represent either the best estimates from the literature, or the only available data for all mammalian species for which data could be found. For any given species, the number of publications reporting plasma IGF-1 is proportional to the human relevance of that species. Laboratory models (e.g. mice, rats, rabbits), livestock
(e.g. cows, pigs, sheep) and primates (e.g. humans, macaques) are all over-represented in the literature. In the case of many of these species, the range of reported plasma IGF-1 data was occasionally rather large and seemed to reflect inter-laboratory variability in addition to 'normal' trait variance. In these instances it was attempted to establish the normal range for a particular species and excluded data that were deemed to be outlying. Table 2.1 contains data from selected studies deemed to be generally representative. Table 2.2 contains summary data for all 36 species for which plasma total IGF-1 data could be obtained. Where multiple plasma IGF-1 values were reported for a given species, a single value was estimated from these values.

In other cases only a single published value could be found for a particular species, so this value was transferred directly to Table 2.2. Adult body masses are either means from the cited studies or, in instances where they were not provided in the cited studies, taken from other sources, including AnAge (de Malgahaes et al., 2005) as indicated. Species' MLSP values in Table 2 were taken from AnAge (de Malgahaes et al., 2005) or, in the case of several primate species absent from this database, from Rowe (1996). Species in Table 2.2 include representatives of seven mammalian orders (Artiodactyla, Carnivora, Lagomorpha, Pinnipedia, Perissodactyla, Rodentia, and Primates). The correlational analyses were performed on data in Table 2.2.
Table 2.1: Plasma IGF-1 values used in this analysis.

<table>
<thead>
<tr>
<th>Common, Strain and Species Name</th>
<th>Data Source</th>
<th>Body Mass (g)</th>
<th>[IGF-1] (ng/ml)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male C57BL/6 mice (Mus musculus)</td>
<td>4 months old, laboratory reared, fed ad libitum, overnight fast prior to blood sampling</td>
<td>27</td>
<td>600</td>
<td>Hempenstall et al., 2010</td>
</tr>
<tr>
<td>Male DBA/2 mice (Mus musculus)</td>
<td>4 months old, laboratory reared, fed ad libitum, overnight fast prior to blood sampling</td>
<td>27</td>
<td>600</td>
<td>Hempenstall et al., 2010</td>
</tr>
<tr>
<td>Female wild / laboratory hybrid mice (Mus musculus)</td>
<td>14 months old, 6 genetic lines of cross-bred mice, laboratory reared</td>
<td>17-35</td>
<td>350-650</td>
<td>Harper et al., 2006</td>
</tr>
<tr>
<td>Wild-derived mice (Mus musculus)</td>
<td>6 months old, laboratory reared</td>
<td>17-20</td>
<td>300-460</td>
<td>Miller et al., 2002</td>
</tr>
<tr>
<td>Gray mouse lemur (Microcebus murinus)</td>
<td>Mean age 2.3 years, laboratory reared</td>
<td>80</td>
<td>592</td>
<td>Terrien et al., 2008</td>
</tr>
<tr>
<td>Golden-mantled ground squirrel (Spermophilus lateralis)</td>
<td>Adult, wild-caught and maintained in laboratory, male and female</td>
<td>150</td>
<td>650</td>
<td>Schmidt and Kelley, 2001</td>
</tr>
<tr>
<td>Wistar rats (Rattus norvegicus)</td>
<td>Adult, laboratory reared, fed ad libitum</td>
<td>210</td>
<td>900</td>
<td>Osorio et al., 1998</td>
</tr>
<tr>
<td>Sprague-Dawley rats (Rattus norvegicus)</td>
<td>14 weeks old, laboratory reared, fed ad libitum</td>
<td>250</td>
<td>960</td>
<td>Garcia et al., 2008</td>
</tr>
<tr>
<td>Sprague-Dawley rats (Rattus norvegicus)</td>
<td>&gt; 6 months old, laboratory reared</td>
<td>450</td>
<td>1400</td>
<td>Lian et al., 2004</td>
</tr>
<tr>
<td>Sprague-Dawley rats (Rattus norvegicus)</td>
<td>Adult, laboratory reared</td>
<td>490</td>
<td>730</td>
<td>Tarasiuk and Segev, 2005</td>
</tr>
<tr>
<td>Marmoset monkeys (Callithrix jacchus)</td>
<td>Adult, laboratory reared</td>
<td>350</td>
<td>500</td>
<td>Thomas et al., 1997</td>
</tr>
<tr>
<td>Guinea pigs (Cavia porcellus)</td>
<td>Sham surgery</td>
<td>430</td>
<td>440</td>
<td>Conlon and Kita, 2001</td>
</tr>
<tr>
<td>Male rabbits (Oryctolagus cuniculus)</td>
<td>Adult, laboratory reared</td>
<td>4200</td>
<td>222</td>
<td>Carter et al., 1997</td>
</tr>
<tr>
<td>New Zealand white rabbits (Oryctolagus cuniculus)</td>
<td>Females, 3.5 months old, laboratory reared</td>
<td>3000*</td>
<td>450</td>
<td>Sirotkin et al., 2009</td>
</tr>
<tr>
<td>Long-tailed macaque (Macaca fascicularis)</td>
<td>Females, 8-20 years old, laboratory reared</td>
<td>3300</td>
<td>670</td>
<td>Shively et al., 2005</td>
</tr>
<tr>
<td>Agile gibbons (Hylobates agilis)</td>
<td>3-4 years old, laboratory reared</td>
<td>4250</td>
<td>500</td>
<td>Suzuki et al., 2003</td>
</tr>
<tr>
<td>Common, Strain and Species Name</td>
<td>Data Source</td>
<td>Body Mass (g)</td>
<td>[IGF-1] (ng/ml)</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-------------</td>
<td>---------------</td>
<td>-----------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Domestic cats (Felis cattus)</td>
<td>2-5 years old, laboratory reared</td>
<td>5500*</td>
<td>200</td>
<td>Campbell et al., 2004</td>
</tr>
<tr>
<td>Domestic cats (Felis cattus)</td>
<td>Male and female, 4.5 years old, neutered</td>
<td>4830</td>
<td>440</td>
<td>Leray et al., 2006</td>
</tr>
<tr>
<td>Sooty mangabey (Cercocebus torquatus atys)</td>
<td>Male and female, adult, laboratory reared</td>
<td>8600</td>
<td>692</td>
<td>Bernstein et al., 2007</td>
</tr>
<tr>
<td>Japanese macaques (Macaca fuscata)</td>
<td>Male and female, free ranging</td>
<td>9800</td>
<td>500</td>
<td>Suzuki and Ishida, 2001</td>
</tr>
<tr>
<td>Japanese macaques (Macaca fuscata)</td>
<td>Male and female, 3 years old, laboratory reared</td>
<td>9000</td>
<td>1000</td>
<td>Suzuki et al., 2000</td>
</tr>
<tr>
<td>Rhesus macaques (Macaca mulatta)</td>
<td>Male and female, zoo populations</td>
<td>9900</td>
<td>355</td>
<td>Bernstein et al., 2007</td>
</tr>
<tr>
<td>Black mangabey (Lophocebus aterrimus)</td>
<td>Male and female, zoo populations</td>
<td>9000</td>
<td>166</td>
<td>Bernstein et al., 2007</td>
</tr>
<tr>
<td>Pudu deer (Pudu puda)</td>
<td>Male, 2-5 years old, kept in pens</td>
<td>12000*</td>
<td>225</td>
<td>Bartos et al., 1998</td>
</tr>
<tr>
<td>Domestic dogs (Canis lupus familiaris)</td>
<td>Male beagles, 1 year old</td>
<td>11000</td>
<td>210</td>
<td>Debreuil et al., 1996</td>
</tr>
<tr>
<td>Domestic dogs (Canis lupus familiaris)</td>
<td>Many varieties and ages</td>
<td>25000</td>
<td>300</td>
<td>Spichiger et al., 2006</td>
</tr>
<tr>
<td>Gelada (Theropithecus gelada)</td>
<td>Male and female, zoo populations, 9-36 years old</td>
<td>15500</td>
<td>417</td>
<td>Bernstein et al., 2007</td>
</tr>
<tr>
<td>Drill (Mandrillus leucophaeus)</td>
<td>Male and female, zoo populations, 10-28 years old</td>
<td>16500</td>
<td>380</td>
<td>Bernstein et al., 2007</td>
</tr>
<tr>
<td>Guinea baboon (Papio hamadryas papio)</td>
<td>Male and female, zoo population, 8-22 years old</td>
<td>19000</td>
<td>269</td>
<td>Bernstein et al., 2007</td>
</tr>
<tr>
<td>Olive baboon (Papio hamadryas anubis)</td>
<td>Male and female, adults, laboratory reared</td>
<td>19500</td>
<td>138</td>
<td>Bernstein et al., 2007</td>
</tr>
<tr>
<td>Mandrill (Mandrillus sphinx)</td>
<td>Male and female, zoo populations, 9-34 years old</td>
<td>31600</td>
<td>589</td>
<td>Bernstein et al., 2007</td>
</tr>
<tr>
<td>Sheep (Ovis aries)</td>
<td>Male Soay, adult, castrated, housed in pens</td>
<td>35000</td>
<td>300</td>
<td>Rhind et al., 2000</td>
</tr>
<tr>
<td>Common, Strain and Species Name</td>
<td>Data Source</td>
<td>Body Mass (g)</td>
<td>[IGF-1] (ng/ml)</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-------------</td>
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<td>----------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Sheep (Ovis aries)</td>
<td>Male Soay, adult rams, housed in pens</td>
<td>39000</td>
<td>920</td>
<td>Lincoln et al., 2001</td>
</tr>
<tr>
<td>Sheep (Ovis aries)</td>
<td>Female Finn and Cambridge, 9 months old</td>
<td>36000</td>
<td>200</td>
<td>Spicer et al., 1993</td>
</tr>
<tr>
<td>Goats (Capra hircus)</td>
<td>Adult females, lactating</td>
<td>45000</td>
<td>55</td>
<td>Nielsen et al., 1990</td>
</tr>
<tr>
<td>Chimpanzees (Pan troglodytes)</td>
<td>Adult males, laboratory raised, 11-16 years old</td>
<td>62000</td>
<td>519</td>
<td>Videan et al., 2009</td>
</tr>
<tr>
<td>Humans (Homo sapiens)</td>
<td>Adult males and females</td>
<td>75000</td>
<td>190</td>
<td>Fontana et al., 2008</td>
</tr>
<tr>
<td>Humans (Homo sapiens)</td>
<td>Healthy males 20-40 years old</td>
<td>75000</td>
<td>210</td>
<td>Leifke et al., 2000</td>
</tr>
<tr>
<td>Reindeer (Rangifer tarandus)</td>
<td>Adult males and females, paddock reared</td>
<td>80000*</td>
<td>350</td>
<td>Bubenik et al., 1998</td>
</tr>
<tr>
<td>Black bear (Ursus americanus)</td>
<td>Females 2-20 years old, held in pens</td>
<td>90000*</td>
<td>390</td>
<td>Donahue et al., 2006</td>
</tr>
<tr>
<td>Pigs (Sus scrofa)</td>
<td>Adult males and females, reared in pens</td>
<td>105000</td>
<td>130</td>
<td>Suzuki et al., 2004</td>
</tr>
<tr>
<td>Pigs (Sus scrofa)</td>
<td>Males and females, 11 months old</td>
<td>160000</td>
<td>95</td>
<td>Hilleson-Gayne and Clapper, 2005</td>
</tr>
<tr>
<td>Stellar sea lion (Eumetopias jubatus)</td>
<td>Females, 2.5-6 years old, held in captivity</td>
<td>180000</td>
<td>225</td>
<td>Jeanniard Du Dot, 2007</td>
</tr>
<tr>
<td>Grizzly bear (Ursus arctos)</td>
<td>Adult males and females, &gt;3 years old, free-ranging</td>
<td>200000*</td>
<td>235</td>
<td>Gau and Case, 2002</td>
</tr>
<tr>
<td>Red deer (Cervus elaphus)</td>
<td>Adult males, 2-9 years old, paddock reared</td>
<td>200000*</td>
<td>300</td>
<td>Bartos et al., 2009</td>
</tr>
<tr>
<td>Elk (Cervus canadensis)</td>
<td>Females, 1.5-7 years old, paddock reared</td>
<td>200000*</td>
<td>50</td>
<td>Cook et al., 2001</td>
</tr>
<tr>
<td>Polar bears (Ursus maritimus)</td>
<td>Females ~12 years old, free-ranging</td>
<td>300000*</td>
<td>50</td>
<td>Lennox and Goodship, 2008</td>
</tr>
<tr>
<td>Muskoxen (Ovibos moschatus)</td>
<td>Adult females, free-ranging</td>
<td>300000*</td>
<td>30</td>
<td>Adamczewski et al., 1998</td>
</tr>
<tr>
<td>Common, Strain and Species Name</td>
<td>Data Source</td>
<td>Body Mass (g)</td>
<td>[IGF-1] (ng/ml)</td>
<td>Reference</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>-------------</td>
<td>--------------</td>
<td>----------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Horses (Equus caballus)</td>
<td>Females, 6-23 years old</td>
<td>530000</td>
<td>160</td>
<td>Heidler et al., 2003</td>
</tr>
<tr>
<td>Horses (Equus caballus)</td>
<td>Female Haflinger, 7-15 years old</td>
<td>460000</td>
<td>150</td>
<td>Deichsel et al., 2005</td>
</tr>
<tr>
<td>Horses (Equus caballus)</td>
<td>Male and Female, ages 8-12 years</td>
<td>500000*</td>
<td>240</td>
<td>Noble et al., 2007</td>
</tr>
<tr>
<td>Cows (Bos taurus)</td>
<td>Adults, lactating</td>
<td>450000*</td>
<td>80</td>
<td>Castigliego et al., 2009</td>
</tr>
<tr>
<td>Cows (Bos taurus)</td>
<td>Holstein Fresian</td>
<td>575000</td>
<td>90</td>
<td>Roberts et al., 2005</td>
</tr>
<tr>
<td>Bulls (Bos taurus)</td>
<td>Angus-cross steers</td>
<td>262000</td>
<td>185</td>
<td>Waggoner et al., 2009</td>
</tr>
</tbody>
</table>

* not reported in cited studies, so these body masses represent average values for adults of that species taken from AnAge (de Magalhaes et al., 2005). ‡ data from Rowe (1996). All other MLSP data from AnAge.
Table 2.2: Adult body mass, maximum lifespan (MLSP) and mean plasma total IGF-1 in the 36 mammalian species analyzed in this study.

<table>
<thead>
<tr>
<th>Species (common name)</th>
<th>Adult body mass (g)</th>
<th>MLSP (y)</th>
<th>Mean [IGF-1] (ng/ml plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>23</td>
<td>4</td>
<td>450 (100)</td>
</tr>
<tr>
<td>Gray mouse lemur</td>
<td>80</td>
<td>18</td>
<td>592 (6)</td>
</tr>
<tr>
<td>Ground squirrel</td>
<td>150</td>
<td>10</td>
<td>650 (13)</td>
</tr>
<tr>
<td>Rat</td>
<td>350</td>
<td>5</td>
<td>975 (30)</td>
</tr>
<tr>
<td>Marmoset monkey</td>
<td>350</td>
<td>12†</td>
<td>500 (6)</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>430</td>
<td>12</td>
<td>440 (6)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>3600*</td>
<td>9</td>
<td>340 (25)</td>
</tr>
<tr>
<td>Long-tailed macaque</td>
<td>3300</td>
<td>39</td>
<td>670 (12)</td>
</tr>
<tr>
<td>Agile gibbon</td>
<td>4250</td>
<td>49</td>
<td>500 (2)</td>
</tr>
<tr>
<td>Domestic cat</td>
<td>5200*</td>
<td>30</td>
<td>320 (16)</td>
</tr>
<tr>
<td>Japanese macaque</td>
<td>9500</td>
<td>38.5</td>
<td>750 (194)</td>
</tr>
<tr>
<td>Rhesus macaque</td>
<td>9900</td>
<td>40</td>
<td>355 (5)</td>
</tr>
<tr>
<td>Black mangabey</td>
<td>9000</td>
<td>33‡</td>
<td>166 (3)</td>
</tr>
<tr>
<td>Sooty mangabey</td>
<td>8600</td>
<td>18‡</td>
<td>692 (203)</td>
</tr>
<tr>
<td>Pudu deer</td>
<td>12000*</td>
<td>18</td>
<td>225 (6)</td>
</tr>
<tr>
<td>Domestic dog</td>
<td>18000</td>
<td>24</td>
<td>255 (48)</td>
</tr>
<tr>
<td>Gelada baboon</td>
<td>15500</td>
<td>36</td>
<td>417 (15)</td>
</tr>
<tr>
<td>Drill</td>
<td>16500</td>
<td>39</td>
<td>380 (10)</td>
</tr>
<tr>
<td>Guinea baboon</td>
<td>19000</td>
<td>40‡</td>
<td>269 (73)</td>
</tr>
<tr>
<td>Olive baboon</td>
<td>19500</td>
<td>45‡</td>
<td>138 (199)</td>
</tr>
<tr>
<td>Mandrill</td>
<td>31600</td>
<td>40</td>
<td>589 (26)</td>
</tr>
<tr>
<td>Sheep</td>
<td>37000</td>
<td>23</td>
<td>400 (36)</td>
</tr>
<tr>
<td>Goat</td>
<td>45000</td>
<td>21</td>
<td>35 (40)</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>62000</td>
<td>60</td>
<td>519 (10)</td>
</tr>
<tr>
<td>Human</td>
<td>75000</td>
<td>122</td>
<td>200 (618)</td>
</tr>
<tr>
<td>Reindeer</td>
<td>80000*</td>
<td>22</td>
<td>350 (9)</td>
</tr>
<tr>
<td>Black bear</td>
<td>90000*</td>
<td>34</td>
<td>390 (5)</td>
</tr>
<tr>
<td>Pig</td>
<td>135000</td>
<td>27</td>
<td>115 (220)</td>
</tr>
<tr>
<td>Stellar sea lion</td>
<td>180000</td>
<td>33</td>
<td>225 (8)</td>
</tr>
<tr>
<td>Grizzly bear</td>
<td>200000*</td>
<td>40</td>
<td>235 (23)</td>
</tr>
<tr>
<td>Red deer</td>
<td>200000*</td>
<td>32</td>
<td>300 (8)</td>
</tr>
<tr>
<td>Elk</td>
<td>200000*</td>
<td>22</td>
<td>50 (43)</td>
</tr>
<tr>
<td>Polar bear</td>
<td>300000*</td>
<td>44</td>
<td>50 (12)</td>
</tr>
<tr>
<td>Muskoxen</td>
<td>300000*</td>
<td>28</td>
<td>30 (18)</td>
</tr>
<tr>
<td>Horse</td>
<td>500000</td>
<td>57</td>
<td>190 (21)</td>
</tr>
<tr>
<td>Cow</td>
<td>500000</td>
<td>20</td>
<td>110 (29)</td>
</tr>
</tbody>
</table>

* see Table 2.1 for note on body mass. ‡ numbers in parentheses indicate measurement sample sizes. † data from Rowe (1996). All other MLSP data from AnAge.

In the 36 mammalian species examined, the correlation of plasma IGF-1 with adult body mass was highly significant (p < 0.0005) and negative (r = -0.62, Fig. 2.2),
indicating that lower plasma IGF-1 are actually associated with increasing species body mass.

\[ y = -0.2033x + 7.5785 \]
\[ r = -0.62 \]

**Fig. 2.2:** Plasma total IGF-1 concentrations are negatively correlated with species' adult body mass. Each data point represents a single mean value for a species. Correlation is significant \((p = 2.9 \times 10^{-5})\).

However, because the correlational analysis used data from multiple species, with shared phylogenetic history, it was necessary to evaluate the phylogenetic signal contained within the data. I used Felsenstein's phylogenetically independent contrasts (FIC; Garland, 2005; Speakman, 2005) to re-evaluate the relationship between IGF-1 and body mass using an estimated phylogeny of all 36 species. This analysis generally weakened the strength of the correlation and the outcome was dependent upon how the analysis was performed. FIC analysis of raw (not Ln-transformed) data using the estimates of relative branch lengths depicted in Fig. 2.1 (based on published phylogenies), gave a weaker correlation between plasma IGF-1 and body mass \((r = -0.42, \text{Fig. } 2.3a)\) that nonetheless remained statistically significant \((p = 0.005)\).
The analysis was repeated using Ln-transformed data and branch lengths equal to one, as in the speciation model of character change (Martins and Garland, 1991; Price et al., 1997), and the correlation was weakened ($r = -0.27$, $p = 0.059$, Fig. 2.3b). Thus, phylogenetic history appears to contribute to the correlation between plasma IGF-1 and body mass. However, there is nonetheless an effect of body mass per se, with larger species generally having lower plasma IGF-1.

As IGF-1 signalling is strongly implicated in longevity (reviewed in Kenyon, 2010), I sought to determine whether there was a relationship between plasma IGF-1 and MLSP. An initial analysis revealed no evidence for correlation of these traits (Fig. 2.4a). However, for the 36 species included in this analysis, MLSP and body mass are highly correlated ($r = 0.65$, $p < 0.001$, Fig. 2.4b). Therefore, the residuals of the MLSP versus body mass plot were correlated with the residuals of MLSP versus plasma IGF-1, to remove the effect of body mass, and then subjected to FIC analysis (Fig. 2.4c). This analysis also indicated no correlation between MLSP and plasma IGF-1 ($r = 0.1$).
Therefore body mass, but not MLSP, is correlated with plasma IGF-1 in mammalian species.

The data set is particularly rich in primate species, with 15 individual species varying in average adult mass from 80g to 75000g. This permits an analysis of IGF-1 correlations exclusively within this clade. Within primates, plasma total IGF-1 appeared to be negatively correlated with species body mass \( r = -0.36 \) and also with species MLSP \( r = -0.40 \) (results not shown). Neither correlation was statistically significant at the \( p < 0.05 \) level, though both were at the \( p < 0.1 \) level. However, both correlations were highly dependent upon the human data point. Exclusion of human data lowered correlation coefficients to \( r = -0.27 \) and -0.25, respectively and rendered \( p > 0.1 \).

2.4 Discussion

It is clear that there is substantial inter-laboratory variability in measurements of plasma free IGF-1 even for highly inbred strains of a particular species. For example, IGF-1 levels for 6-7 month old Sprague-Dawley rats of similar body mass have been reported as 730 ng/ml (Tarasiuk and Segev, 2005) and 1400 ng/ml (Lian et al., 2004). Such substantial differences, which are observed within multiple individual species and strains, are unlikely to reflect primarily the use of recombinant IGF-1 standards originating from a species other than that being measured. Amino acid sequence is highly conserved amongst mammals (100% in most species included in this study), and recombinant rat, human and bovine IGF-1 standards are commercially available and used routinely. Instead, the inter-laboratory variability probably reflects more general differences in analytical methods. A standardized protocol for IGF-1 measurement would be useful in overcoming this problem in the future.
Fig. 2.4: Correlation of MLSP with plasma total IGF-1 concentration. (a) correlation of Ln-transformed data is not significant ($p = 0.281$). (b) MLSP and body mass are significantly correlated in the data set ($p = 1 \times 10^{-5}$), so (c) data was re-analyzed using residual analysis and FIC to account for independent effects of body mass and phylogeny, respectively. This correlation was also not significant ($p = 0.241$). Solid trend line represents a significant relationship; dotted trend line represents a non-significant relationship.

However, by using multiple reported measurements of IGF-1 for individual species (where possible) the effects of this 'noise' was minimized so that underlying trends were not completely obscured, and the significant negative correlation between body mass and plasma IGF-1 was detectable. Within a species, increased IGF-1 signalling and/or
expression of specific IGF-1 variants play(s) significant roles in determining adult body mass. Inbred strains of mice (Yuan et al., 2009) and dogs (Spichiger et al., 2006) with reduced plasma IGF-1 show concomitantly reduced body masses. Presumably this reflects IGF-1’s mitogenic capacity to stimulate the generation of cells and tissue. Similarly, reduced plasma IGF-1 in aged animals has been linked to reduced tissue regenerative potential (Scicchitano et al., 2009; Kooijman et al., 2009). From this perspective, it is counter-intuitive that very large mammalian species maintaining substantial masses of body tissue would have relatively lower circulating IGF-1 levels.

Circulating IGF-1 originates primarily in the liver, an organ that is proportionately smaller and less metabolically active in larger mammalian species (see Porter, 2001 and references therein). So, while liver tissue constitutes approximately 5.5% of body mass in the smallest species included here (mice), it is only about 0.5% of body mass in the largest species (e.g. horses or cows). In addition, the rate of oxygen consumption by an individual horse or cow hepatocyte is only about one-tenth that of a mouse hepatocyte (Porter, 2001). Therefore, the product of these two values should be almost 100-times lower in a horse than in a mouse. If the rate of IGF-1 production and secretion in hepatocytes is roughly proportional to their overall metabolic activity, this would predict significantly reduced circulating levels of the hormone.

It should be noted, however, that plasma levels of other metabolically important hormones (e.g. thyroxine, Hulbert and Else, 2004) do not scale with body mass. In addition, the levels of IGF-1 binding proteins (IGFBP) in plasma as they relate to species’ body masses are unknown. As IGFBPs affect the half-life of plasma IGF-1 (Lee and Gorospe, 2010), differences in IGFBP abundance could contribute ultimately to
lower steady-state IGF-1 levels. Similarly, growth hormone (GH) levels are unknown for the species included in this study, however growth hormone levels do strongly regulate IGF-1 secretion. Given the pulsatile nature of GH secretion from the pituitary (Sherlock and Toogood, 2007), and differences in the timing and amplitude of the GH secretion cycle in nocturnal or crepuscular species (e.g. many rodents) versus diurnal species, this information may be difficult to acquire reliably. Like liver though, pituitary mass as a proportion of body mass is indirectly correlated with species body mass (Stahl, 1965), so larger species will have proportionately smaller pituitary glands. This could result in a reduced rate of GH secretion in larger species. In summary, while I can robustly conclude that plasma IGF-1 levels are indirectly correlated with species body mass, I do not know mechanistically why this is so.

The lower circulating IGF-1 levels in larger mammalian species could also contribute to their reduced mass-specific metabolic rates (Rolfe and Brown, 1997). In a rat model of adult onset IGF-1 deficiency, a reduction of plasma IGF-1 by about 35% was associated with 20-40% lower rates of glucose utilization, and lower ATP levels, in various brain regions (Sonntag et al., 2006). Brain tissue accounts for up to 20% of whole body metabolic rate in rats and humans (Rolfe and Brown, 1997). Therefore, this could make an appreciable contribution to the observed differences in mass-specific metabolic rates, particularly if other tissues are similarly affected.

IGF-1 is also a powerful mitogen, with roles in regulating growth of various tissues, including muscle and brain. In adults, IGF-1 is implicated in the regeneration of damaged tissue (e.g. see Ten Broek et al., 2010), and remodelling of existing tissue as in neural plasticity (Sonntag et al., 2005). In adult mammals, reduced circulating IGF-1
levels associated with advanced age have been implicated in the reduced capacities for tissue regenerative repair and remodelling (Ten Broek et al., 2010). Thus, the lower levels of circulating IGF-1 in larger mammalian species might be expected to contribute to slower rates of proliferative growth. While this would be expected to slow the rate of wound healing and tissue repair, it may equally be important in avoiding the permissive environment for cancer growth created by high IGF-1 levels (e.g. Dunn et al., 1997, Wu et al., 2002). Plasma IGF-1 is reduced typically about 40% in CR rodents, and this corresponds to reduced incidences of a variety of tumours (see Sonntag et al., 2006 and references therein). This effect can be reversed by direct injection of IGF-1 in CR animals (Ramsey et al., 2002). Epidemiological studies of humans also indicate a correlation between circulating IGF-1 levels and cancer growth and metastasis (Khandwala et al., 2000). Thus, lower plasma IGF-1 levels in larger species may contribute to the cancer resistance that is associated with longevity. Seluanov et al. (2007) demonstrated a negative correlation between telomerase activity and body mass in rodents, which presumably is protective against unregulated cell division. There is evidence that IGF-1 stimulates telomerase activity in some cell types (e.g. Wetterau et al., 2003; Moverare-Skrtic et al., 2009). It is possible the lower IGF-1 levels of larger species contribute to these observed reductions in telomerase activity.

Reduced IGF-1 signalling is also strongly associated with increased stress resistance in invertebrates and mammals, mediated at least in part via interactions with FOXO transcription factors (Kenyon, 2010). Most mutant mouse strains with increased longevity show concomitantly enhanced stress resistance at whole animal and cellular levels (Robb et al., 2009). Kapahi et al. (1999) demonstrated a robust direct correlation
between stress resistance of skin fibroblasts and MLSP in mammalian species. The reduced plasma IGF-1 of larger species may play a role in mediating these differences in cellular stress resistance. Investigation of elements of the IGF-1 intracellular signalling pathway in the inter-species context will provide further insight into this possibility.

The contribution of IGF-1 to the physiological regulation of body mass is achieved by both local (paracrine) and systemic (endocrine) effects. Delineating the relative contributions of paracrine and endocrine IGF-1 signalling has been contentious. Whereas Yakar et al. (1999) provided evidence that IGF-1 actions in mice are entirely paracrine, more recently Stratikopoulos et al. (2008) have shown approximately equal contributions of systemic and local IGF-1 to body mass determination. In rats, systemic injection of IGF-1 is capable of rescuing the effects of congenital GH/IGF-1 deficiency during development (Sonntag et al., 2005), which also indicates the importance of endocrine IGF-1 signalling. Unfortunately, it is unknown if tissue IGF-1 levels scale similarly to plasma IGF-1, due to a lack of published data across a broad range of species. However, it would be interesting to make such measurements in the future.

It is interesting that the correlation between plasma IGF-1 and species body mass may be detectable within primates alone. Though this correlation was not statistically significant, this may be due to the relatively low statistical power associated with the current dataset of only 15 primate species. Bernstein et al. (2007) similarly found evidence for a weak (r = -0.20, not statistically significant) negative correlation between plasma IGF-1 and body mass in females of eight papionin primate species. Application of a standardized IGF-1 measurement protocol to primate plasma samples may reveal real relationships between IGF-1 and body mass or MLSP.
In summary, while intra-species comparisons have revealed a positive correlation between plasma total IGF-1 and body mass, I have shown the opposite trend in an inter-species context. This negative correlation between circulating IGF-1 levels and species body mass is interesting because of the significant roles of IGF-1 in metabolism, cancer, cellular stress resistance, and longevity. Larger mammalian species are generally also longer-lived, and it is tempting to speculate that reductions of plasma IGF-1 contribute to important aspects of the large mammal phenotype. The reductions in plasma IGF-1 in larger mammals may promote a hormonal environment conducive to longevity.
Chapter 3. Antioxidant enzyme activities are not broadly correlated with longevity in 14 vertebrate endotherm species

Hypothesis

Intracellular antioxidant enzymes have a role in conferring longevity in long-lived species and/or strains.

Objective

The primary objective of this project was to determine if intracellular antioxidant enzymes play a role in longevity in both an inter-specific and intra-specific context.

Publications of results


Contributions

I performed all experiments, phylogenetic and statistical analysis and chapter write-up.
3.1 Introduction

Since the publication of Harman’s free radical theory of aging (Harman, 1956), the contribution of reactive oxygen species (ROS) and oxidative damage to cellular macromolecules to animal aging and longevity has attracted significant interest amongst biologists. Data have accumulated indicating that longevity is positively correlated with resistance to oxidative stress (Kapahi et al., 1999; Salmon et al., 2005; Labinskyy et al., 2006; Harper et al., 2007), which is consistent with Harman’s original theory. Identification of the molecular mechanisms conferring the enhanced stress resistance associated with longevity remains a major goal. As the most proximal mechanism for dealing with ROS is direct and immediate detoxification, antioxidant enzymes have been well studied in this context. However, the use of genetic manipulation to under- or over-express antioxidant enzymes in a variety of animal models of aging and longevity has produced somewhat varied results. While in some instances lifespan extension has been achieved via overexpression of one or more antioxidant enzymes, this outcome is often dependent upon species, or the particular laboratory strain of a species (Pérez et al., 2009b). The developmental timing and tissue specificity of transgenic over-expression are also important aspects that influence the outcome.

An alternative to using genetic manipulations to identify molecular mechanisms conferring longevity is the use of broad comparative studies. Whereas genetic manipulations query whether altering expression of a gene or several genes can alter lifespan, the comparative approach attempts to determine what molecular characteristics have been selected for during the evolution of longevity. A number of comparative studies have investigated whether mitochondrial ROS production (Labinskyy et al., 2006;
Lambert et al., 2007), cellular resistance to exogenous ROS (Kapahi et al., 1999; Salmon et al., 2008), or the effectiveness of antioxidant defences correlate with animal species’ lifespans (Tolmasoff et al., 1980; Cutler, 1991; Sohal et al., 1990; Brown and Stuart, 2007). These studies have revealed potentially interesting and important insights into how long-lived species might minimize ROS production or maximize resistance to oxidative stress. However, the confident interpretation of most of these results is limited by the use of small sample sizes. In some cases, correlative analyses of antioxidant enzymes and lifespan have been performed on as few as five species. Limited sample sizes (number of individual species) are more prone to bias due to occasional outlying data points, which increase the chance of accepting a hypothesis that should be rejected.

In addition to the limits imposed by small sample sizes, two other major problems exist with all comparative studies of antioxidant enzymes done to date. These are the confounding effects of body mass and the non-independence of data points representing individual species, due to their phylogenetic relatedness (see Speakman, 2005 for review). All extant animals share some of their evolutionary history with a common ancestor; therefore animal species need to be treated as non-independent data. An appropriate approach, which has been used in some comparative studies of longevity (Lambert et al., 2007; Moosmann and Behl, 2008) relies on the transformation of raw data into Felsenstein’s phylogenetic independent contrasts (Felsenstein, 1985) to account for phylogenetic relatedness.

Despite the problems outlined above, which are readily overcome via modifications to study design and data analysis, the comparative approach has tremendous value as a means of identifying mechanisms that have been selected for
during the evolution of longevity. Between species differences in longevity can be as great as two orders of magnitude within the vertebrates alone. These differences thus greatly exceed the more subtle differences observed in, for example, long-lived genetically mutant mice in which maximum lifespan (MLSP) is typically increased on the order of 50% (Brown-Borg et al., 1996; Flurkey et al., 2001). Thus, the magnitude of the molecular mechanistic differences might also be expected to be particularly large in the longest-lived species.

Here, I report the results from a comprehensive comparative study of antioxidant enzyme capacities in 14 species of vertebrate endotherms (Table 3.1; Fig. 3.1).

Fig. 3.1: Phylogeny of the 14 species used in this study. The tree represent an approximate consensus from four phylogenies (Stuart et al., 2002; Springer and Murphy, 2007; Hackett et al., 2008; Prasad et al., 2009), and branch lengths are best estimates from these phylogenies.

I measured the maximal activities of the five major intracellular antioxidant enzymes: cytosolic superoxide dismutase (CuZnSOD), mitochondrial superoxide dismutase
(MnSOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR). For most species, measurements were made in three organs in which dysfunction and failure are associated with aging and death: brain, heart and liver. I corrected for the potentially confounding effect of body mass on the interpretation of data by analysing residuals from a regression on body size. I also corrected for the non-independence of species data points using Felsenstein’s phylogenetically independent contrasts (FIC; see Garland et al., 2005 for review). This therefore represents the most comprehensive and statistically validated such study performed to date. I included in the study a single experimental model of an intra-specific difference in longevity, the long-lived Snell dwarf mouse. The Snell dwarf mouse harbours a mutation within the Pit-1 gene, and is similar to the Ames dwarf mouse (mutation in Prop-1 gene) in having dramatically reduced body size accompanied by longevity extension of up to 60% compared to normal-sized littersmates (Brown-Borg et al., 1996; Flurkey et al., 2001). Both strains possess enhanced stress resistance at the whole animal and/or cellular level (Salmon et al., 2005; Bokov et al., 2009). I found no evidence, in either the inter-specific or the intra-specific comparisons, of a broad multi-tissue upregulation of antioxidant enzyme activities associated with the evolution of longevity. However, I did observe select positive correlates of longevity amongst antioxidant enzymes in brain tissue specifically.

3.2 Experimental Procedures

3.2.1 Materials
Chemicals were obtained from Bioshop (Burlington, ON, Canada) and Sigma-Aldrich (Oakville, ON, Canada; including Fluka and Caledon). BioRad protein dye was obtained from BioRad Laboratories (Hercules, CA, USA). Prestained broad range protein marker
was obtained from BioLabs (New England, MA, USA). Memcode reversible protein
stain kit was obtained from Pierce Biotechnology (Rockland, IL, USA). Antibodies to
human CuZnSOD were purchased from Abcam (Cambridge, MA, USA) and antibodies
to rat MnSOD were purchased from Stressgen Biotechnologies (Ann Arbor, MI, USA).
Infrared dye-conjugated secondary antibodies to rabbit were purchased from Rockland
Immunochemicals (Gilbertsville, PA, USA).

3.2.2 Animals
Between three and eight individuals of each of twelve mammalian and two avian species
were used in this study (Table 3.1). Species were selected on the bases of: (1)
phylogenetic position; (2) MLSP; (3) routine diet and (4) availability. With respect to
phylogenetic position, I confined my analysis to species with a similar overall body plan
and physiology, i.e. vertebrate endotherms. Within this constraint, I sampled species
within and between orders. I also attempted to maximize the range of MLSP represented
by species included in the study. The inclusion of rodents and human samples established
a MLSP range from 3.5 years to over 100 years. I also included species with
exceptionally long lifespans for their body mass, such as the big brown bat, zebra finch
and human. I was, however, careful to note instances in which trends might have been
driven by single outlier species, particularly from the human samples (see Speakman,
2005). The nature of the study meant that I was unable to directly control for differences
in diet specific to a given species. However, the species collection includes
Table 3.1: Sex, age, mass and maximum lifespan (MLSP) of the mammalian and avian species. Species MLSP data are from AnAge (de Magalhaes et al., 2005).

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Scientific Name</th>
<th>Number and Sex</th>
<th>Approximate Age</th>
<th>Body Mass (kg)</th>
<th>MLSP (yrs)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mammalia</strong></td>
<td>Mus musculus</td>
<td>6F (+/dw)</td>
<td>6-7 months</td>
<td>0.02996</td>
<td>2.7</td>
<td>Jackson Laboratories (Bar Habor, ME, USA)</td>
</tr>
<tr>
<td></td>
<td>Mus musculus</td>
<td>6F (dw/dw)</td>
<td>6-7 months</td>
<td>0.0096</td>
<td>3.9</td>
<td></td>
</tr>
<tr>
<td>C57BL/6Ner1BR mouse</td>
<td>Mus Musculus</td>
<td>4F</td>
<td>4 months</td>
<td>0.0276</td>
<td>3.5</td>
<td>Charles River (Wilmington, MA, USA)</td>
</tr>
<tr>
<td></td>
<td>Mus Musculus</td>
<td>4M</td>
<td>3 months</td>
<td>0.55</td>
<td>5</td>
<td>Charles River (Wilmington, MA, USA)</td>
</tr>
<tr>
<td>Norway rat</td>
<td>Rattus norvegicus</td>
<td>6M</td>
<td>3 months</td>
<td>0.55</td>
<td>5</td>
<td>University of Manitoba Field Research Station (Carmen MB, Canada)</td>
</tr>
<tr>
<td>13-lined ground squirrel</td>
<td>Spermophilus tridecemlineatus</td>
<td>8F</td>
<td>Unknown</td>
<td>0.2050</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>Oryctolagus cuniculus</td>
<td>4F</td>
<td>3.5 months</td>
<td>2.6</td>
<td>11.8</td>
<td>Local abattoir (Fort Erie, ON, Canada)</td>
</tr>
<tr>
<td></td>
<td>Cavia porcellus</td>
<td>1M</td>
<td>Unknown</td>
<td>1.05</td>
<td>12</td>
<td>Rockland (Gilbertsville, PA, USA)</td>
</tr>
<tr>
<td>Big brown bat</td>
<td>Eptesicus fuscus</td>
<td>3F</td>
<td>6-12 months</td>
<td>0.025</td>
<td>19</td>
<td>McMaster University Bat Laboratory (Hamilton, ON, Canada)</td>
</tr>
<tr>
<td></td>
<td>Eptesicus fuscus</td>
<td>2M</td>
<td>6-12 months</td>
<td>0.025</td>
<td>19</td>
<td>Local abattoir (Fort Erie, ON, Canada)</td>
</tr>
<tr>
<td>Sussex Sheep</td>
<td>Ovis aries</td>
<td>5F</td>
<td>6-12 months</td>
<td>29.5</td>
<td>19.6</td>
<td>Local hunters (Cobden, ON, Canada)</td>
</tr>
<tr>
<td>White-tailed deer</td>
<td>Odocoileus virginianus</td>
<td>3M</td>
<td>2.5-3.5 years</td>
<td>110</td>
<td>21.6</td>
<td>Equake-Bio, Inc. (Kerrville, TX, USA)</td>
</tr>
<tr>
<td>Domestic Dog</td>
<td>Canis familiaris</td>
<td>3M</td>
<td>2-8 years</td>
<td>11</td>
<td>24</td>
<td>Local abattoir (Fort Erie, ON, Canada)</td>
</tr>
<tr>
<td>Yorkshire/ Hampshire Pig</td>
<td>Sus scrofa</td>
<td>2F</td>
<td>6 months</td>
<td>99.9</td>
<td>27</td>
<td>Local abattoir (Fort Erie, ON, Canada)</td>
</tr>
<tr>
<td></td>
<td>3M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black angus/ Charlet Cow</td>
<td>Bos taurus</td>
<td>4F</td>
<td>1.5-2.5 years</td>
<td>379.5</td>
<td>30</td>
<td>Local abattoir (Fort Erie, ON, Canada)</td>
</tr>
<tr>
<td></td>
<td>1M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>Homo sapiens</td>
<td>2F</td>
<td>26-37</td>
<td>68</td>
<td>122</td>
<td>Zenbio (Research Triangle Park, NC, USA)</td>
</tr>
<tr>
<td></td>
<td>2M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Aves</strong></td>
<td>Coturnix japonica</td>
<td>2F</td>
<td>4-5 months</td>
<td>0.264</td>
<td>6</td>
<td>Cro Quail Farms Inc (St. Anne's, ON, Canada)</td>
</tr>
<tr>
<td></td>
<td>2M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zebra finch</td>
<td>Taeniopygia guttata</td>
<td>3F</td>
<td>1 year</td>
<td>0.0166</td>
<td>14.5</td>
<td>Trent University Animal Care Facility (Peterborough, ON, Canada)</td>
</tr>
<tr>
<td></td>
<td>3M</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

67
graminivores, nucivores, insectivores, omnivores and carnivores and no trends were
noted that related to dietary preference. I also included wild-caught species where
possible to control for effects of domestication. In most cases, exact ages and sex were
known; however, this was not always possible to determine depending on source. All
animals were healthy young adults.

3.2.3 Tissue collection
Normal C57BL mice were euthanized by cervical dislocation following which brain,
heart and liver tissues were immediately excised, flash frozen in liquid nitrogen and
stored at -80°C. Snell normal and dwarf mice were anaesthetized with isoflurane,
injected with a mixture of 50 µg/g ketamine and 5 µg/g xylazine and then euthanized by
incision into the lower abdomen, heart and brain tissues were removed 30 - 45 min post-
mortem. These tissues were flash frozen in liquid nitrogen and stored at -80°C. Norway
laboratory rats were euthanized with pentabarbitol sodium injection (Euthanyl®, 2 µg/g
body mass). Brain, heart, and liver tissues were removed and flash frozen in liquid
nitrogen and stored at -80°C. Tissues from 13-lined ground squirrels were collected at
the University of Western Ontario (London, ON, Canada; see Chapter 6). Active (i.e.
non-hibernating) animals were euthanized by Euthanyl overdose (270 mg/ml, 0.2 ml/100
g), brain, heart and liver tissue were rapidly removed, flash-frozen in liquid nitrogen, and
stored at -80°C. Big brown bats were from a colony housed at McMaster University
(Hamilton, ON, Canada). Bats were euthanized using 0.6 mg/g body wt pentabarbitol
sodium followed by decapitation. Brain, heart and liver tissues were excised and flash
frozen in liquid nitrogen and stored at -80°C. Zebra finch and Japanese quail tissues were
collected at Trent University (Peterborough, ON, Canada). Birds were euthanized with 2
(finch) or 0.6 (quail) mg/g body wt pentobarbital sodium (Euthasol®), followed by
decapitation. Brain, heart and liver tissues from both bird species were removed and
flash frozen in liquid nitrogen and stored at -80°C. Guinea pigs were euthanized, tissues
rinsed in 1X PBS and immediately frozen in liquid nitrogen and shipped to Brock
University (St. Catharines, ON, Canada) on dry ice. Similarly, dogs were euthanized and
excised tissues immediately frozen in liquid nitrogen and then shipped to Brock
University (St. Catharines, ON, Canada) on dry ice. Domesticated livestock were
collected during normal processing at a local abattoir, with the exception of rabbits which
were collected during processing from a local farmer. In all cases, brain, heart and liver
tissues were collected from these animals within approximately 30 min post mortem.
Due to the organ size of the domesticated livestock and rabbits, tissue samples were
isolated from the following locations; for brain, from the cortex and striatum; for heart,
from the right ventricle; and for the liver, from the tip of the left lobe. The sample site
for the heart, brain and liver were similar in all domesticated livestock, including the
rabbit. Antioxidant enzyme activities were measured separately for cortex and striatum
tissue, however values were similar between both sites and therefore the data was
compiled. Tissues were frozen on-site in dry ice then brought to Brock University (St.
Catharines, ON, Canada) where they were stored at -80°C. White-tailed deer were killed
by hunters in Cobden (ON, Canada) following which brain, heart and liver tissue was
collected within 1 h post mortem and frozen at -20°C for one month, then transferred to
Brock University where they were stored at -80°C. Tissue sampling sites were unknown
for the white-tailed deer. Human liver samples (ZenBio, Research Triangle Park, NC,
USA) were isolated from recently deceased patients following cardiac arrest and/or head
trauma. Sampling sites and time prior to tissue isolation and subsequent freezing were unknown for these samples.

3.2.4 Tissue homogenization
Brain, heart and liver tissues were homogenized in homogenization buffer (10 mM KH$_2$PO$_4$ (pH 7.4), 20 mM EDTA, 30 mM KCl, 0.1% Triton X-100, 10% glycerol). Tissues were homogenized at 4°C with a PowerGen 125 homogenizer (Fisher Scientific, Ottawa, ON, Canada) on full speed for 10 s for 3 cycles with 10 s rests between each cycle. Samples were centrifuged for 10 min at 500 g (4°C). Protein concentrations of the supernatants were measured using the Bradford technique with a BioRad protein kit. Tissue homogenates were stored at -80°C.

3.2.5 Immunodetection of MnSOD and CuZnSOD in Snell mouse tissues
Heart (30 μg) and brain (20 μg) protein extract were separated by SDS-PAGE and electrotransferred to a PVDF membrane. Membranes were stained with Memcode reversible protein stain to ensure even transfer. Membranes were incubated in blocking solution (5% w/v skim milk in 1X PBS) for 45 min. Following blocking, membranes were incubated with anti-CuZnSOD (1:1000 v/v) or anti-MnSOD (1:1000 v/v) overnight in blocking buffer at 4°C. Following repeated washing, membranes were incubated with infrared dye conjugated secondary antibody for 1 h at room temperature. Membranes were visualized using the Odyssey infrared imaging system from LI-COR Biosciences (NE, USA). Protein level quantification was done using Odyssey imaging system software, version 1.0.
3.2.6 In-gel superoxide dismutase assay
SOD isoform activities were assayed as in Robb et al. (2008a,b). Briefly, brain, heart and liver tissue homogenates equal amounts of protein were resolved by native polyacrylamide gel electrophoresis at 20mA at 4°C for 2.5 to 3.5 h. Resolution time was dependent upon species and tissues. Gels were incubated in a solution of 1.23 mM nitro blue tetrazolium. Gels were briefly rinsed with deionized water and then stained with a solution containing a superoxide generating system: 28 mM TEMED and 4.6 x 10^-2 mM riboflavin in potassium phosphate buffer (pH 7.8). Gels were rinsed with deionized water and illuminated under fluorescent light until the background developed a uniform blue-violet colour upon which the bands were clearly visible. Bands represented areas of SOD activity in which nitro blue tetrazolium was not reduced by superoxide due to its dismutation. An in-gel standard curve was resolved using a dilution series of pure bovine liver CuZnSOD (Sigma), and the activities of tissue homogenates were calculated by interpolation. The addition of 5 mM KCN to the staining solution differentiates CuZnSOD from MnSOD due to the inhibition of CuZnSOD by KCN. Analysis of SOD activity of individual protein bands was done by scanning the gels on an HP Scanjet G4050 (Mississauga, ON, Canada) and quantifying band intensity using BioRad’s Quantity One® software.

3.2.7 Other enzyme assays
Catalase, glutathione peroxidase and glutathione reductase assays were performed at 30°C, using a Varian Cary 100 Bio UV-Visible Spectrophotometer. For all assays, the background change in absorbance was subtracted from the change in absorbance following the initiation of the reaction. Conditions for the glutathione peroxidase (GPx) activity assay were: 50 mM potassium phosphate buffer (pH 7.0), 0.4 mM EDTA, 0.15
mM β-NADPH, 1 unit glutathione reductase, and 1 mM reduced glutathione. The reaction was initiated with the addition of 0.20 mM hydrogen peroxide; the following change in absorbance was measured at 340 nm 6 min. Glutathione reductase (GR) activity was measured in a solution containing 75 mM KH$_2$PO$_4$ buffer (pH 7.6), 2.6 mM EDTA, and 0.09 mM NADPH. The addition of 1 mM oxidized glutathione initiated the reaction and a change in absorbance over 6 min was measured at 340 nm. GR activity assays were occasionally measured using a Bio-Tek Power Wave plate reader spectrophotometer, in which cases protein homogenates were limited. Conditions were the same as above and the reaction was carried out at 30°C. Catalase (CAT) activity was measured in a solution of 25 mM potassium phosphate buffer and 33 mM hydrogen peroxide. The reaction was initiated by the addition of 80 μg of protein homogenate and the following change in absorbance was measured at 240 nm for 4 min. Conditions for the citrate synthase (CS) activity assay were: 0.5 mM 5,5'-dithiobis(2-nitrobenzoic) acid, 0.1 mM acetyl-Coenzyme A, 0.05% Triton X-100, 50 mM Tris (pH 8.0). A background change in absorbance was measured prior to the addition of 0.5 mM oxaloacetate which initiated the reaction; the following change in absorbance over 6 min was measured at 412 nm. Occasionally, when protein homogenates were limited, CS activity assays were measured using a Bio-Tek Power Wave plate reader spectrophotometer. Conditions were the same as above and the assay was carried out at 30°C.

### 3.2.8 Statistical analyses
Correlation analysis is as in Chapter 2. In addition, the phylogenetic tree was constructed from four phylogenies from which branch length estimates were taken (Stuart et al., 2002; Springer and Murphy, 2007; Hackett et al., 2008; Prasad et al., 2009) (Fig. 3.1).
However, the analysis was repeated with all branch lengths set to one, as with a speciational model of character change (Martins and Garland, 1991; Price et al., 1997). Statistical analyses within the intra-species context (Snell dwarf mice) used a two-tailed t-test to compare normal and dwarf mice. A p-value of 0.05 was considered significant. Outlier data points were calculated from species means using Dixon’s Q parameters at 95% confidence intervals (Rorabacher, 1991).

3.3 Results

CuZnSOD activity data from 14 mammalian and avian species were analysed with respect to MLSP. CuZnSOD activity in liver, heart and brain showed no evidence of correlation with species MLSP (Fig. 3.2a-c). Neither residual analysis nor the application of Felsenstein’s phylogenetically independent contrasts to the residuals changed this conclusion (Fig. 3.2d, e shows liver; Table 3.2 shows heart and brain). There was also no evidence that CuZnSOD activity was correlated with body mass (Supplemental Table 3.1; includes all relationships between enzyme activities and body mass). CuZnSOD levels were examined in an intra-specific model of mammalian longevity, the long-lived Snell dwarf mouse.

There were no differences observed between Snell dwarf and normal mice in either heart or brain CuZnSOD protein levels (Fig. 3.2f). CuZnSOD thus appears to be constitutively expressed in liver, heart and brain tissue of endotherms irrespective of the lifespan of the species or laboratory strain.
Fig. 3. CuZnSOD does not correlate with lifespan. CuZnSOD activity in liver (p = 0.238) (a), heart (p = 0.338) (b) or brain (p = 0.254) tissue (c) does not correlate with species MLSP. Values are ln-transformed and each data point consists of measurements from 3-8 individuals of each species. (d) Residual analysis of liver CuZnSOD activity and animal MLSP (p = 0.411). (e) FIC analysis of liver CuZnSOD activity and MLSP (p = 0.213). (f) CuZnSOD levels are not elevated in tissue of young adult Snell dwarf mice compared to normal littermates. White bars represent Snell normal and black bars represent Snell dwarf. Values are means ± SEM of duplicate measurements from 6 animals per experimental group. A two tailed t-test revealed no significant difference between normal and dwarf mice (heart, p value = 0.088; brain, p value = 0.356). Solid trend line represents a significant relationship; dotted trend line represents a non-significant relationship.
MnSOD activity in liver tissue was positively correlated with MLSP (Fig. 3.3a). This relationship was driven by a human data point that was particularly high. Rorabacher’s (1991) technique for identifying outliers in the raw data based on Dixon’s Q-parameter confirmed that the human data point was an outlier. Although, I do not know why the human data point was an outlier, this may be due to the unique sampling protocol for human tissues, where time post-mortem is longer and not known exactly. Human data points are often outliers in comparative studies for a variety of reasons, including exceptional documentation of birth and death records (Speakman, 2005). Removal of the human data point abolished the correlation (Fig. 3.3b; residual and FIC analyses for liver with and without human data point in Supplemental Fig. 1a-d). MnSOD activity in heart tissue did not correlate with MLSP (Table 3.2). However, a positive correlation between MnSOD and MLSP was observed for brain tissue (Fig. 3.3c). FIC (Fig. 3.3d) analyses of the residuals confirmed the positive correlation between brain MnSOD activity and MLSP. This was not due to a general scaling of mitochondrial abundance with MLSP. MnSOD is localized exclusively within the mitochondrial matrix, so between-species differences in cellular mitochondrial abundance would influence scaling behaviour of any mitochondrial protein.

Although the scaling of mitochondrial abundance with species MLSP should be negative (Porter, 2001) I nonetheless measured citrate synthase activity (CS) as a proxy for mitochondrial number and found no relationship between brain CS activity and MLSP (Fig. 3.3e shows brain; see Supplemental Table 3.2 for heart and liver tissue; see Supplemental Table 3.3 for relationship between CS activity and species body mass). Also, MnSOD did not correlate with CS activity (Fig. 3.3f shows brain), further
indicating that the MnSOD correlation was not due simply to increased mitochondrial abundance in long-lived species.
Fig. 3.3: Correlation between MnSOD activity and animal MLSP. (a) MnSOD activity within liver tissue correlates with animal MLSP (p value = 0.013). (b) The correlation between MnSOD activity and animal MLSP is lost after the removal of the human data point (p value = 0.227). (c) MnSOD activity within brain tissue correlates with animal MLSP (p value = 0.001). (d) The relationship between brain MnSOD activity and MLSP remains following residual and FIC analysis (p value = 0.007). (e) Brain CS activity does not significantly correlate with species MLSP (p value = 0.228). (f) Brain CS activity does not significantly correlate with brain MnSOD activity (p value = 0.206). (g) MnSOD protein levels are higher in Snell normal mice than in long-lived dwarf littermates (only brain is significant (p value = 0.021; heart, p value = 0.325). In (a-f) values are ln-transformed and each data point consists of a single measurement from 3-8 animals for each species. In (g) white bars represent Snell normal mice and black bars represent Snell dwarf mice. Values are means ± SEM of duplicate measurements from 6 animals per experimental group. * = significantly different (p < 0.05) using a two-tailed t-test. Solid trend line represents a significant relationship; dotted trend line represents a non-significant relationship.

Thus, MnSOD activity within brain tissue homogenates was strongly correlated with species MLSP, and the relationship is not driven by body mass or phylogenetic constraints. In the Snell dwarf mouse comparison, MnSOD protein levels in heart and brain tissue actually appeared to be marginally lower in dwarf than normal individuals, reaching statistical significance in brain (p = 0.021; Fig. 3.3g).

CAT activity was also found to correlate positively with animal MLSP only in brain tissue (Fig. 3.4a, see Supplemental Fig. 3.2a for body mass). Residual and FIC analyses confirmed the positive correlation between brain CAT activity and MLSP (Fig. 3.4b). In contrast, there was no correlation between liver CAT activity and species MLSP (Table 3.2). CAT activity in heart tissue appeared to correlate with animal MLSP (although this was not significant) (Fig. 3.4c). This relationship was actually driven by a significant correlation of heart CAT activity with body mass (Fig. 3.4d). Once body mass was removed in the residual analysis, the relationship between heart CAT activity
and animal MLSP was lost (Fig. 3.4e, Supplemental Fig 3.2b). No differences in heart or brain CAT activity were observed between Snell dwarf and normal mice (Fig. 3.4f).

**Table 3. 2:** Statistical analysis of linear regressions, enzyme activity as a function of lifespan.

<table>
<thead>
<tr>
<th>Antioxidant Enzyme Activity</th>
<th>Correlation Coefficient</th>
<th>Slope</th>
<th>p value</th>
</tr>
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<tbody>
<tr>
<td><strong>Heart CuZnSOD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>-0.244</td>
<td>-0.1842</td>
<td>0.221</td>
</tr>
<tr>
<td>Residual + FIC</td>
<td>0.147</td>
<td>0.1469</td>
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<tr>
<td><strong>Brain CuZnSOD</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>-0.0031</td>
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</tr>
<tr>
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<td>-0.0883</td>
<td>0.372</td>
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<tr>
<td><strong>Heart MnSOD</strong></td>
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<td></td>
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<td>0.466</td>
<td>0.193</td>
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<tr>
<td>Residual</td>
<td>0.157</td>
<td>0.1912</td>
<td>0.322</td>
</tr>
<tr>
<td>Residual + FIC</td>
<td>-0.173</td>
<td>-0.2402</td>
<td>0.315</td>
</tr>
<tr>
<td><strong>Liver CAT</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>No correction</td>
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<td>-0.085</td>
<td>0.374</td>
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<tr>
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<td><strong>Heart GPx</strong></td>
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<td></td>
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</tr>
<tr>
<td>No correction</td>
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<td>-1.3903</td>
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<td>-0.784</td>
<td>0.199</td>
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<td>Residual + FIC</td>
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<td><strong>Brain GPx</strong></td>
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<tr>
<td>Residual</td>
<td>0.086</td>
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<td>Residual + FIC</td>
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<td><strong>Liver GPx</strong></td>
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<td></td>
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<tr>
<td>No correction</td>
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<td>Residual</td>
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<tr>
<td><strong>Brain GR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>0.122</td>
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<td>0.345</td>
</tr>
<tr>
<td>Residual + FIC</td>
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<tr>
<td><strong>Liver GR</strong></td>
<td></td>
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<tr>
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<tr>
<td>Residual + FIC*</td>
<td>-0.569</td>
<td>-1.0269</td>
<td>0.026</td>
</tr>
</tbody>
</table>

Residual = removal of body mass; Residual + FIC = removal of body mass and phylogenetic relatedness. * = relationship is negative correlation
Fig. 3.4: Correlation between CAT activity and animal MLSP. (a) Brain CAT activity correlates with animal MLSP (p value = 0.008). (b) Brain CAT activity remains correlated with MLSP following residual and FIC analyses (p value = 0.002). (c) Heart CAT activity appears to correlate with animal MLSP (p value = 0.098), however this is due to the correlation between (d) heart CAT activity and species body mass (p value = 0.036). (e) The effect of body mass in (c) is removed by plotting the residuals (p value = 0.404). In (a-e) values are ln transformed and each data point consists of duplicate measurements from 3-8 animals for each species. (f) No differences in CAT activity between Snell dwarf and normal mice (heart, p value = 0.408; brain, p value = 0.954). In (f) white bars represent Snell normal mice and black bars represent Snell dwarf mice. Values are means ± SEM of duplicate measurements from 6 animals per experimental group. Solid trend line represents a significant relationship; dotted trend line represents a non-significant relationship.
GPx activity did not correlate with animal MLSP for brain (Fig. 3.5a; Table 3.2), heart or liver tissue (Table 3.2). Interestingly, there appeared to be a slight negative correlation between GPx activity and body mass in all three tissues. However these correlations did not reach significance (Supplemental Table 3.1). GPx activity was significantly higher in Snell dwarf heart tissue compared to their normal littermate mice (p < 0.05); however no difference was observed in brain tissue (Fig. 3.5b).

GR activity did not correlate with animal MLSP in brain (Fig. 3.6a; Table 3.2), heart or liver (Table 3.2). Interestingly, GR activity correlated negatively with species MLSP within liver tissue (Table 3.2), however this was only observed once the data was corrected for both body mass and phylogenetic relatedness. It appeared that this correlation was driven by the data point that represents the branching of the two avian samples from the mammalian species.

**Fig. 3.5:** GPx activity does not correlate with animal MLSP. (a) Brain GPx activity does not correlate with animal MLSP. (b) Heart GPx activity, but not brain, is higher in Snell dwarf mice compared to normal littermates. In (a) all values are ln-transformed and each data point consists of duplicate measurements from 3-8 animals for each species. Dotted trend line represents a non-significant relationship. In (b) white bars represent Snell normal mice and black bars represent Snell dwarf mice. Values are means ± SEM of duplicate measurements from 6 animals per experimental group. ‘*’ = significantly different (p < 0.05) using a two-tailed t-test.
Although Dixon’s Q-parameter (Rorabacher, 1991) did not confirm this data point as an outlier the correlation was abolished following its removal from the analysis (data not shown). Similarly, there were no differences in heart or brain GR activity between Snell normal and dwarf mice (Fig. 3.6b).

**Fig. 3. 6:** GR activity does not correlate with animal MLSP. (a) Brain GR activity is not correlated with animal MLSP (p value = 0.391). (b) GR activity in heart (p value = 0.25) and brain (p value = 0.133) does not differ between Snell dwarf and normal mice. In (a) values are ln-transformed and each data point consists of duplicate measurements from 3-8 animals for each species. In (b) white bars represent Snell normal mice and black bars represent Snell dwarf mice. Values are means ± SEM of duplicate measurements from 6 animals per experimental group. Dotted trend line represents a non-significant relationship.

3.4 Discussion

Results from studies in which antioxidant enzyme levels are manipulated via genetic modification have been varied, and thus present an inconsistent view of the connection between ROS detoxification and longevity. Taken together, the results from dozens of studies in which antioxidant enzyme expression has been manipulated via genetic modification indicate that any effects on lifespan are highly dependent upon species, developmental timing of overexpression, or parallel overexpression of multiple enzymes. However, the results do not present a compelling and consistent case for a pro-longevity capability of antioxidant enzymes, particularly in mammals (reviewed in Pérez
et al., 2009b). The comparative approach used in the present study avoids some of the pitfalls inherent to genetic manipulations, such as unbalancing ROS detoxification pathways by the overexpression of a single enzyme. It is striking then that this different approach has produced a very similar conclusion, i.e. that there is no compelling evidence for global increases in five major antioxidant enzyme activities in several tissues of exceptionally long-lived mammals or birds.

CuZnSOD, as the major cytosolic catalyst of superoxide dismutation, has been well studied using genetic manipulations. In Drosophila melanogaster, targeted overexpression of CuZnSOD in motor neurons has been shown to extend lifespan up to 60% (Parkes et al., 1998). However, global CuZnSOD overexpression is effective in extending D. melanogaster MLSP only in laboratory strains with relatively short lifespans, suggesting that the overexpression is simply compensating for an inherent susceptibility to ROS in those particular strains (Orr and Sohal, 2003). In mice, CuZnSOD knockout results in an approximately 30% reduction of MLSP (Elchuri et al., 2005; Pérez et al., 2009b), but overexpression does not increase MLSP (Huang et al., 2000, Pérez et al., 2009b). In the nematode Caenorhabditis elegans, disruption of the gene encoding a cytosolic SOD has no effect on lifespan (Yang et al., 2007). However, C. elegans possess multiple SODs (Giglio et al., 1994; Hunter et al., 1997; Fujii et al., 1998; Jensen and Culotta, 2005) and the functions, and intracellular locations, of the different isoforms are not well understood.

The correlation of CuZnSOD activity with MLSP has been studied previously. Tolmasoff et al. (1980) and Ono and Okada (1984) reported that total SOD activity in various tissues correlated positively with MLSP of mammalian species. However, the
data generated in these reports were either based on very few species (e.g. five; Ono and Okada, 1984) or not corrected for phylogenetic relatedness of species (e.g. Tolmasoff et al., 1980; Ono and Okada, 1984). The relatively comprehensive approach taken here allows me to resolve this issue for endothermic vertebrates. Measurements in 14 mammalian and avian species clearly indicate the absence of correlation between longevity and CuZnSOD activity in brain, heart or liver. These are critical, highly oxidative organs and would thus be expected to generate significant amounts of ROS. Indeed, brain and heart are notably vulnerable to oxidative stress, particularly in aged individuals (see Mariani et al., 2005; Judge and Leeuwenburgh, 2007 for reviews). Thus, there is no evidence that the increase in species MLSP is achieved by increasing CuZnSOD activity in highly oxidative tissues that are vulnerable to oxidative stress.

I included measurements of Snell normal and long-lived dwarf mice as an intra-specific test of the association between enhanced antioxidant enzyme activities and increased lifespan. Although the approximately 50% increase in lifespan of Snell dwarf mice compared to their normal littermates is modest compared to the inter-species differences in MLSP, this intra-specific comparison benefits from minimal genetic variability, and identical dietary and environmental conditions. I found no evidence for increased CuZnSOD protein in heart or brain tissue of the long-lived Snell dwarf mice relative to normal littermates. These results are thus consistent with studies in which the transgenic overexpression of CuZnSOD has had no effect on mouse lifespan. Thus three lines of evidence: genetic manipulation, inter-species comparisons and an intra-species comparison, all indicate the absence of a role for CuZnSOD in mammalian and avian longevity.
The present study is the first to specifically determine the activity of the other major intracellular SOD, MnSOD, in a broad inter-species context. Previous comparative studies of SOD have simply measured total activity, or KCN inhibitable activity, in tissue homogenates (Tolmasoff et al., 1980; Sohal et al., 1990; Lopez-Torres et al., 1992; Pérez-Campo et al., 1993). As MnSOD is an exclusively mitochondrial enzyme, and mitochondrial abundance scales negatively with species body mass in most tissues (Porter, 2001), it is also necessary to take this into account when assessing relative MnSOD levels. Similar to CuZnSOD, I found no correlation between the activity of liver or heart MnSOD and animal MLSP. An apparently positive correlation of liver MnSOD with MLSP was dependent upon the human data point, which was notably high. Omitting this data point from the analysis rendered the correlation non-significant. I interpret this as indicating that there is no real relationship between MLSP and liver MnSOD activity, but simply particularly high MnSOD activity in human liver. While I do not know the reason for this, it is not due to high mitochondrial abundance, based on relatively moderate CS activities in the same samples (Supplemental Fig. 3.3). In the Snell dwarf mouse MnSOD levels in brain and heart appeared slightly lower than in normal-sized littermates, reaching significance in the brain. Thus I conclude that, similar to CuZnSOD, the evolution of longevity in mammals and birds has not selected for globally increased MnSOD activity. Again, this is generally in agreement with results from genetic manipulations. While MnSOD overexpression can extend lifespan in flies (Sun et al., 2002; Curtis et al., 2007), deletion of a mitochondrial SOD gene actually increases lifespan in C. elegans (Van Raamsdonk and Hekimi, 2009). MnSOD<sup>−/−</sup> mice
have normal lifespans (Van Remmen et al., 2003), as do some MnSOD overexpressors (Jang et al., 2009 but see Hu et al., 2007).

Interestingly, despite the absence of a relationship in liver and heart, a relatively robust correlation of brain MnSOD activity with species MLSP (not explained simply by differences in mitochondrial abundance) was found in brain tissue. Similarly, catalase activity in brain, but not liver or heart, was positively correlated with MLSP. These results indicate that longevity is associated with increased activity of a complete ROS detoxification pathway in brain tissue. Transgenic overexpression of MnSOD alone confers neuroprotection in mice (Keller et al., 1998; Dumont et al., 2009), and the magnitude of the MnSOD elevation in brain tissue of longer-lived species is sufficient that it could be similarly neuroprotective, particularly in concert with higher CAT activities. Thus, my data indicate that longer-lived species might have enhanced neuroprotective capacity due to MnSOD and CAT activity. This would not necessarily be expected to confer extended longevity, given the stochasticity of age-related organ failures in aging, with a multitude of possible targets. Nonetheless, it is an interesting observation that warrants further investigation. For example, I do not know if the increased MnSOD and CAT activities are present in particular cell types, or are elevated in all cell types within brain tissue.

Intracellular H$_2$O$_2$ detoxification also occurs via the GPx/GR coupled reaction utilizing glutathione and NADPH. Global overexpression of GPx1 or GPx4 is protective against a wide range of oxidative stressors, both in vivo and in vitro (Ran et al., 2004; Lei and Cheng, 2005; Ran et al., 2006; Dabkowski et al., 2008). However, genetic manipulation of either GPx isoform gives results that are inconsistent with a role for
increased GPx activity in lifespan extension (reviewed in Pérez et al., 2009b). Similarly, neither total cellular GPx nor GR activities in any organ were positively correlated with MLSP in the present study. A previous study (López-Torres et al., 1993) suggested a negative correlation of liver GPx activity with MLSP. However, of the eight species used, three were ectotherms, and the enzyme activities calculated for these species were not corrected for body temperature differences between ectotherms and endotherms. My results also indicate only one significant difference in GPx or GR activities between Snell normal and dwarf mice, i.e. a slight elevation of GPx activity in heart tissue of Snell dwarf mice. Thus, there is little evidence that an upregulation of GPx/GR is associated with extended longevity in the Snell dwarf mice. Taken together with the results of published studies, my results suggest no link between GPx or GR activities and longevity.

The current data set includes both hibernating and non-hibernating mammalian species, and this allows me to address a previously stated hypothesis that hibernating mammalian species may constitutively express antioxidant enzymes at high levels (reviewed in Carey et al., 2003). During hibernation animals experience severe fluctuations in tissue blood flow and metabolic rates during torpor/arousal transitions (Carey et al., 2003). This constitutes a significant metabolic stress, and it has been suggested that hibernators either constitutively express higher levels of antioxidant enzymes than non-hibernating species, or that hibernators upregulate antioxidant enzymes to protect against oxidative damage. Indeed, hibernators have been reported to avoid oxidative stress associated with arousal (Ma et al., 2005) and, similar to dwarf mice, show evidence of cellular stress resistance (Lindall et al., 2005; Kurtz et al., 2006;
Dave et al., 2006). I also show that 13-lined ground squirrels do not upregulate intracellular antioxidant enzyme activities in brain, heart or liver tissue during hibernation (Chapter 6). The present data set indicates that the 13-lined ground squirrels, as well as another species that routinely hibernates (big brown bat), do not maintain constitutively high intracellular antioxidant enzyme activities compared to species that do not hibernate. However, a broader study that includes a greater number of hibernators and non- hibernators is required to confirm the observation that antioxidant enzyme capacity is not greater in hibernators.

Many studies have demonstrated the cellular protection conferred by overexpression of individual antioxidant enzymes, both in vitro and in vivo. This includes protection against many of the common disorders associated with advanced age, such as ischemia-reperfusion injuries, neurodegeneration, and cancer. Despite this cellular protective capability, these experimental manipulations have generally failed to extend lifespan, at least in mice (Pérez et al., 2009b). In inter-specific and intra-specific experimental models of longevity, enhanced cellular resistance to oxidative stress is a very common observation (Kapahi et al., 1999; Salmon et al., 2005; Labinskyy et al., 2006; Harper et al., 2007). While it seems intuitive that this oxidative stress resistance might be achieved by increased expression of antioxidant enzymes in long-lived species, experimental evidence generally indicates that this does not occur (e.g. Andziak et al., 2005; Chapter 5). In the present study, out of 15 possible correlations (five antioxidant enzymes in each of three tissues), only two statistically significant positive correlations were identified. In addition, there were no correlations between antioxidant enzyme activities and body mass, furthering the argument that antioxidant enzymes have not co-
evolved with longevity, as generally larger animals live longer. Thus, in the vast majority of cases, there is no relationship between antioxidant enzyme activities and MLSP in the species studied here and elsewhere (Pérez et al., 2009b).

It is important to note that other strategies are possible for minimizing oxidative damage that might reduce MLSP in animal species. Life-history strategies that avoid stresses related to ROS overproduction (e.g. Costantini, 2008), metabolic adjustments that reduce the rate of respiratory ROS production (Lambert et al., 2007), adaptation of membrane unsaturation index to reduce the incidence of peroxidative damage (Costantini, 2008) may all be selected more strongly than intracellular antioxidant enzymes yet achieve the same end. Also, the antioxidant capacity of individual species may be influenced by dietary antioxidants (reviewed by Monaghan et al., 2009) that would not be accounted for in the present study. In future studies, it will be important to quantify a range of oxidative stress biomarkers as well as to identify other molecular mechanisms conferring cellular stress resistance in long-lived animals.
3.6 Supplemental Data

**Table S3. 1:** Statistical analysis of linear regressions, enzyme activity as a function of mass.

<table>
<thead>
<tr>
<th>Antioxidant Enzyme Activity</th>
<th>Correlation Coefficient</th>
<th>Slope</th>
<th>p value</th>
</tr>
</thead>
<tbody>
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<td>CuZnSOD</td>
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<td></td>
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</tr>
<tr>
<td>Heart</td>
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<td>GPx</td>
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<td>Heart</td>
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<td>-0.014</td>
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<tr>
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<td>-0.015</td>
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<td>Liver</td>
<td>-0.397</td>
<td>-0.086</td>
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<tr>
<td>Heart</td>
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<tr>
<td>Brain</td>
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<tr>
<td>Liver</td>
<td>0.300</td>
<td>0.040</td>
<td>0.151</td>
</tr>
</tbody>
</table>

**Table S3. 2:** Statistical analysis of linear regressions, CS activity as a function of MLSP.

<table>
<thead>
<tr>
<th>MLSP</th>
<th>Correlation Coefficient</th>
<th>Slope</th>
<th>p value</th>
</tr>
</thead>
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<tr>
<td>Heart</td>
<td>0.2168</td>
<td>0.3305</td>
<td>0.249</td>
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<tr>
<td>Liver</td>
<td>-0.3276</td>
<td>-0.4898</td>
<td>0.137</td>
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</table>

**Table S3. 3:** Statistical analysis of linear regressions, CS activity as a function of body mass.

<table>
<thead>
<tr>
<th>Mass</th>
<th>Correlation Coefficient</th>
<th>Slope</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>-0.2311</td>
<td>-0.0288</td>
<td>0.234</td>
</tr>
<tr>
<td>Brain</td>
<td>-0.4109</td>
<td>-0.0426</td>
<td>0.081</td>
</tr>
<tr>
<td>Liver</td>
<td>-0.6286</td>
<td>-0.1072</td>
<td>0.010</td>
</tr>
</tbody>
</table>
Fig. S3.1: Correlations between MnSOD activity and species MLSP. (a) Residual analysis of liver MnSOD activity with the inclusion of the human data point (p value = 0.017). (b) FIC analysis of liver MnSOD with the inclusion of the human data point (p value = 0.098). (c) Residual analysis of liver MnSOD activity following the removal of the human data point (p value = 0.258). (d) FIC analysis of liver MnSOD activity following the removal of the human data point. Values are ln-transformed and each data point consists of measurements from 3-8 individuals of each species. Solid trend line represents a significant relationship; dotted trend line represents a non-significant relationship.
Fig. S3. 2: The activity of brain and heart CAT. (a) Brain CAT activity is not significantly correlated with species body mass (p value = 0.068). (b) FIC analysis of heart CAT activity and species MLSP (p value = 0.248). Values are ln-transformed and each data point consists of measurements from 3-8 individuals of each species. Dotted trend line represents a non-significant relationship.

Fig. S3. 3: Citrate synthase activity in the human is relatively moderate compared to the other species in this data set.
Chapter 4. DNA base excision repair in mammals and birds is negatively correlated with body mass, but has no relationship to species maximum lifespan

Hypothesis

Base excision repair enzymes have a role in conferring longevity in long-lived species and/or strains.

Objective

The primary objective of this project was to determine if base excision repair enzyme activities are positively correlated with longevity in both an inter-specific and intra-specific context.

Contributions

I performed all experiments, phylogenetic and statistical analysis and chapter write-up.
4.1 Introduction

When the rate of ROS production exceeds that of neutralization, oxidative damage will accumulate within proteins, lipids and DNA. The accumulation of DNA damage however may be the most detrimental to cell viability due to the risk that replication and transcription of the genome will be compromised, thus affecting all aspects of cell function. The exposure of mammalian DNA to endogenous genotoxic agents is estimated to account for as many as 10,000 DNA lesions per cell per day (Lindahl, 1993). Common forms of DNA oxidative damage include strand breaks, base loss, and base modifications (Barnes and Lindahl, 2004). The failure to properly remove and repair DNA lesions can result in genomic instability, mutagenesis, transcriptional blockage and/or apoptotic cell death.

DNA repair deficiencies are associated with replicative senescence and premature aging syndromes, including Werner and Cockayne syndrome (Weirich-Schwaiger et al., 1994; reviewed by Lombard et al., 2005). These observations suggest that competent DNA repair pathways are essential to achieving normal lifespan. A corollary of this statement is that longer-lived animal species, or long-lived strains of a particular species, should have greater DNA repair capacities, thus allowing them to maintain genomic integrity over longer periods of time. There have been several reports offering evidence for this hypothesis. Hart and Setlow (1974), Francis et al. (1981), Hall et al. (1984), and Licastro and Walford (1985) have all demonstrated positive correlations between species maximum lifespan (MLSP) and the rate of UV-induced DNA damage repair in fibroblasts and lymphocytes. The pyrimidine dimers and photoproducts associated with UV radiation are removed by global nucleotide excision repair (NER). Although the
accumulation of pyrimidine dimers and photoproducts leads to significant genomic instability, this type of damage is typically localized to the outer dermis. In comparison, virtually all cells are continuously exposed to reactive oxygen species (ROS) which are produced as normal by-products of an aerobic metabolism (reviewed in Lambert and Brand, 2009). Therefore, removal of lesions arising from endogenous ROS production may be of ubiquitous importance.

Many forms of oxidative damage, and also alkylative damage and spontaneous base loss are repaired by enzymes of the base excision repair (BER) pathway. BER is subdivided into short- and long-patch pathways that are catalyzed by a number of enzymes. The ‘core’ BER pathway (Robertson et al., 2009) includes DNA glycosylases, which initiate BER through the recognition and removal of specific oxidative lesions; apurinic/apyrimidinic (AP) endonuclease (APE), which processes resultant abasic sites; polymerase β (polβ) which inserts the correct nucleotide(s); and a DNA ligase which seals the nick, thereby completing repair. Two steps in BER are potentially ‘rate limiting’ to the overall processing capacity. APE possesses a 3’-phosphoesterase activity, which is believed to be rate-limiting specifically following oxidation-induced DNA base loss (Izumi et al., 2000). Polβ possesses a putatively rate-limiting deoxyribophosphatase (dRP) activity (Srivastava et al., 1998). Cabelof et al. (2003a) have demonstrated the dependence of BER activity on polβ in polβ haploinsufficient mice. Therefore, measurements of APE and polβ activities provide insight into overall BER capacity.

There is evidence that DNA BER is upregulated concomitant with increased longevity. Caloric restriction (CR), the most widely recognized lifespan-extending
intervention, increases polβ (Cabelof et al., 2003b) and overall nuclear short-patch BER activities (Stuart et al., 2004) in mouse tissues. Similarly, APE activity in brain tissue of CR rats is upregulated relative to ad libitum fed controls (Kisby et al., 2010). These results suggest that BER activity may influence longevity. Therefore it is somewhat surprising that a 50% reduction in either APE or polβ activity do not reduce lifespan in mice (Meira et al., 2001; Cabelof et al., 2006), even though reduced activity is associated with elevated DNA strand breaks and increased spontaneous mutagenesis compared to control mice (Meira et al., 2001; Huamani et al., 2004; Cabelof et al., 2006).

Although experiments with genetically modified mice offer valuable insights into mechanisms underlying animal lifespan, there are limitations associated with this approach, including the fact that they may provide insight relevant only to murine biology. Here I use a different approach to determining the relationship between BER activity and longevity by comparing the activities of the two core BER enzymes, APE and polβ, in brain and liver tissues of 15 vertebrate endotherm species (Table 4.1; Fig. 4.1) with naturally disparate lifespans. Surprisingly, I find no evidence that the activities of APE or polβ are positively correlated with MLSP. Rather, polβ activity in both brain and liver tissues was negatively correlated with species body mass. I also included in this study a long-lived mouse strain, the Snell dwarf, to further investigate whether mouse models of extended longevity are generally characterized by having enhanced BER activity. Both Snell and Ames dwarf mice are resistant to a variety of physiological stressors (Salmon et al., 2005; Bokov et al., 2009), consistent with an increased capacity for DNA repair. In contrast to the multi-species comparative study, I find that APE and
polβ activities are indeed elevated in some tissues of Snell dwarf mice relative to normal littermates.

Fig. 4.1: Phylogeny of the 15 species used in this study. The tree is a compilation of four phylogenies (Stuart et al., 2002; Springer and Murphy, 2007; Hackett et al., 2008; Prasad et al., 2009). Branch lengths are best estimates from these phylogenies.

4.2 Experimental Procedures

4.2.1 Materials
Chemicals were purchased from Sigma-Aldrich (Oakville, Ontario, Canada), Fisher Scientific (Mississauga, Ontario, Canada) and Bioshop (Burlington, Ontario, Canada). BioRad protein dye was purchased from BioRad laboratories (Hercules, California, USA). Oligonucleotides were purchased from Midland Certified Reagent Co. (Midland, Texas, USA).
4.2.2 Animals
Between three and eight individuals of each of thirteen mammalian and two avian species were used in the inter-specific component of this study (Table 4.1). See Chapter 3 for the basis of species selection.

4.2.3 Tissue collection
Tissues were collected as in Chapter 3. In addition, hamsters and gerbils were euthanized and tissues were frozen in liquid nitrogen and shipped to Brock University (St. Catharines, ON, Canada) on dry ice.

4.2.4 Isolation of nuclear fractions
Frozen brain and liver tissue were used to isolate nuclear fractions as in Cabelof et al. (2002). Tissue was powdered using a mortar and pestle and rinsed twice in 1X PBS. Nuclear isolation was essentially as described by Stuart et al. (2004). Powder tissue was further minced in 20 volumes of MSHE buffer (0.21M mannitol, 70mM sucrose, 10mM HEPES pH 7.4, 1mM EGTA, 2mM EDTA), 5mM DTT and 1μg/ml aprotonin were added immediately prior to use and homogenized with at least 10 strokes of a Potter-Elvejhem homogenizer. Homogenization was carried out on ice. Samples were centrifuged for 10 min at 500 g (4°C). The nuclear pellet was resuspended in lysis buffer A (20mM HEPES, pH 7.5, 1mM EDTA, 5% glycerol, 0.5% Triton X-100, 300mM KCl), 5mM DTT, 1 μg/ml aprotonin, 1mM PMSF were added immediately prior to use. Resuspended samples were incubated in ice with intermittent vortexing for 30 min and then centrifuged for 1 h at 20,000 g. The concentration of the supernatants was measured using the Bradford technique with a BioRad protein kit and nuclear protein homogenates were stored at -80°C.
4.2.5 Base Excision Repair Assays

APE activity was measured by incubating 100ng protein with 1pmol of [γ-32P]-tetrahydrofuran-containing 30-mer oligonucleotide (THF; Table 4.2). The % incision of the THF oligonucleotide was determined at 5, 10, 15 min at 37°C in a 10μl reaction buffer (50mM HEPES (pH 7.5), 50mM KCl, 100μg/ml BSA, 100mM MgCl₂, 10% glycerol, and 0.05% Triton X-100). Reactions were terminated with the addition of 10μl formamide loading dye (80% formamide, 10mM EDTA, 1 mg/ml xylene cyanol, and 1mg/ml bromophenol blue) followed by incubation at 90°C for 10 min. Two controls were run in tandem with each APE activity assay: an oligonucleotide without the abasic site analog (THF-control; Table 4.2); and the absence of cell lysate (data not shown). Reaction products were resolved by electrophoresis at 15 W for 1 h 30 min in 20% polyacrylamide gels containing 7M urea. Gels were visualized with PhosphoImager (Fujifilm FLA-3000) and analyzed with ImageGuage™ software. APE activity was quantified as the intensity of the product bands relative to the combined intensity of the product and substrate bands, essentially as in Stuart et al. (2004).

Polβ gap filling activity was measured by incubating 1μg protein with 1pmol of an oligonucleotide with a single nucleotide gap (GAP; Table 4.2) for 10, 20, 30 min at 37°C, in a total volume of 10μl reaction buffer (40mM HEPES, 0.1mM EDTA, 5mM MgCl₂, 0.2mg/ml BSA, 50mM KCl, 1mM DTT, 40 mM phosphocreatine, 100μg/ml phosphocreatine kinase, 3% glycerol, 2mM ATP, 40μM dCTP, and 4μCi [α-32P]dCTP). Reactions were terminated with the addition of 5μg proteinase K, 1μl 10% SDS and incubation at 55°C for 30 min. DNA was precipitated with 1μg glycogen, 4μl 11M ammonium acetate and 90% ethanol overnight (-20°C). Samples were washed with 75% ethanol, centrifuged (16,000g and
4°C) and speed vacuumed. Dried DNA was then resuspended in 10μl formamide loading buffer and incubated at 90°C for 10 min. Two controls were run in parallel with each polβ activity assay: the identical oligonucleotide with no gap (GAP-control; Table 4.2); and exclusion of cell lysate (Fig. 4.3a). In addition, on each gel an internal standard (4μg) was included to control for inter-gel variation. Gels were visualized in a similar manner as above, however polβ activity was quantified as the increase in signal intensity due to incorporation of [α-32P]dCTP. Complete incorporation and ligation activity was measured using the conditions as above for polβ activity. A higher shifted band (30-mer) represented complete repair of the gapped oligonucleotide. Gels were visualized as above and DNA ligase activity was quantified as the increase in signal intensity due to the incorporation of [32P]dCTP.

4.2.6 Statistical analyses
Statistics were analyzed as in Chapter 2 and 3.
<table>
<thead>
<tr>
<th>Common Name</th>
<th>Scientific Name</th>
<th>Number and Sex</th>
<th>Approximate Age</th>
<th>Body Mass (kg)</th>
<th>MLSP (yrs)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammalia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Snell mouse</td>
<td><em>Mus musculus</em></td>
<td>6 F (+/dw)</td>
<td>6-7 months</td>
<td>0.02996</td>
<td>2.7</td>
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<tr>
<td></td>
<td></td>
<td>6 F (dw/dw)</td>
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<td>3.9</td>
<td></td>
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<tr>
<td>C57BL/6Ncr1BR mouse</td>
<td><em>Mus Musculus</em></td>
<td>4 F 4 M</td>
<td>4 months</td>
<td>0.0276</td>
<td>3.5</td>
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<td>Syrian hamster</td>
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<td>4-8 months</td>
<td>0.130</td>
<td>3.9</td>
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<td><em>Rattus norvegicus</em></td>
<td>6 M</td>
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<td>0.55</td>
<td>5</td>
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<td>Mongolian gerbil</td>
<td><em>Meriones unguiculatus</em></td>
<td>unknown</td>
<td>4-8 months</td>
<td>0.070</td>
<td>6.3</td>
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<td>13-lined ground squirrel</td>
<td><em>Spermophilus tridecemlineatus</em></td>
<td>8 F 3M</td>
<td>Unknown</td>
<td>0.2050</td>
<td>7</td>
<td>University of Manitoba Field Research Station (Carmen MB, Canada)</td>
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<tr>
<td>Rabbit</td>
<td><em>Oryctolagus cuniculus</em></td>
<td>4 F 1 M</td>
<td>3.5 months</td>
<td>2.6</td>
<td>11.8</td>
<td>Local abattoir (Fort Erie, ON, Canada)</td>
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<tr>
<td>Guinea Pig</td>
<td><em>Cavia porcellus</em></td>
<td>Unknown</td>
<td>Unknown</td>
<td>1.05</td>
<td>12</td>
<td>Rockland (Gilbertsville, PA, USA)</td>
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<td>Big brown bat</td>
<td><em>Eptesicus fuscus</em></td>
<td>3 F 2 M</td>
<td>6-12 months</td>
<td>0.025</td>
<td>19</td>
<td>McMaster University Bat Laboratory (Hamilton, ON, Canada)</td>
</tr>
<tr>
<td>Sussex Sheep</td>
<td><em>Ovis aries</em></td>
<td>5 F</td>
<td>6-12 months</td>
<td>29.5</td>
<td>19.6</td>
<td>Local abattoir (Fort Erie, ON, Canada)</td>
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<tr>
<td>White-tailed deer</td>
<td><em>Odocoileus virginianus</em></td>
<td>3 M</td>
<td>2.5-3.5 years</td>
<td>110</td>
<td>21.6</td>
<td>Local hunters (Cobden, ON, Canada)</td>
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<tr>
<td>Domestic Dog</td>
<td><em>Canis familiaris</em></td>
<td>3 M</td>
<td>2-8 years</td>
<td>11</td>
<td>24</td>
<td>Equitech-Bio, Inc. (Kerrville, TX, USA)</td>
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<tr>
<td>Yorkshire/ Hampshire Pig</td>
<td><em>Sus scrofa</em></td>
<td>2 F 3 M</td>
<td>6 months</td>
<td>99.9</td>
<td>27</td>
<td>Local abattoir (Fort Erie, ON, Canada)</td>
</tr>
<tr>
<td>Black angus/ Charlet Cow</td>
<td><em>Bos taurus</em></td>
<td>4 F 1 M</td>
<td>1.5-2.5 years</td>
<td>379.5</td>
<td>30</td>
<td>Local abattoir (Fort Erie, ON, Canada)</td>
</tr>
<tr>
<td>Human</td>
<td><em>Homo sapiens</em></td>
<td>2 F 2 M</td>
<td>26-37</td>
<td>68</td>
<td>122</td>
<td>Zenbio (Research Triangle Park, NC, USA)</td>
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<tr>
<td>Aves</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Japanese quail</td>
<td><em>Coturnix japonica</em></td>
<td>2 F 2 M</td>
<td>4-5 months</td>
<td>0.264</td>
<td>6</td>
<td>Cro Quail Farms Inc (St. Anne’s, ON, Canada)</td>
</tr>
<tr>
<td>Zebra finch</td>
<td><em>Taeniopygia guttata</em></td>
<td>3 F 3 M</td>
<td>1 year</td>
<td>0.0166</td>
<td>14.5</td>
<td>Trent University Animal Care Facility (Peterborough, ON, Canada)</td>
</tr>
</tbody>
</table>
**Table 4.2:** Oligonucleotides used to measure BER enzyme activity. THF, tetrahydrofuran; Comp, complementary sequence; GAP, double-stranded oligonucleotide containing single nucleotide gap.

<table>
<thead>
<tr>
<th>Name</th>
<th>THF Control</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP endonuclease</td>
<td>5' ATATACCGCGGCCCAGGTCAAGCTTATT 3'</td>
<td></td>
</tr>
<tr>
<td>THF</td>
<td>5' ATATACCGCGGCCCAGGTCAAGCTTATT 3'</td>
<td></td>
</tr>
<tr>
<td>THF Comp</td>
<td>5' ATATACCGCGGCCCAGGTCAAGCTTATT 3'</td>
<td></td>
</tr>
<tr>
<td>Polymerase β</td>
<td>5' CTGCAGCTGATGCCTACCGATCTCCCCCCGGGTTAC 3'</td>
<td></td>
</tr>
<tr>
<td>GAP</td>
<td>15mer - 5' CTGCAGCTGATGCCTACCGATCTCCCCCCGGGTTAC 3'</td>
<td></td>
</tr>
<tr>
<td>GAP Comp</td>
<td>18mer - 5' P-TACCGATCTCCCCCCGGGTTAC 3'</td>
<td></td>
</tr>
</tbody>
</table>

4.3 Results

APE catalyzes the first of two putative rate-limiting steps in the short-patch BER pathway. Therefore overall BER capacity may be influenced by the activity of this key enzyme. Measurements of APE activity in nuclear fractions isolated from brain and liver tissues of 13 mammalian and 2 avian species were analyzed with respect to species MLSP and body mass. There was no evidence for a correlation of APE activity in brain (Fig. 4.2a shows brain representative gel) or liver with species MLSP (Fig. 4.2b, c). This result was also obtained when the potentially confounding effects of body mass and phylogenetic position were accounted for (Table 4.3 reports liver; Fig. 4.2d, e shows brain). Animal species cannot be treated as independent data, as all extant animals share some evolutionary history with a common ancestor. To account for phylogenetic relatedness, raw data was transformed into Felsenstein’s phylogenetic independent contrasts (FIC; Felsenstein, 1985). There was also no significant correlation between APE activities in brain or liver and species body mass (Table 4.4).
Fig. 4.2: APE activity does not correlate with MLSP. APE activity in (a) brain (p value = 0.399) and (b) liver (p value = 0.433) does not correlate with MLSP. Values are In-transformed and each data point consists of measurements from 3-8 individuals representing one species. (c) Residual analysis of brain APE activity and animal MLSP (p value = 0.312). (d) FIC analysis of brain APE activity and MLSP (p value = 0.161). Dotted trend line represents a non-significant relationship. (e) APE activity is higher in brain (p value = 0.002) and heart (p value = 0.18) tissue of young adult Snell dwarf compared to normal littermates. White bars represent Snell normal and black bars represent Snell dwarf. Values are means ± SEM of duplicate measurements from 5 animals per experimental group. * = significantly different (p < 0.005) using a two-tailed t-test. Dotted trend line represents a non-significant relationship.
Measurements of APE in Snell normal and dwarf mice revealed apparently higher activities in both brain and heart tissues of dwarf mice compared to normal controls (Fig. 4.2e), however while this apparent difference was statistically significant for brain ($p = 0.002$), it was not for heart ($p = 0.18$). This latter result was influenced by two of five individuals with particularly elevated APE activities, which introduced substantial variance about the mean.

Polβ gap filling activity in an oligonucleotide containing a single nucleotide gap was measured via the incorporation of $[^{32}\text{P}]\text{dCTP}$. In these assays, either one or two products were visible on electrophoresed acrylamide gels: the band representing incorporation of $[^{32}\text{P}]\text{dCTP}$ into the oligonucleotide, and in some instances an additional band representing both $[^{32}\text{P}]\text{dCTP}$ incorporation and subsequent ligation. The first product represents the activity of polβ exclusively, whereas the formation of the second product requires the combined activities of both Polβ and DNA ligase (Fig. 4.3a). Quantification of $[^{32}\text{P}]\text{dCTP}$ incorporation revealed no correlation of brain polβ activity with MLSP (Fig. 4.3b; $p = 0.061$), nor between liver polβ activity and MLSP (Fig. 4.3c; $p = 0.061$). Analysis of residuals (Fig. 4.3d,e), to remove any underlying effects of body mass on the relationship, revealed that this negative trend was driven by body mass, as it was absent in the residual plot (Fig. 4.3e; and see Fig. 4.4a,b for correlations with species body mass). The trend was also absent following FIC transformation of liver data; however brain residual data re-analysed using FIC revealed a significant negative correlation (Table 4.3).
Fig. 4.3: Polβ nucleotide incorporation activity negatively correlates with MLSP. (a) Gel showing incorporation (18-mer) and incorporation plus ligation (34-mer); NP = no protein extract. Polβ activity in brain (p value = 0.061) (b) and liver (p value = 0.061) (c) correlates negatively with MLSP, however significance is not reached in either tissue. The negative correlation between Polβ activity and MLSP in brain (p value = 0.469) (d) and liver (p value = 0.276) (e) is lost after residual analysis. Values are In-transformed and each data point consists of measurements from 3-8 individuals representing one species. Solid trend line represents a significant relationship; dotted trend line represents a non-significant relationship. (f) Polβ activity in Snell mouse tissue. Polβ activity is significantly higher in dwarf heart tissue (0.019) compared to normal littermates. Values are means ± SEM of duplicate measurements from 5 animals per experimental group. ** = significantly different (p < 0.05) using a two-tailed t-test.
Polβ nucleotide incorporation activity in both brain (p value = 0.0004) and liver (p value = 0.043) tissue homogenates showed negative correlations with species body mass (Fig. 4.4a,b). The correlation remained for brain following FIC analysis, but became non-significant for liver (Table 4.4). Comparisons of polβ in tissues of Snell dwarf mice and normal controls showed significantly higher activities in heart tissue (Fig. 4.3f; p value = 0.019).

The product representing [32P]dCTP incorporation and subsequent ligation of the oligonucleotide was sufficient to be accurately quantified in liver tissue but not brain, presumably owing to lower ligase activity in the latter. Analysis of this fully ligated product in liver tissue of all species revealed a similar negative correlation with MLSP (p value = 0.003; Fig. 4.5a). However, this negative correlation of incorporation and ligation activity also appeared to be mainly driven by body mass (p value = 0.014; Fig. 4.5b). Although plotting the residuals did not remove the significant correlation, the relationship was weakened (Table 4.3) and following FIC analysis of residuals the correlation was not observed (Table 4.3). FIC analysis of the correlation between incorporation + ligation activity and mass also did not reveal a significant relationship (Table 4.4). Ligase activity was believed to be lower in Snell normal and dwarf tissues and could not be quantified.
Table 4.3: Results of correlational analysis of enzyme activities as a function of lifespan.

<table>
<thead>
<tr>
<th>BER Enzyme Activity</th>
<th>Correlation Coefficient</th>
<th>Slope</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver APE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>-0.305</td>
<td>-0.202</td>
<td>0.144</td>
</tr>
<tr>
<td>Residual + FIC</td>
<td>-0.026</td>
<td>-0.018</td>
<td>0.465</td>
</tr>
<tr>
<td>Brain Polβ (incorporation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual + FIC</td>
<td>-0.462</td>
<td>-0.093</td>
<td>0.047</td>
</tr>
<tr>
<td>Liver Polβ (incorporation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual + FIC</td>
<td>-0.288</td>
<td>-0.374</td>
<td>0.17</td>
</tr>
<tr>
<td>Liver Polβ (incorporation + ligation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>-0.477</td>
<td>-0.338</td>
<td>0.042</td>
</tr>
<tr>
<td>Residual + FIC</td>
<td>-0.189</td>
<td>-0.103</td>
<td>0.268</td>
</tr>
</tbody>
</table>

Table 4.4: Results of correlational analysis of enzyme activities as a function of body mass.

<table>
<thead>
<tr>
<th>BER Enzyme Activity</th>
<th>Correlation Coefficient</th>
<th>Slope</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain APE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Versus mass</td>
<td>0.041</td>
<td>0.0083</td>
<td>0.44</td>
</tr>
<tr>
<td>FIC analysis</td>
<td>-0.103</td>
<td>-0.0229</td>
<td>0.36</td>
</tr>
<tr>
<td>Liver APE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Versus mass</td>
<td>0.251</td>
<td>0.0602</td>
<td>0.19</td>
</tr>
<tr>
<td>FIC analysis</td>
<td>-0.229</td>
<td>-0.0776</td>
<td>0.23</td>
</tr>
<tr>
<td>Brain Polβ (incorporation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIC analysis</td>
<td>-0.591</td>
<td>-3.978</td>
<td>0.012</td>
</tr>
<tr>
<td>Liver Polβ (incorporation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIC analysis</td>
<td>-0.047</td>
<td>-0.3554</td>
<td>0.44</td>
</tr>
<tr>
<td>Liver Polβ (incorporation + ligation)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FIC analysis</td>
<td>0.053</td>
<td>0.1759</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Fig. 4.4: Incorporation activity negatively correlates with species body mass. Incorporation activity within brain (p value = 0.0004) (a) and liver (p value = 0.043) (b) tissue homogenates is significantly and negatively correlated with species body mass. Values are ln-transformed and each data point consists of measurements from 3-8 individuals representing one species. Solid trend line represents a significant relationship.
Fig. 4.5: Incorporation plus ligation activity negatively correlates with species MLSP and body mass. Incorporation plus ligation activity within liver tissue homogenate is significantly and negatively correlated with species MLSP (p value = 0.003) (a) and species body mass (p value = 0.014) (b). Values are In-transformed and each data point consists of measurements from 3-8 individuals representing one species. Solid trend line represents a significant relationship.

4.4 Discussion

Although it has long been postulated that enhanced DNA repair capacity has co-evolved with longevity, direct comparative tests of the hypothesis have typically employed only a few actively mitotic cell types (e.g. fibroblasts) and focused upon UV-lesions repaired by NER (e.g. Hart and Selow, 1974; Francis and Regan, 1981; Hall et al., 1984; Licastro and Walford, 1985; Cortopassi and Wang, 1996). Such UV-induced lesions are not relevant to the vast majority of metazoan somatic cell genomes, shielded as they are from UV radiation. In contrast, somatic cells are with few exceptions continually exposed to the endogenous oxidants produced during normal mitochondrial respiration, particularly in highly oxidative tissues such as brain, heart and liver. Although NER and double strand break repair pathways play roles in the repair of oxidative damage (reviewed by Slupphaug et al., 2003), the BER pathway appears to be
important in the repair of single oxidized bases and abasic sites that occur commonly (Croteau and Bohr, 1997).

Reports of BER pathway activity in the context of MLSP are limited and have to date focused primarily on fibroblasts. Brown and Stuart (2007) reported the absence of a positive correlation between MLSP and either APE or polβ activities in dermal fibroblasts from nine mammalian species. In contrast, Austad (2010) has reported preliminary results from six mammalian species suggesting that BER pathway activity in fibroblasts is positively correlated with MLSP, based on superior repair of γ-irradiation damage in longer-lived species. However, in addition to lesions that are substrates for BER, γ-irradiation introduces lesions requiring NER and double strand break repair responses (Hafer et al., 2007; Asaithamby and Chen, 2009), as well as causing non-specific protein and membrane damage (Pleshakova et al., 1998; Srinivasan et al., 2009). Therefore, differences in the apparent rate of DNA repair following γ-irradiation may not reflect exclusively differences in BER activities, but also the previously reported differences in multiplex stress resistance (Kapahi et al., 1999).

While numerous investigators have reported correlations between MLSP and DNA repair capacity in fibroblasts and lymphocytes, these probably have limited relevance to tissues, such as brain, heart and liver, which are comprised primarily of highly oxidative post-mitotic cells. In these cells, the primary contribution of BER to cellular homeostasis is presumably preservation of transcriptional fidelity and prevention of transcriptional stalling. My data represents the first multi-species measurements of BER in such oxidative tissues, and the complete lack of correlation with MLSP is a surprising result. However, the multiple two-species comparisons that can be made
within the dataset also support the conclusion that enhanced BER is not generally associated with longevity. For example, in brain and liver there was no evidence for elevated APE activity in long-lived bat or finch species relative to the shorter-lived size-matched mouse. Similarly, there was no consistent pattern for elevation of polβ in long-lived species, as seen in comparisons between finch and mice, or finch and the shorter-lived quail, though higher activities were observed in both brain and liver tissues of bats relative to mice. These mixed results are consistent with the interpretation that various strategies for preventing or repairing oxidative DNA damage may have evolved, and that these are specific to particular clades. They do not, however, support the hypothesis that enhanced BER activity is requisite for achieving longevity.

The decline in polβ activity with increasing body mass may relate to concomitant reductions in rates of intracellular ROS production. Mitochondrial abundance (i.e. volume density) scales inversely with species body mass in many cells and tissues (Else and Hulbert, 1985; Porter and Brand, 1995). There is evidence that mitochondrial superoxide production also scales inversely with body mass. Replotting of the mitochondrial superoxide production data from Ku et al. (1993) versus the mean body mass of each of the seven mammalian species used in this study gives a highly significant correlation ($r = -0.9$) of the two variables (not shown here). Although this was not confirmed in a more recent study of heart mitochondria (Lambert et al., 2007), there is nonetheless evidence that both body mass and MLSP influence mitochondrial ROS production. Reduced cellular mitochondrial abundance per cell combined with reduced rates of ROS production per mitochondrion would be expected to significantly reduce the amount of potentially damaging intracellular ROS produced per unit time. Given that
most intracellular antioxidant enzymes are constitutively expressed and not correlated with body mass (Chapter 3), the rate at which oxidative DNA damage occurs should therefore be significantly reduced as body mass increases. This would explain the observation that levels of nuclear DNA oxidative damage (e.g. 8oxodG within heart tissue homogenates) are generally lower in larger species (Fig. 4.6) despite my observation that BER activities are not greater.

![Graph showing significant negative correlation between 8-oxodG levels within heart nuclear DNA and species body mass. Estimates of nuclear DNA 8-oxodG levels (from Barja and Herrero, 2000) were natural log-transformed and plotted against natural log-transformed species average body mass, yielding a significant negative correlation (p < 0.025).]

**Fig. 4.6:** Significant negative correlation between 8-oxodG levels within heart nuclear DNA and species body mass. Estimates of nuclear DNA 8-oxodG levels (from Barja and Herrero, 2000) were natural log-transformed and plotted against natural log-transformed species average body mass, yielding a significant negative correlation (p < 0.025).

It is interesting that the activities of protein repair/recycling mechanisms, such as 20S/26S proteasome, show a similar negative correlation with body mass (Salway et al., 2010). This is also consistent with the hypothesis that the incidence of macromolecular oxidative damage (to both proteins and DNA) generally decreases with increasing body mass, and therefore the rates of recycling/repair required to maintain cellular homeostasis and viability are also reduced.

Another potential explanation for the inverse scaling of polβ with body mass might be that the proportion of actively mitotic cells in brain and liver (and presumably
other tissues) is dependent upon body mass. BER enzyme activities are upregulated at specific times during the cell cycle (Chaudhry, 2007) and are generally elevated in tissues containing higher proportions of actively mitotic cells (see Karahalil et al., 2002). Narciso et al. (2007) demonstrated that a significant reduction in BER capacity occurs during differentiation of actively mitotic myoblasts to post-mitotic myotubes. It is interesting that larger mammalian species generally have lower circulating levels of insulin-like growth factor-I (IGF-1), a mitogenic hormone (Chapter 2). Similarly, the activity of telomerase (in various tissues), which is associated with cell replication, also correlates negatively with body mass (Seluanov et al., 2007). Both observations are consistent with there being a general reduction in the proportion of actively mitotic cells in tissues as body mass increases, though I am not aware of data that could confirm this.

The observation of generally elevated APE activities in tissues of young adult Snell dwarf mice is potentially significant for its role in BER as well as its function as a redox-sensitive transcription factor. Leiser and Miller (2010) recently demonstrated the induction of Nrf2, a transcription factor involved in regulating the antioxidant response to facilitate stress resistance, in fibroblasts of Snell dwarf mice. Interestingly, Nrf2 and APE1 (ref-1) are interacting partners in heart tissue, where they contribute to an adaptive induction of stress resistance (Gurusamy et al., 2007). Snell dwarf fibroblasts are generally characterized by their rapid induction of stress responsive genes (Sun et al., 2009) and activities (Chapter 5). Elevated APE (ref-1) activities in Snell dwarf brain, and possibly heart, are consistent with this rapid response poised. Although the apparent induction of APE activity in Snell dwarf mouse heart tissue was not statistically significant, a rather dramatic elevation of polβ activity in this tissue indicates that BER
pathway activity is substantially enhanced. This would be expected to contribute to cardiac stress resistance that, although not tested in Snell dwarf mice, is present in similar murine models of longevity (Yan et al., 2007; Mitchell et al., 2010).

In contrast to heart, brain polβ activity was actually lower in Snell dwarf mice (compared to normal), despite elevated APE activities in this organ. Differences between dwarf and normal mice were not due to regional sampling heterogeneity, as in both instances whole brains were excised and pulverized to homogeneity while frozen, and thereafter sampled from for my measurements. However, it is possible that reduced polβ activities relate to altered cellular composition of Snell brain tissue due to hormonal deficiencies (Noguchi, 1988).

The generally elevated BER activities observed in Snell dwarf mice parallel observations made in CR mice, in which BER is enhanced in multiple tissues (Cabellof et al., 2003b; Stuart et al., 2004). Elevated BER activities associated with CR manifest early in the lifespan, and are observable in young adults. This may contribute to two of the most interesting aspects of the phenotype shared by dwarf and CR mice: stress resistance, which also manifests early in the lifespan (Bokov et al., 2009; Tatar et al., 2003), and longevity.

In conclusion, I found no evidence for the expected positive correlation between MLSP and DNA BER amongst vertebrate endotherms. Rather, increasing adult body mass was negatively correlated with apparent BER activity, reminiscent of similar relationships observed for mitochondrial ROS production, nuclear DNA damage (Fig. 5), and proteasome (Salway et al., 2010) activity. Taken together, these trends are consistent with the interpretation that increasing body mass reduces the incidence of intracellular
macromolecular oxidative damage and therefore concomitantly reduces the need for repair processes. The effect of DNA BER on longevity generally may in fact be surprisingly marginal. In the light of previous results showing that genetic modification reducing either APE1 or polβ activity by approximately 50% did not significantly reduce lifespan of mice, despite subtly increasing cancer incidence (Meira et al., 2001; Huamani et al., 2004; Cabelof et al., 2006). An important caveat of the present study, however, is its focus on large oxidative tissues that are comprised primarily of terminally differentiated post-mitotic cells. Perhaps relationships between DNA BER capacity and longevity are observable only in actively mitotic cells, although a previous study (Brown and Stuart, 2007) does not support this hypothesis. Alternatively, it is possible that mitochondrial DNA (mtDNA) repair specifically is enhanced in longer-lived species. Given the proximity of mtDNA to the source of ROS and the ongoing replication of mtDNA throughout the lifespan, it may be especially vulnerable to the oxidative damage that would limit cellular lifespans. This will be an important area for future study.
Chapter 5. Mechanisms of stress resistance in Snell dwarf mouse fibroblasts: enhanced antioxidant and DNA base excision repair capacity, but no differences in mitochondrial metabolism

Hypothesis

Intracellular antioxidant enzymes have a role in conferring stress resistance in dermal fibroblasts isolated from long-lived Snell dwarf mice.

Objective

The primary objective of this project was to determine if intracellular antioxidant enzymes play a role in cellular stress resistance observed in dermal fibroblasts isolated from long-lived Snell dwarf mice.

Publications of results


Contributions

Performed all antioxidant enzyme and base excision repair experiments, statistical analysis and chapter write-up.
5.1 Introduction

Cellular resistance to physiological stress is correlated with animal lifespan within species (Honda and Honda, 1999; Murakami et al., 2003; Yan et al., 2007) and between species (Kapahi et al., 1999; Harper et al., 2007). Stress resistance is also greater in some states of metabolic quiescence that are associated with extended lifespan (Wang and Kim, 2003). Both stress resistance and lifespan can be extended via genetic mutations (Honda and Honda, 1999; Yan et al., 2007; Migliaccio et al., 1999; Barsyte et al., 2001; Salmon et al., 2005), demonstrating the plasticity of the phenotype and allowing the identification of specific genes involved. In *C. elegans*, a series of insulin-like growth factor-1 (IGF-1) signaling pathway (*daf*) mutants have been studied to uncover the regulation of lifespan by this hormone. The basic architecture and function of this pathway appears to be conserved in mammals, as mice deficient in the IGF-1 receptor (IGF-1R\(^{-/-}\)) are also stress resistant and long-lived (Holzenberger et al., 2003). Several other strains of mice affected by mutations that occur upstream of this pathway are also long-lived. In Snell dwarf mice, a mutation within the *Pit-1* gene, which encodes a transcription factor required for the normal development of the anterior pituitary, results in deficient levels of growth hormone (GH), thyroid stimulating hormone (TSH) and prolactin (PRL) (Li et al., 1990; Camper et al., 1990). One of the most notable secondary effects of the *Pit-1* mutation is the reduction of basal levels of IGF-1 (Hsieh et al., 2002). As a result, these mice have provided useful models for the study of mechanisms of stress resistance that confer longevity.

Dermal fibroblasts isolated from Snell dwarf mice can withstand levels of a variety of oxidative and non-oxidative stressors that are lethal to cells derived from their
normal-sized littermates (Murakami et al., 2003; Salmon et al., 2005). In addition, the Snell dwarf mouse fibroblasts are relatively resistant to the growth crisis and cell death induced by passage in standard culture conditions with atmospheric (20%) rather than physiological (3%) levels of O₂ (Maynard and Miller, 2006). Thus, dermal fibroblasts derived from the Snell dwarf mice are resistant to the oxidative stress that appears to drive growth crisis and transformation in mouse fibroblasts (Parrinello et al., 2003). However, little is known about the specific mechanisms through which cellular stress resistance is enhanced within the dwarf phenotype, and direct measurements of the levels and activities of potential proteins involved in the stress resistance mechanism have rarely been made.

Cellular oxidative stress resistance can be conferred by the antioxidant enzymes that detoxify reactive oxygen species (ROS) originating endogenously (for example via mitochondrial respiration) or exogenously. Cytosolic (CuZnSOD) and mitochondrial (MnSOD) superoxide dismutase isoforms convert superoxide radicals into hydrogen peroxide, which can subsequently be detoxified by glutathione peroxidase (GPx; found within various cellular compartments including mitochondria) and catalase (CAT; localized to peroxisomes).

In the event that ROS escape detoxification, however, DNA repair enzymes may play a critical role in preserving genomic integrity and thus conferring stress resistance. Base excision repair (BER) is the main pathway for repair of oxidative damage; it is also involved in repairing alkylative (caused by, for example, methyl methanosulfonate) and other forms of DNA damage. Though a number of subpathways are recognized to exist (Sung and Demple, 2006; Srivastava et al., 1998), two enzymes are thought to play
central roles in BER. The apurinic/apyrimidinic (AP) endonuclease processes abasic sites generated either by removal of damaged bases (via DNA glycosylase activity) or spontaneous base loss. AP endonuclease has been found to also possess the rate limiting activity of a 3'-phosphoesterase specifically following oxidative-induced DNA base loss (Izumi et al., 2000). Polymerase β is a small DNA polymerase dedicated to repair. Resynthesis of DNA at AP endonuclease-generated gaps is catalyzed by polymerase β. The activity of this enzyme is also particularly informative regarding overall BER activity as it possesses the rate limiting activity of a deoxyribophosphatase (dRP) (Srivastava et al., 1998).

Here I have used Snell dwarf mouse fibroblasts to investigate the molecular mechanisms that allow these cells to resist oxygen toxicity. I explore two hypotheses: (1) that antioxidant enzyme activities are upregulated, and (2) that DNA BER enzyme activities are upregulated in the dwarf mouse fibroblasts. I addressed these questions via measurements of protein levels and activities of the key antioxidant and BER enzymes in Snell normal and dwarf fibroblasts cultured at 3% or 20% oxygen, in the presence and absence of serum, and with or without exposure to the pro-oxidants, paraquat (PQ) and hydrogen peroxide (H₂O₂). Possible differences between normal and dwarf mouse cells in mitochondrial content and activity were also accounted for.

5.2 Experimental Procedures

5.2.1 Materials

Chemicals were purchased from Sigma-Aldrich (Oakville, Ontario, Canada), Fisher Scientific (Mississauga, Ontario, Canada) and Bioshop (Burlington, Ontario, Canada). BioRad protein dye was purchased from BioRad laboratories (Hercules,
California, USA). Prestained broad range protein marker was purchased from BioLabs (New England, Massachusetts, USA). MemCode reversible protein stain kit was purchased from Pierce Biotechnology (Rockland, Illinois, USA). MnSOD and CuZnSOD human antibodies were purchased from Stressgen (Victoria, British Columbia, Canada). Infrared dye-conjugated secondary antibodies to rabbit were purchased from Rockland Immunochemicals (Gilbertsville, Pennsylvania, USA). Oligonucleotides were purchased from Midland Certified Reagent Co. (Midland, Texas, USA). Purified human AP endonuclease and human polymerase β were purchased from Trevigen (Gaithersburg, Maryland, USA). MitoTrackerTM Green and MitoTrackerTM Red were purchased from Molecular Probes (now Invitrogen, Carlsbad, CA, USA).

5.2.2 Preparation of Snell Mice Dermal Fibroblasts
Tail skin biopsies from 8 Snell normal control mice (+/+ or dw/+ genotype) and 8 Snell dwarf mice (dw/dw homozygotes) were collected, diced and split into 2 samples; both were digested overnight in collagenase, one in an atmospheric O₂ incubator (20%), and the other in a 3% O₂ incubator as in (Maynard et al., 2006). Cultures were passaged on the same days, ensuring that all treatments were initiated at the same time for both growth conditions. For 3% and 20% O₂, each cell line was split into four 100 mm tissue culture dishes (∼ 6x10⁶ cells/dish) and were treated as follows; A) 24 h attachment, complete media (confluent), B) treatment A plus 24 h serum deprivation, C) treatment B plus 2 h incubation with 15µM hydrogen peroxide (H₂O₂), D) Treatment B plus 24 hour incubation with 15µM paraquat (PQ). The concentrations of H₂O₂ and PQ were chosen to provide a sublethal level of oxidative stress and therefore avoid the contribution of dead cells to the harvested sample. Following treatments, cells were rinsed with cold...
phosphate buffered saline (PBS); scraped, centrifuged and excess PBS was removed. Cell pellets were stored at -80°C for several months.

5.2.3 Preparation of Cell Lysates

Cell pellets were lysed by incubation in ice cold lysis buffer (10mM Tris pH 8.0, 150mM NaCl, 2mM EDTA, 2mM dithiothreitol (DTT), 0.4mM phenylmethylsulfonylfluoride (PMSF), 40% glycerol and 0.5% NP40) for 1 h with periodic sonication (Ultrasonic Inc. Sonicator W-375; setting 3). Following incubation, cell lysates were centrifuged at 13,000g (4°C) for 10 min (Fisher Scientific accuspin™ MicroR). Protein concentration was determined using the Bradford technique with a BioRad protein kit. Whole cell lysates were stored at -80°C.

5.2.4 Western Blots

Western blots were performed as in Chapter 3. 20μg of cell lysates was resolved. Band signal intensities were measured for each sample and normalized to an internal standard (a single Dwarf sample cultured at 3% O2 confluence). MnSOD and CuZnSOD protein levels were calculated from a standard curve constructed from the internal standard.

5.2.5 Antioxidant and Metabolic Enzyme Assays

All antioxidant enzyme assays were performed as in Chapter 3. Conditions for the cytochrome c oxidase (COX) activity assay were: 25mM potassium phosphate buffer with 0.5% Tween 20 (pH 7.2). 10μg protein was added to obtain a background rate of absorbance change before the reaction was initiated with the addition of 50μM fully reduced cytochrome c and the instantaneous change in absorbance was measured at 550nM. Reduced cytochrome c was prepared by adding sodium dithionite to an aqueous
solution of cytochrome c until fully reduced, then passing the solution through a Sephadex G-25 (Sigma-Aldrich) column to remove the excess dithionite. Lactate dehydrogenase (LDH) activity was measured in a solution containing 20mM HEPES buffer (pH 7.3), 0.2mM NADH, and 20-30μg of protein to obtain a background absorbance. The reaction was initiated with the addition of 10mM pyruvate after which a change in absorbance was measured at 340nm.

5.2.6 Base Excision Repair Assays

AP endonuclease activity and polymerase β activity were measured as in Chapter 4. Oligonucleotides are reported in Table 4.2.

5.2.7 Assessment of mitochondrial abundance and membrane potential

The mitochondrial content and membrane potential of fibroblasts from Snell dwarf and normal littermate controls were measured by flow cytometry using MitoTracker™ probes. Briefly, confluent cells grown at 20% O2 were placed in complete and serum-free media for 24 hours, after which the cells were washed thoroughly in DMEM media, without serum, immediately prior to their incubation with MitoTracker™ Green (100nM) and MitoTracker™ Red (250nM) probes for 30 min. The cells were then washed twice with ice-cold PBS before being scraped into test tubes. The cells were analyzed on a Beckman-Dickson FACSCalibur with excitation at 488nm and emission at 530nm for MitoTracker™ Green and 585nm for MitoTracker™ Red and gated for single cells. MitoTracker™ Green accumulates within mitochondria independent of membrane potential and therefore acts to measure mitochondrial content, whereas MitoTracker™ Red accumulation within mitochondria is dependent upon membrane potential.
5.2.8 Statistical Analysis

Data were analyzed by repeated measures two-factor (effect of genotype and effect of oxygen level) ANOVA using Systat v.12. Post-hoc comparisons between means were done by Sidak’s significant differences method. All data are presented as means ± standard error of the mean (SEM). A p-value of < 0.05 was considered significant. Where p-values are close to, but greater than, 0.05 they are provided in the text.

5.3 Results

5.3.1 Antioxidant and BER enzymes in normal and dwarf mouse fibroblasts in complete and serum-free media

Fibroblasts from Snell dwarf mice are relatively resistant to the oxygen-mediated growth inhibition and replicative failure compared to cells from their normal littermates (Maynard et al., 2006). Overexpression of intracellular SOD isoforms confers stress resistance and prevents apoptotic cell death (He et al., 2004; Chen et al., 2000), and the activities of these enzymes are also negatively correlated with cellular senescence in culture (Blander et al., 2003), so I determined whether an upregulation of SOD activities might contribute to the stress resistance of dwarf mouse cells. However, there was no effect of genotype on MnSOD or CuZnSOD levels under any of the experimental conditions (Fig. 5.1). As MnSOD localizes exclusively within the mitochondrial matrix, differences in mitochondrial content of cells under different experimental conditions could affect MnSOD levels. COX activity and MitoTracker™ green fluorescence was measured to estimate cellular mitochondrial content (see below). Even when standardized to mitochondrial content (Supplemental Fig. 5.1), there was no effect of genotype on MnSOD levels under any of the experimental conditions shown in Fig. 5.1. O₂ level and serum deprivation, however, did affect cellular MnSOD levels.
levels were approximately 2-fold higher in cells grown at 20% versus 3% O₂ (Fig. 5.1b; complete media). This was partially a result of an apparent increase in cellular mitochondrial abundance based on COX activity (see below). On the other hand, neither O₂ level nor serum deprivation affected CuZnSOD protein levels in either normal or dwarf mouse cells (Fig. 5.1c).

Interestingly, greater resistance (higher LD₅₀) of Snell dwarf fibroblasts, compared to normal littermates, to exogenous stressors, such as PQ and H₂O₂, is only evident in the absence of serum (Murakami et al., 2003), because exogenous serum increases resistance dramatically in both Snell and control cell lines. I therefore investigated SOD levels in fibroblasts cultured in serum deprived (SD) media in the presence and absence of an additional oxidative stressor. There was no genotype-specific response to SD with respect to either MnSOD or CuZnSOD levels. However, SD led to a significant increase in MnSOD levels in both normal and dwarf fibroblasts (Fig. 5.1b). As SD had no apparent effect on mitochondrial content (see below), this suggests that the amount of MnSOD protein per mitochondrion increased approximately 2- to 5-fold (Fig. 5.1b). In contrast, CuZnSOD protein levels were unaffected by SD (Fig. 5.1c).
Fig. 5.1: Antioxidant protein levels and activities in normal and Snell dwarf mouse skin derived fibroblasts cultured at physiological (3%) and standard (20%) oxygen in complete and serum-free media. (a) Representative western blot showing MnSOD and CuZnSOD expression; lower panel shows the membrane stained for equal loading and transfer. (b) MnSOD relative protein concentration from normal (empty bars) and Snell dwarf (filled bars) mouse fibroblasts. (c) CuZnSOD relative protein levels in normal and Snell dwarf mouse fibroblasts. In (b) and (c), bars represent mean protein levels ± SEM measured in six independent cell lines for each genotype. (d) GPx activity of normal and Snell dwarf mouse fibroblasts. (e) CAT activity from normal and Snell dwarf mouse fibroblasts. In (d) and (e), bars represent mean enzyme activity ± SEM from eight independent cell lines measured in duplicate for each genotype. Statistical comparisons between means were by repeated measures ANOVA with Sidak post-hoc test.
Intracellular detoxification of H₂O₂ is catalyzed primarily by GPx and CAT. While the latter enzyme is localized to peroxisomes, isoforms of GPx are distributed in various subcellular compartments, including mitochondria (Del Maestro and McDonald, 1989). Genetic disruption of GPx1 induces characteristics of senescence in cultured fibroblasts (de Haan et al., 2004), suggesting it may also play a role in mediating resistance to senescence. Also, mice overexpressing the phospholipid hydroperoxide, GPx4, are protected from cellular injury during ischemia-reperfusion stress (Dabokowski et al., 2008). In contrast to the SODs, GPx activity was affected by genotype (p < 0.01), being elevated in dwarf mouse cells under several experimental conditions (Fig. 5.1d). In addition, GPx activity was also affected by O₂ level, and the interaction between genotype and O₂ level was significant (p < 0.001), indicating that the normal and dwarf mouse fibroblasts differed in their ability to upregulate GPx in response to increasing O₂ levels. In contrast, there was no effect of either genotype or oxygen level on CAT activity, though it was reduced by SD in cells of both genotypes (Fig. 5.1e).

Because cumulative DNA damage has been associated with aging and cellular senescence, I hypothesized that enhanced DNA repair in the Snell dwarf fibroblasts may increase their resistance to detrimental DNA oxidative damage. BER is the major pathway of DNA oxidative damage repair in active cells, and so I measured two key enzymes within this pathway using in vitro assays. AP endonuclease activity, which catalyzes the processing of abasic sites in DNA, did not differ between genotypes under any of the conditions shown in Fig. 5.2 (a, b). However, it was significantly affected by experimental condition, being reduced by SD (versus complete medium) in cells grown at 20% O₂. Polymerase β catalyzes the incorporation of a nucleotide in the gap produced by
AP endonuclease activity. The interaction between genotype and O₂ level for polymerase β activity was almost significant (p = 0.08), suggesting that normal and dwarf cells may differ in their ability to upregulate polymerase β activity in response to oxidative stress. Also, in complete medium, dwarf mouse cells cultured at 3% O₂ had approximately 70% greater polymerase β activity than those of normal mice (Fig. 5.2d).

**Fig. 5.2:** Base excision repair activities of normal and Snell dwarf mouse skin derived fibroblasts cultured at physiological (3%) and standard (20%) oxygen conditions in complete and serum-free media. (a) Representative gel showing AP endonuclease activity, measured in whole cell lysates (100 ng) containing 1 pmol THF-containing double-stranded oligonucleotide and incubated for 5, 10, 15 min; NP = no protein control (for summary of experimental controls see Materials and Methods). (b) AP endonuclease activity of normal and dwarf Snell mice skin fibroblasts. (c) Representative gel showing measurement of polymerase β activity in whole cell lysates (0.5 µg). Incorporation of [³²P]dCTP into GAP oligonucleotide (see Table 5.1) is visible as the progressive accumulation of an 18-mer oligonucleotide, at 10, 20, 30 min. (d) Relative polymerase β activity in normal and Snell dwarf mouse skin fibroblasts. In (b) and (d) bars represent mean activity ± SEM measured in lysates from eight independent cell lines for each genotype; in (b) measurements were done in duplicate. Identity of bars and statistical analysis as in Fig. 5.1.
There were also significant effects of experimental condition on polymerase β activity (Fig. 5.2c, d), with effects of both O₂ and SD. As has been observed in other mammalian fibroblasts (Brown and Stuart, 2007), polymerase β activity appeared to be elevated (approximately 80%) in normal mouse fibroblasts grown in complete medium at 20% O₂ versus 3% O₂ (Fig. 5.2d; complete). However, this effect did not reach statistical significance in the current set of comparisons.

5.3.2 Effects of exogenous oxidants on antioxidant and BER enzymes

Following SD for 24h, Snell normal and dwarf fibroblasts were exposed to sublethal concentrations of oxidants; either 15µM PQ for 24h or 15µM H₂O₂ for 2h. These concentrations were determined based upon previous studies of lethal doses (Kapahi et al., 1999; Maynard et al., 2006). PQ exposure slightly, but significantly, reduced MnSOD protein levels in both normal and dwarf mouse fibroblasts (Fig.5.3a; see Supplemental Fig. 5.1 for mitochondrial abundance standardization) grown at 20% O₂. Under this experimental condition, MnSOD protein levels were almost 10% higher in dwarf mouse, versus normal, cells. PQ exposure did not affect CuZnSOD protein levels (Fig. 5.3b). Although there was no effect of genotype on GPx activity under these conditions, both PQ and H₂O₂ exposure significantly reduced GPx activity in both normal and dwarf fibroblasts (Fig. 5.3c). There was also a significant interaction between genotype and experimental condition (p < 0.05) in respect to CAT activity, which appears to have been driven by higher CAT activity in dwarf relative to normal cells following exposure to either PQ or H₂O₂. However, only the H₂O₂ effect reached statistical significance (p < 0.05; Fig. 5.3d).
Fig. 5.3: Effect of paraquat and hydrogen peroxide on antioxidant protein levels and activities of normal and Snell dwarf mouse skin derived fibroblasts cultured at standard (20%) oxygen conditions in serum-free media. (a) MnSOD relative protein concentration. (b) CuZnSOD relative protein concentration. In (a) and (b), bars represent mean SOD protein levels ± SEM from six samples for each genotype. (c) GPx activity of normal and dwarf Snell mice fibroblasts. (d) CAT activity from normal and Snell dwarf mouse fibroblasts. In (c) and (d), bars represent mean enzyme activity ± SEM from eight samples for each genotype, measured in duplicate. Identity of bars and statistical analysis as in Fig. 5.1.

Genotype also affected AP endonuclease activity in SD cells in the presence or absence of exogenous stressors (p < 0.05; Fig. 5.4a) with dwarf cells having apparently higher activities under all experimental conditions, though only in the instance of H₂O₂ exposure was the dwarf versus normal comparison statistically significant. In contrast, polymerase β activity was not affected by genotype in SD cells in the presence or absence of exogenous stressors, though a trend toward higher activities in the dwarf cells was evident (p = 0.08; Fig. 5.4b). Polymerase β activity however was affected by the presence of exogenous oxidants (p < 0.05); there is a suggestion that dwarf cells may be
more responsive to $\text{H}_2\text{O}_2$ treatment, but the interaction between genotype and stress was not significant.

Fig. 5.4: Effect of paraquat and hydrogen peroxide on base excision repair activities of normal and Snell dwarf mouse skin derived fibroblasts cultured at standard (20%) oxygen conditions in serum-free media. (a) AP endonuclease activity. (b) Polymerase $\beta$ activity; bars represent mean activity ± SEM from eight independent cells line for each genotype; in (a) measurements were done in duplicate. Identity of bars and statistical analysis as in Fig. 5.1. PQ = paraquat; see Materials and Methods for details of oxidant exposure.

5.3.3 Mitochondrial and fermentative metabolism in normal and dwarf mouse fibroblasts

Previous studies have shown that fibroblasts derived from Snell dwarf mice are more resistant to stressors that interact directly with mitochondria, such as PQ. This raises the possibility that the superior stress resistance of dwarf mouse fibroblasts could be due to reduced mitochondrial activity, such that the impact of metabolic and oxidative poisons that involve mitochondrial activation is less. To investigate this possibility, mitochondrial content and activity was analyzed under several of the experimental conditions studied above. Using COX activity as a proxy of mitochondrial number/activity, the only apparent effect of genotype was that COX activity was lower in
dwarf compared to normal fibroblasts grown to confluence in serum containing media at 20% O₂ (p < 0.05; Fig. 5.5a).

Total cellular mitochondrial content and mitochondrial inner membrane potential were also estimated in both normal and dwarf mouse fibroblasts using the fluorescent probes MitoTracker™ Green (total mitochondria) and MitoTracker™ Red (membrane potential). These results are shown in Figure 5.5 (b, c), and indicate no significant differences between dwarf and normal fibroblast mitochondrial content or membrane potential in cells grown in the presence or absence of serum. The ratio of the membrane potential signal to the total mitochondria signal (Fig. 5.5d; mean membrane potential per mitochondrion), was also calculated to correct mitochondrial membrane potential to mitochondrial abundance. However, this value also did not differ between genotypes, under either experimental condition investigated. The addition of 10μM FCCP, which reduces or abolishes the inner membrane potential, served as a negative control, and this ratio was also not different between normal and dwarf cells (Fig. 5.5d). Taken together with the COX data, this suggests that mitochondrial activity of normal and dwarf mouse fibroblasts is similar under most experimental conditions.

Fibroblasts in culture rely partially on glycolytic pathways to meet energy requirements, even in the presence of relatively high O₂ levels (Vazquez et al., 2010). Therefore I also measured the activity of lactate dehydrogenase (LDH), an indicator of anaerobic metabolic flux, as an indirect test of whether dwarf and normal mouse fibroblasts differed in their reliance on fermentative metabolism for ATP production. In complete media, there was a significant effect of genotype on LDH activity, which was approximately 15% lower in dwarf mouse cells growing at either 3% or 20% O₂.
**Fig. 5.** Mitochondrial content and membrane potential in normal and Snell dwarf mouse skin derived fibroblasts cultured in complete and metabolically stressful conditions. (a) Cytochrome c oxidase (COX) activity; (b) Mitochondrial content as measured by fluorescence of MitoTracker Green™ binding in normal (empty bars) and dwarf (filled bars) fibroblasts in complete media or following 24h SD. (c) Mitochondrial membrane potential as measured by fluorescence of MitoTracker Red™ binding in cells in complete media or following 24h SD. (d) The ratio of mitochondrial membrane potential to mitochondrial content (mean membrane potential per mitochondrion). (e) Lactate dehydrogenase (LDH) activity. (a) and (e) are means ± SEM of eight independent cell lines for each genotype, measured in duplicate. (b-d) are means ± SEM of 4 experiments. Differences between experimental conditions are not indicated in a and e, though there were significant effects of O₂ level, SD and exogenous oxidants on COX and LDH activities. LDH activity was significantly greater in normal, compared to dwarf mouse cells growing in complete media (Effect of genotype by ANOVA; p < 0.05).
LDH activities were lower in both genotypes at 20% O$_2$, which is expected as fibroblasts upregulate oxidative phosphorylation under these conditions (Leiser et al., 2006). Exposure to exogenous oxidative stressors affected LDH activity similarly in both genotypes ($p < 0.001$; Fig. 5.5e).

5.4 Discussion

Fibroblasts cultured from dermal tissue of Snell dwarf mice are resistant to multiple forms of stress (Marakami et al., 2003; Salmon et al., 2005; Leiser et al., 2006) and oxygen-induced growth crisis or replicative failure (Maynard and Miller, 2006). I initially hypothesized that the oxidative stress resistance of dwarf mouse fibroblasts might be mediated by a systematic upregulation of antioxidant enzymes to reduce ROS levels, and DNA repair defenses, to remove oxidative damage. The experimental results reported here are partially supportive of this hypothesis. Interestingly, some molecular targets identified in other models of extended longevity were not differentially expressed in Snell dwarf cells. Also, my data support the contribution of multiple, relatively subtle, differences in metabolism, antioxidant status and DNA BER capacity to the overall phenotype of stress resistance.

MnSOD has been identified as a molecular mediator of animal longevity in a wide variety of contexts. It is positively correlated with stress resistance in fibroblasts from mammalian species of widely ranging lifespans (compare Kapahi et al., 1999 and Brown and Stuart, 2007), upregulated in long-lived type 5 adenylyl cyclase mice (Yan et al., 2007), in C. elegans daf-2 mutants (Honda and Honda, 2008), and by the polyphenol resveratrol (Robb et al., 2008a; Robb et al., 2008b), which promotes longevity in some animals (reviewed by Baur and Sinclair, 2006). Transgenic overexpression of MnSOD is
effective in preventing or limiting mitochondrial permeability transition and cell death under a variety of stressful conditions Silva et al., 2005; Solaini and Harris, 2005; Warner et al., 2004). Despite this association of MnSOD activity with longevity in disparate biological contexts, I found limited evidence to support a role for this enzyme in the stress resistant phenotype of the dwarf mouse cells. MnSOD protein levels were found to be higher in dwarf compared to normal fibroblasts only following PQ exposure.

Conversely, experimental conditions, including O₂ level and SD, significantly affected MnSOD levels in both the normal and dwarf mouse cells. For example, growth of cells at 20% O₂ caused a 2-fold increase in MnSOD levels over cells grown at 3% O₂. This appears to have been partially caused by mitochondrial proliferation in fibroblasts exposed chronically to atmospheric oxygen, an effect which has been reported previously (Brown et al., 2007). The effect of elevated (i.e. greater than atmospheric) oxygen tensions on mitochondrial biogenesis has also been demonstrated in vivo (Gutsaeva et al., 2006; Suliman et al., 2007). An induction of MnSOD in response to SD was also observed in both normal and dwarf mouse cells. This appeared to represent a true increase in the level of this enzyme per mitochondrion, as apparent cellular mitochondrial content did not differ between cells cultured in complete media compared to those cultured in SD media under atmospheric oxygen conditions. This elevation of MnSOD levels is consistent with reports that increased MnSOD activity regulates the transition into quiescence in mouse embryonic fibroblasts (Sarsour et al., 2008). Elevation of MnSOD activity can trigger a transition from proliferative growth to replicative senescence in culture (Sarsour et al., 2008; Behrend et al., 2005).
The above results suggest that elevation of MnSOD makes only a minor contribution to the stress resistant phenotype of dwarf mouse fibroblasts. Similarly, CuZnSOD, which confers enhanced stress resistance in mammals (Solaini et al., 2007; Warner et al., 2004), can extend longevity in Drosophila (reviewed by Phillips et al., 2000), and is associated with replicative senescence in culture (Blander et al., 2003), was unaffected by either experimental treatment or genotype in my experiments. Taken together, this suggests that differences in superoxide dismutation are not a major factor underlying differences in stress resistance between normal and Snell dwarf fibroblasts.

Interestingly, however, there was more evidence that enzymatic detoxification of $\text{H}_2\text{O}_2$ was greater in Snell dwarf fibroblasts compared to those from normal littermates, and this could be important in conferring stress resistance. Elevated GPx activities may also contribute to the resistance of dwarf mouse cells to \textit{in vitro} growth crisis when grown at 20% $\text{O}_2$ (Maynard and Miller, 2006). The importance of GPx in this context is illustrated by experiments in which genetic disruption of \textit{GPx1} elicits characteristics of senescence in cultured fibroblasts (de Hann et al., 2006). The elevation of GPx activity in Snell dwarf cells deprived of serum may also be important in imparting resistance to oxidative stress. GPx activity has been positively correlated with cellular stress resistance in a number of studies. For example, \textit{GPx1} -/- mice are susceptible to paraquat exposure (de Haan et al., 1998) and ischemia/reperfusion brain injury (Crack et al., 2001). Similarly, MEFs cultured from GPx1 deficient mice have increased levels of apoptotic cell death under standard culture conditions and following exposure to $\text{H}_2\text{O}_2$ (de Haan et al., 2003). In contrast, GPx1 overexpression protects mouse fibroblasts from UV-induced DNA damage (Baliga et al., 2007) and protects murine neuronal cells from
H$_2$O$_2$-induced cell death (McLean et al., 2005), perhaps by inhibiting cytochrome c release from mitochondria (Hoehn et al., 2003).

There are multiple GPx isoforms (reviewed by Margis et al., 2008) and, although GPx1 is the most ubiquitous, abundant, and active towards reduction of hydrogen peroxide, the phospholipid hydroperoxide glutathione peroxidase (GPx4) appears to play a similar role in promoting stress resistance. Lung fibroblasts isolated from GPx4 heterozygous mice are susceptible to H$_2$O$_2$-mediated cell death (Garry et al., 2008). GPx4 overexpression reduces ischemia/reperfusion associated cardiac dysfunction (Dabkowski et al., 2008) and protects mouse fibroblasts from oxidant-induced apoptosis (Ran et al., 2004). Mitochondria isolated from GPx4 transgenic mice are resistant to diquat exposure, perhaps due to increased protection of the mitochondrial inner membrane and the maintenance of membrane potential (Liang et al., 2007). I was unable to detect GPx4 activity in the Snell normal and dwarf cell lysates. It is, however, likely that the differences in total GPx activity I observed were due primarily to GPx1, based on the fact that fibroblasts have extremely low GPx4 activity (Garry et al., 2008), and negligible GPx activity towards H$_2$O$_2$ is measurable in GPx1 -/- mice (de Haan et al., 1998).

Interestingly, differences in GPx activity between normal and Snell dwarf cells disappeared following exposure to PQ or H$_2$O$_2$, when activity in cells from both genotypes was reduced. Lower GPx activities in both normal and dwarf cells are consistent with oxidative stress-induced inactivation of GPx1, which has been previously reported in pulmonary microvascular endothelial cells exposed to paraquat (Takizawa et al., 2007). Interpretation of this reduction in GPx activity may also be complicated by the
possibility that PQ and/or H$_2$O$_2$ may have injured the cells, causing loss of cytosolic material. In any case, the initially higher GPx activity of dwarf mouse cells prior to oxidant exposure may be a significant factor in their subsequent survival.

While there was no effect of genotype on CAT activity in cells grown in serum-containing media, CAT activity was significantly higher in dwarf mouse cells following exposure to H$_2$O$_2$ in serum-free media, and showed a similar (but not significant) trend in cells exposed to PQ. While the contribution of CAT activity to oxidative stress resistance is likely to be less significant than that of GPx, owing to its lower specific activity under all conditions, this does suggest that Snell dwarf mouse cells have a generally superior ability to detoxify H$_2$O$_2$ via multiple pathways. This may be a significant factor in the multiplex stress resistance of the Snell dwarf fibroblasts. As even stressors that are not directly oxidative can induce redox stress secondarily (Kultz, 2005), this capacity could affect resistance to multiple toxins.

Dermal fibroblasts derived from Snell dwarf mice are more resistant than their normal littermates to multiple genotoxic agents, including cadmium, paraquat, hydrogen peroxide, UV light, and DNA methylating agents (Murakami et al., 2003; Slamon et al., 2005). Most of these compounds can produce lesions that are substrates for DNA BER, and therefore increased resistance to such stressors could be a direct result of greater DNA BER activity. Reduced polymerase $\beta$ and AP endonuclease activities are associated with reduced BER and increased DNA damage, mutation and cell death caused by oxidizing and alkylating agents (Chen et al., 1998; Cabelof et al., 2002; Cabelof et al., 2003). However, I did not observe constitutively elevated BER enzyme activities in Snell dwarf cells under all experimental conditions, though some important
differences between genotypes were noted. Perhaps most interesting was the apparently
greater ability of the dwarf mouse cells (compared to controls) to increase AP
endonuclease and polymerase β (p = 0.08) activities in response to H₂O₂ exposure. This
suggests that the dwarf mouse cells are better able to upregulate BER in response to
H₂O₂-induced stress.

The BER results support the contention that dwarf mouse cells might possess
superior DNA repair capacity, at least under some experimental conditions. It has
previously been shown that Snell dwarf mouse dermal fibroblasts repair UV-induced
DNA lesions more rapidly than normal mouse fibroblasts, indicating enhanced nucleotide
excision repair (NER) in the former (Salmon et al., 2008). Taken together, the BER and
NER results suggest that that dwarf mouse cells possess an enhanced ability to repair a
wide variety of DNA lesions, and that this contributes to their stress resistance. The
participation of other DNA repair pathways, such as mismatch repair (MMR) and double
strand break (DSB) repair would also be interesting targets of further investigation in the
Snell dwarf model, particularly as enzymes involved in MMR and DSB repair are
consistently identified in proteomic surveys of the cellular stress response (Kultz, 2005).

It is interesting that, in addition to their oxidative stress resistance, Snell dwarf
fibroblasts are resistant to several metabolic stressors (Leiser et al., 2006). The breadth
of this stress resistant phenotype points to the potential involvement of mitochondria,
organelles that have been broadly connected to aging and cell death (reviewed by
Wallace, 2005). Molecules such as PQ (see Cochemé et al., 2008) achieve their toxicity
primarily via interactions with mitochondria. Also, mitochondria are known to undergo
adaptive remodeling in such disparate models of extended longevity as p66shc−/− mice
(Pinton et al., 2007) and caloric restriction (López-Lluch et al., 2006; Anderson et al., 2008), and such remodeling could alter the activation of metabolic and oxidative stressors. I considered, therefore, that similar mitochondrial remodeling might occur in the Snell dwarf context.

COX activity as well as MitoTracker™ fluorescence measured in both normal and dwarf cells under different experimental conditions revealed no differences in mitochondrial content or membrane potential between genotypes. This suggests that the dwarf fibroblasts were no less reliant than normal fibroblasts on oxidative phosphorylation for the maintenance of ATP pools, and nor were they in general less metabolically active. The parallel measurements of LDH, an indicator of anaerobic metabolism, suggested few apparent differences in the recruitment of anaerobic pathways, though LDH activity was slightly reduced in dwarf cells (relative to normal) growing in complete media. The significance of this observation is not known, but the results generally indicate that if Snell dwarf fibroblasts are less metabolically active, the difference lies primarily in reduced fermentative metabolism. On the other hand, dwarf mouse cells replicate more rapidly and reach higher densities in culture than those of normal mice (Maynard and Miller, 2006), which also argue against the idea that dwarf cells have lower rates of anabolic metabolism. It may nonetheless be interesting in the future to examine aspects of mitochondrial function such as ROS production and propensity to undergo permeability transition and/or apoptotic release of cytochrome c, as these properties are known to be modulated in cells of other long-lived mouse mutants (eg Pinton et al., 2007).
In summary, Snell dwarf mouse fibroblasts appear to maintain levels of metabolic activity similar to those of their normal littermates. The capacity to dismutate superoxide radicals is also similar in normal and dwarf mouse cells. Potentially important differences in dwarf mouse cells, relative to normal mouse cells, included elevated activities of H$_2$O$_2$ detoxifying enzymes, and an ability to induce DNA BER activities in response to H$_2$O$_2$ exposure. Taken together with the results of previous studies, these results are not consistent with the existence of a single mechanism conferring stress resistance to the Snell dwarf dermal fibroblasts. Rather, they suggest that relatively subtle differences in a number of cellular processes contribute to the stress resistant phenotype.
5.6 Supplemental Data

![Graph showing relative MnSOD level/COX activity](image)

**Fig. S5. 1:** Genotype does not affect MnSOD protein levels that have been corrected for mitochondrial abundance (COX activity). Bars represent mean enzyme activity ± SEM from eight samples for each genotype, measured in duplicate. Identity of bars and statistical analysis as in Fig. 5.1.
Chapter 6. Contribution of intracellular antioxidant enzymes to stress resistance during hibernation

Hypothesis

Intracellular antioxidant enzymes have a role in conferring stress resistance in tissues isolated from animals in quiescent states.

Objective

The primary objective of this project was to determine if intracellular antioxidant enzymes play a role in cellular stress resistance observed in highly oxidative tissues isolated from hibernating 13 lined ground squirrels compared to their respective active controls.

Publications of results


Contributions

I performed all antioxidant enzymes experiments, statistical analysis and chapter write-up.
6.1 Introduction

Cellular reactive oxygen species (ROS) metabolism has been the focus of intense interest, due in part to the demonstration that overexpression of antioxidant enzymes confers resistance to stress, disease (reviewed in Solaini and Harris, 2005; Blomgren and Hagberg, 2006) and, in some instances, increased lifespan (e.g., Phillips et al., 2000). Amongst the intracellular antioxidant enzymes, the mitochondrial (MnSOD) and cytosolic (CuZnSOD) superoxide dismutases are perhaps most strongly linked to stress and disease resistance and to extended lifespan. For example, MnSOD is upregulated in various tissues of the long-lived type 5 adenylyl cyclase knockout mice (Yan et al., 2007), in long-lived Caenorhabditis elegans mutants (Honda and Honda, 2002), and by the polyphenol resveratrol (Robb et al., 2008a,b), which promotes extended longevity in some animals (see Baur and Sinclair, 2006 for review).

Interestingly, superoxide dismutase activities are also upregulated in a number of species as they enter a metabolically depressed dormant state, such as the hypometabolic C. elegans dauer larva (reviewed in Honda and Honda, 2002), and estivating terrestrial snails (Hermes-Lima and Storey, 1995). Whether enhanced antioxidant capacity is a general strategy practiced by animals entering a dormant state remains to be determined, but recent studies have demonstrated enhanced stress resistance in brain and liver tissue of hibernators (Lindell et al., 2005; Dave et al., 2006; Christian et al., 2008). Also, one of the major intracellular signaling pathways mediating formation of the stress resistant and long-lived dauer larva in C. elegans is regulated similarly in hibernating 13-lined ground squirrels (Spermophilus tridecemlineatus). Akt, which phosphorylates FOXO and thus inhibits its transcriptional activity, is repressed in brain during hibernation (Cai et al., 2006).
This suggests that, during hibernation, FOXO would actively stimulate transcription of its target genes, which include those encoding antioxidant enzymes. Therefore, it is important to establish whether intracellular antioxidant enzymes are indeed upregulated in hibernating mammals and play a role in conferring stress resistance.

Several authors (Hermes-Lima, 2002; Carey et al., 2003) have suggested that hibernators and estivators upregulate intracellular antioxidant enzymes to protect against ischemia-reperfusion (I-R) injury during torpor-arousal transitions. Even relatively brief periods of ischemia-reperfusion (I-R) can be fatal to cells in highly oxidative tissues like heart and brain characterized by rapid ATP turnover (see Solaini and Harris, 2005; Blomgren and Hagberg, 2006 for reviews). Transgenic overexpression of MnSOD, which catalyzes the first step in \( \text{O}_2^\cdot^- \) detoxification in mitochondria, can protect murine heart (Chen et al., 1998) and brain (Murakami et al., 1998; Kim et al., 2002) from I-R injury. In contrast, MnSOD deficiency increases vulnerability of both tissues to oxidant-mediated death (Kim et al., 2002; Asimakis et al., 2002). This protective effect may be mediated by the ability of MnSOD to prevent mitochondrial permeability transition and cell death under a variety of stressful conditions (Silva et al., 2005; Solaini and Harris, 2005; Warner et al., 2004). Thus, hibernators such as the 13-lined ground squirrel might upregulate intracellular antioxidant enzymes to protect highly oxidative tissues from I-R injury during repeated bouts of torpor and arousal. Other antioxidant enzymes also can be protective in this context. CuZnSOD could effectively dismutate superoxide produced in the cytosol by, for example, xanthine oxidase, which is activated in rodents during I-R (Warner et al., 2004). Similarly, transgenic overexpression of other antioxidant enzymes
such as glutathione peroxidase and catalase confer resistance to I-R damage in mouse brain tissue and might play a similar role during hibernation (Warner et al., 2004).

Several authors have investigated antioxidant enzyme activities in hibernators (reviewed in Carey et al., 2003). However, these studies have generally not focused on brain and heart tissue, though these are most vulnerable to I-R injury in other mammals, or they have quantified SOD activities directly in crude tissue homogenates using spectrophotometry. Spectrophotometric assays of SOD isoforms are particularly problematic, due to the presence of multiple sources of potential interference (Beyer and Fridovich, 1987; Spitz and Oberley, 1989). To address this problem, assays that employ native gel electrophoresis to separate SOD isoforms from interfering molecules prior to quantification have been developed (Beauchamp and Fridovich, 1971). In addition, quantification by Western blot is useful, as neither SOD isoform has been reported to be physiologically regulated by phosphorylation or by other post-translational modification, so that there is good agreement between the enzyme level and activity. Also, for measurement of MnSOD, it is critical to account for the fact that this isoform is localized exclusively in mitochondria, so that any change in mitochondrial abundance in cells will affect cellular MnSOD levels/activities without changing the enzyme activity ‘per mitochondrion’. For this reason, it is necessary to establish mitochondrial abundance, or volume density, to correctly interpret the significance of altered MnSOD protein levels and/or activities in crude tissue homogenates.

Here, I have used both immunodetection and an in-gel activity assay method to measure the levels and activities of MnSOD and CuZnSOD in heart, brain and liver tissues of hibernating 13-lined ground squirrels. In addition, I have measured the
activities of enzymes involved in the second step of superoxide detoxification, the conversion of $\text{H}_2\text{O}_2$ to water. Using these approaches I tested the hypothesis that hibernation is associated with an upregulation of intracellular antioxidant enzymes in the major oxidative tissues.

6.2 Experimental Procedures

6.2.1 Materials
Chemicals were purchased from Sigma-Aldrich (Oakville, Ontario, Canada; including Fluka and Caledon) and BioShop (Burlington, Ontario, Canada). BioRad protein dye was obtained from BioRad laboratories, (Hercules, California, USA). Prestained broad range protein marker was obtained from BioLabs, (New England, Massachusetts, USA). MemCode reversible protein stain kit was purchased from Pierce Biotechnology, (Rockland, Illinois, USA). Antibodies to human CuZnSOD and human MnSOD were purchased from StressGen Biotechnologies (Assay Designs, Ann Arbor, Michigan, USA). Anti-actin antibody (C-11) was purchased from Santa Cruz Biotechnology. Infrared dye-conjugated secondary antibodies to rabbit and goat were purchased from Rockland Immunochemicals, (Gilbertsville, Pennsylvania, USA).

6.2.2 Animals
Squirrels were wild-caught as apparently young adults during late spring at the University of Manitoba’s field research station in Carmen MB (49°30’N, 98°01’W). Although the age of the animals could not be determined, trapping at this time of year typically yields recently weaned young of the year (Vaughan et al., 2006). After transfer to the University of Western Ontario (London, ON) animal husbandry followed the methods of Muleme et al. (2006). All animals underwent a full hibernation season in the
lab. Monitoring of hibernation and sampling followed the procedure described by Muleme et al. (2006). Hibernating squirrels were sampled towards the end of the first lab hibernation season, 3-4 days into a hibernation bout when both body temperature (~5°C) and metabolic rate (measured as in Muleme et al., 2006) were low and constant. Hibernating squirrels had not eaten for several weeks (hibernating *S. tridecemlineatus* do not eat, even during periodic spontaneous arousals) and weighed between 131g and 154g at the time of sampling. ‘Active,’ non-hibernating squirrels, underwent a hibernation season in the lab but were sacrificed in mid-June when they weighed between 168g and 239g, and were actively gaining weight, but were fasted for 18-24 hours prior to sampling. Active animals were euthanized by Euthanyl overdose (270 mg/ml, 0.2ml/100g) whereas hibernating animals were euthanized by cervical dislocation to prevent inducing arousal. Brain, heart and liver tissue were rapidly excised, flash-frozen in liquid nitrogen, and stored at -80°C.

6.2.3 Tissue Homogenization

Tissues from active and hibernating ground squirrels were homogenized in Buffer A (10mM KH$_2$PO$_4$ (pH 7.4), 20mM EDTA, 30mM KCl, 0.1% Triton X-100, 10% glycerol) to measure SOD activity (in-gel assay) and protein level (immunodetection). For all other measurements of enzyme activities, tissues were homogenized in Buffer B (20mM HEPES (pH7.5), 2mM EDTA, 2mM EGTA, 100mM KCl, 0.5% Triton X-100, 5% glycerol). All homogenization was performed at 4°C with a PowerGen 125 homogenizer (Fisher Scientific, Ottawa, Canada) on full speed for 10s for 3 cycles. Samples were centrifuged for 10 min at 500 g (4°C). Protein concentrations of
supernatants were measured using the Bradford technique with a BioRad protein kit. Homogenates were stored at -80°C.

6.2.4 Western Blots
Western blots were performed as in Chapter 3. 20µg of tissue homogenate was resolved.

6.2.5 In-gel Superoxide Dismutase Activity Assay
MnSOD and CuZnSOD activities were measured as in Chapter 3.

6.2.6 Other Enzyme Assays
All enzyme assays were performed as in Chapter 3.

6.2.7 Statistical Analysis
Within-tissue comparisons of protein levels and/or activities between active and hibernating squirrels were done using the Students t-test.

6.3 Results
The approximately 22kDa MnSOD and 16kDa CuZnSOD subunits were both identified in mouse and squirrel tissues (Fig. 6.1a). MnSOD and CuZnSOD proteins were immunodetected in heart, brain and liver, with actin serving as a loading control (Fig. 6.1b). As has been observed in other rodents (Szymonik-Lesiuk et al., 2003; Meng et al., 2007), SOD levels are particularly high in liver tissue, compared to heart and brain. This is likely related to the role of the liver in xenobiotic metabolism and concomitant ROS production. Importantly, this analysis revealed no significant differences in MnSOD protein levels within any tissue between active and hibernating squirrels (Fig. 6.2a). However, a slight increase (though not statistically significant) in MnSOD level appeared to occur in heart tissue of hibernators. MnSOD is localized exclusively to mitochondria,
so CS activity was determined as an indicator of mitochondrial abundance in active and hibernating animals (Fig. 6.2b). CS activity was approximately 25% greater in hearts of hibernators relative to active squirrels. In contrast, liver CS activity was reduced by approximately 25% in hibernators. However, when MnSOD protein levels were standardized to CS activity, no significant differences in relative protein levels were observed in heart, brain or liver (Fig. 6.2c). Similarly, no differences in CuZnSOD protein levels were found between active and hibernating squirrels (Fig. 6.2d).

Fig. 6.1: Immunodetection of MnSOD and CuZnSOD squirrel tissues. (a) Recognition of the 22 kDa subunit of MnSOD and ~16 kDa subunit of CuZnSOD in mouse and squirrel brain tissue. (b) Representative western blot showing MnSOD, CuZnSOD and actin levels in brain tissue of active and hibernating squirrels. 20 μg of tissue was loaded in each lane.

Immunodetection provides an approximation of SOD activities because neither major isoform has been reported to be physiologically regulated by post-translational modification. However, SOD can be inactivated by high levels of ROS or reactive nitrogen species (Alvarez et al., 2004; Demicheli et al., 2007). If hibernation were associated with oxidative stress and elevated tissue ROS levels, SOD inactivation could occur in vivo, so that SOD abundance as detected by western blot would overestimate the in vivo SOD antioxidant capacity in hibernating tissue.
I therefore employed a direct assay of SOD activity. Native gel electrophoresis effectively separated the two major isoforms of SOD, which were then identified in-gel using the cyanide sensitivity of CuZnSOD to confirm the identity of individual bands (Fig. 6.3a,b). Similar to my immunodetection results, I found no differences in MnSOD (Fig. 6.3c,d) or CuZnSOD (Fig. 6.3e) activities between active and hibernating squirrels. MnSOD activity/CS activity was the same between hibernating and active squirrels in all tissues (Fig. 6.3d), indicating that MnSOD activity per unit mitochondria was maintained during hibernation. Ratios of MnSOD activity/MnSOD protein levels (i.e. specific activities) were also not different (not shown).
Fig. 6.3: MnSOD and CuZnSOD activities are unaltered during hibernation. 
(a) Representative gel showing identification of MnSOD and CuZnSOD bands and inhibition of CuZnSOD activity by cyanide; (b) Representative gel showing SOD standard (bovine heart CuZnSOD) and squirrel liver SOD bands; (c) MnSOD activity in tissues of active and hibernating squirrels; (d) MnSOD/CS (correction of MnSOD activity for citrate synthase activity, a proxy of cellular mitochondrial content); (e) CuZnSOD activities in tissues of active and hibernating squirrels. In all bar graphs, values are means ± SEM of duplicate measurements on 5-6 individuals per experimental group.

Detoxification of hydrogen peroxide produced from either spontaneous or catalyzed superoxide dismutation is supported by the GPx/GR couple that catalyzes the net transfer of an electron from NADPH to H$_2$O$_2$, and by the direct conversion of H$_2$O$_2$ to
O₂ and H₂O by catalase. The maximal activities of GPx and GR were measured in tissues of active and hibernating squirrels. GPx was approximately 2-fold higher in liver, but unchanged in heart or brain tissue, of hibernators (Fig. 6.4). GR activity was approximately 25% lower in brain, but unchanged in other tissues of hibernating squirrels. In contrast, catalase activity was significantly higher in heart and brain of hibernators, and reduced in livers of hibernators (Fig. 6.4).

![Graphs showing activities of GPx, GR, and catalase in heart, brain, and liver of active and hibernating squirrels.]

**Fig. 6.4**: Glutathione peroxidase, glutathione reductase and catalase activities are unaltered during hibernation. (a) glutathione peroxidase; (b) glutathione reductase; and (c) catalase activities in heart, brain and liver of active and hibernating squirrels. Values are means ± SEM of duplicate measurements on 5-6 individuals per experimental group. ‘*’ = significantly different (P < 0.05).

**6.4 Discussion**
I tested the hypothesis that hibernating squirrels adapt to the stress of hibernation by upregulating antioxidant enzyme activities in highly oxidative tissues like heart, brain and liver. To address this hypothesis, I assessed antioxidant enzyme levels and activities during the summer period when the animals are active and during the hibernation season, when this species cycles repeatedly between torpid and aroused states. Few differences were found in the antioxidant enzymes measured between these two states, suggesting that no adaptive upregulation of antioxidant enzymes is sustained in the 13-lined ground squirrel during hibernation. These results contrast with previous reports of hibernation-induced effects on SOD activities in the ground squirrel *Citellus citellus* (Petrovic et al., 1986; Buzadzic et al., 1997). Petrovic et al. (1986) reported increased CuZnSOD activity in brain and brown adipose tissue (BAT), and lower MnSOD activity in liver and brain during hibernation. In contrast, Buzadzic et al. (1997) reported slightly lower total SOD activity in brain of hibernating versus active squirrels sampled in spring. Recently, Morin et al. (2008) reported that CuZnSOD levels in heart increase by up to 50% during entry into hibernation, but by three days in torpor have fallen to the levels equal to those in active squirrels. During arousal from this first period of torpor, CuZnSOD levels are reduced still further, to levels below those of active squirrels (Morin et al., 2008). The disparity between results from these different studies may thus be related to when during the hibernation season tissues are sampled. However, the current data, from torpid animals that had been hibernating for several months, indicate that, if upregulation of CuZnSOD or other antioxidant enzymes occurs early in the hibernation season, it is not maintained. Thus, squirrels arousing from a full season of hibernation, during which I-R
stress would be expected to be high, would not have enhanced antioxidative capacities in the major oxidative tissues.

It is important to note that both quantitative immunodetection and activity assays reported here provided essentially identical results. As neither SOD isoform is thought to be physiologically regulated post-translationally, a good agreement between the two methods of quantification is expected, as seen above. In addition, standardizing to CS activity allows the more meaningful value of MnSOD level/activity per mitochondrion to be obtained. The marginal increase in heart MnSOD activity observed in hibernators (Fig. 6.2) was thus apparently due to a slightly higher mitochondrial content (CS activity), and not an increase in MnSOD per unit mitochondrion. Increased MnSOD activities reported previously in, for example, C. citellus BAT (Buzadzic et al., 1990) may have reflected the proliferation of mitochondria in this thermogenic tissue during cold exposure (Milner et al., 1989; Barger et al., 2006) as there is no evidence that mitochondrial abundance was measured in C. citellus BAT.

Various reports have suggested that intracellular antioxidant activities might increase during the period of arousal from torpor (reviewed in Carey et al., 2003). Though this appears to be the case for blood-borne small molecule antioxidants, which may be imported into cells (see below), it is unlikely to include the intracellular antioxidant enzymes measured here. MnSOD and CuZnSOD are reported to be primarily transcriptionally regulated, and increasing enzyme copy number during arousal would be impractical. Profound changes in metabolic rate occur within the first hour of arousal in this species (Carey et al., 2003; Muleme et al., 2006), when body temperature is low and protein synthesis in brain (Frerichs et al., 1998) and liver (van Breukelen and Martin,
is severely suppressed. Therefore, it is unlikely that a rapid and transient upregulation of enzyme during this period of stress would be a practicable strategy. In rats, transcriptional upregulation of MnSOD in response to oxidative stress is not observed until six hours (Clerch et al., 1996) or more (Ho et al., 1996) post-stress. In addition hypoxia, which may occur during arousal (Ma et al., 2005), is actually associated with a down-regulation of MnSOD levels in rodent cells (Russell et al., 1995). It therefore seems likely that any burst of mitochondrial ROS production during arousal from torpor would already have occurred by the time sufficient MnSOD or CuZnSOD could be produced to counter it.

Although I found no evidence of enhanced intracellular SOD activities in hibernating squirrels, it is known that various antioxidants accumulate in plasma during hibernation and/or arousal. For example, extracellular SOD (ecSOD) (Okamoto et al., 2006; Akita et al., 2007) and catalase (Ohta et al., 2006) accumulate in plasma of the hibernating Syrian hamster (*Mesocricetus auratus*). Also, both *S. tridecemlineatus* (Drew et al., 1999) and the Arctic ground squirrel *S. parryii* (Drew et al., 1999; Tøien et al., 2001) accumulate ascorbate in plasma during hibernation, to levels several-fold above those in active animals. Osborne and Hashimoto (2007) showed in *M. auratus* that ascorbate in brain extracellular fluid is oxidized during arousal from torpor. Thus, increases both in oxidative stress and resistance to this stress appear to occur in the extracellular compartment during arousal. This may be as, or more, important than intracellular oxidative stress, and it is important to make the distinction between intracellular and extracellular oxidative stress. However, it is also possible that some extracellular small-molecule antioxidants move rapidly into cells during arousal. For
example, one route of ascorbate import is via glucose transporters (Liang et al., 2001). A rapid increase in glucose oxidation occurs during arousal (reviewed in Carey et al., 2003), suggesting increased transporter abundance within cell membranes, which might facilitate a rapid import of ascorbate to combat oxidative stress within cells. Thus, small molecule and/or extracellular antioxidants may play an important role during the torpor-arousal transition, and these may provide oxidative stress resistance in the absence of increases in SODs or other intracellular antioxidant enzymes.

Only relatively subtle changes in GPx and GR were observed in hibernators, and these were confined to liver and brain, respectively. The almost 2-fold increase in liver GPx activity measured here was similar to that reported in *Citellus citellus* (Buzadzic et al., 1990). However, this was a tissue-specific phenomenon, with no similar increase in brain or heart tissue, suggesting no role for GPx in countering oxidative stress in brain tissue. Also similar to Buzadzic et al. (1990), I observed a significant decrease in liver catalase activity during hibernation, though interestingly it was higher in both brain and heart tissue of hibernators. It is difficult to interpret these changes in catalase activity exclusively as pre-adaptations to potential oxidative stress, as peroxisomal proliferation may have occurred during hibernation. Peroxisomes are an important site of lipid oxidation (Wanders, 2004), and torpid ground squirrels are highly reliant on the metabolism of lipid fuels (Carey et al., 2003). Nonetheless, higher catalase activities could increase oxidative stress resistance of heart and brain tissue during hibernation.

Thus, taken together, my results do not support a major role for either intracellular isoform of SOD, nor for GPx or GR in augmenting oxidative stress resistance of heart or brain tissue during hibernation in 13-lined ground squirrels. A role for catalase is
possible. Additionally, other antioxidant enzymes or small molecule antioxidants, may play more important roles. For example, Eddy et al. (2005) identified a putative thioredoxin peroxidase that is upregulated during hibernation in heart tissue of squirrels (Morin and Storey, 2007) and bats (Myotis lucifugus).

The central thesis underlying the expectation that upregulation of tissue antioxidant enzymes occurs during hibernation has been that the profound shifts in tissue perfusion and metabolic activity between torpor and arousal would create transiently hypoxic or anoxic conditions that might enhance mitochondrial ROS production during both the hypoxic event and following reperfusion. In this context, it is interesting that liver (Lindell et al., 2005), intestinal (Fleck and Carey, 2005; Kurtz et al., 2006) and brain (Dave et al., 2006) tissue all show enhanced resistance to I-R injury during hibernation in squirrels. Similarly, Ma et al. (2005) demonstrate an absence of oxidative damage in brain tissue of arousing Arctic ground squirrels (Ma et al., 2005), though the level of malondialdehyde, a marker of oxidative lipid damage, is elevated two-fold in liver mitochondria isolated from hibernating S. tridecemlineatus compared to summer active controls (Gerson et al., 2008). My data indicate for liver, brain and heart that a global upregulation of intracellular antioxidant enzymes does not mediate this phenotype. It will be interesting to identify what other possible adaptive mechanisms confer tissue stress resistance during hibernation.

Given that the levels/activities of intracellular antioxidants during hibernation do not appear to change, one alternative explanation for the apparent absence of oxidative stress and damage during the torpor-arousal transition is that the I-R associated ROS production that occurs under these conditions in non-hibernators may simply be avoided
in hibernators, either by avoiding profound tissue hypoxia or by tolerating it without initiation of aberrant ROS production. In Arctic ground squirrels, the torpid state is associated with both high arterial PO$_2$ and low hypoxia inducible factor-$\alpha$ (HIF-$\alpha$) expression (Ma et al., 2005). Plasma hypoxemia occurs during arousal from torpor, and tissue HIF-$\alpha$ levels increase during this period, but only to levels seen in active animals. Thus, evidence supporting the idea that profound tissue hypoxia occurs during torpor and/or arousal is limited. Even if transient hypoxia did occur in tissues of hibernators, it may not elicit the same burst of mitochondrial ROS that occurs in non-hibernators (Duranteau et al., 1998; Chandel et al., 1998). In other mammals, mitochondrial superoxide production during hypoxia appears to be mediated primarily by respiratory complex III (Guzy and Schumacker, 2006). Perhaps differences in complex III structure and/or function in hibernators allow them to avoid this. However in daily torpor, a phenotype similar to hibernation, flux through complex III is not significantly reduced in liver mitochondrial isolated from torpid *Phodopus sungorus* compared with active, normothermic controls (Brown et al., 2007). Studies of the effects of hypoxia on cultured squirrel cells may provide insight into whether they respond similarly to those of rats to a transient hypoxia mimicking I-R.
Chapter 7. Contribution of intracellular antioxidant enzymes to stress resistance during estivation

Hypothesis

Intracellular antioxidant enzymes have a role in conferring stress resistance in tissues isolated from animals in quiescent states.

Objective

The primary objective of this project was to determine if intracellular antioxidant enzymes play a role in cellular stress resistance observed in highly oxidative tissues isolated from estivating slender lungfish compared to their respective active controls.

Publications of results


Contributions

I performed all antioxidant enzymes experiments, statistical analysis and chapter write-up.
7.1 Introduction

African lungfish (genus *Protopterus*) are considered bimodal breathers; however in the adults gills are degenerated and these individuals rely upon their lungs for gas exchange (see Burggren and Johnson, 1986). This capacity for pulmonary breathing allows lungfish to survive without oxygenated water, and they have been reported to survive drought for periods as long as several years by estivating (Smith, 1931). To estivate, lungfish burrow into the mud, encapsulate themselves within a cocoon of mud and secreted mucus, and assume an inactive state characterized by low metabolic rate. While estivating, the fish are resistant to transient hypoxia, high temperatures (above 40°C), and a variety of other environmental stresses (Smith, 1931).

A similarly enhanced stress resistance is observed in other species capable of a regulated and prolonged hypometabolic state. The diapausing *Caenorhabditis elegans* (dauer larva; reviewed in Honda and Honda, 2002) is resistant to a wide range of environmental stresses. Recent studies have shown also that brain and liver tissues of hibernating squirrels are characterized by increased oxidative stress resistance (Lindell et al., 2005; Dave et al., 2006; Christian et al., 2008). An adaptive response to oxidative stress often includes increased synthesis and/or activity of intracellular antioxidant enzymes, which can protect cellular macromolecules from potentially lethal stress-induced damage. Overexpression of mitochondrial (MnSOD) or cytosolic (CuZnSOD) superoxide dismutase both increase oxidative stress resistance (Murakami et al., 1997; Shan et al., 2007; Jang et al., 2009). Overexpression of the superoxide dismutases (SODs), glutathione peroxidase (GPx) and catalase (CAT) also act to protect against ischemia associated cytochrome c release from mitochondria, thereby limiting the
occurrence of apoptotic cell death that can occur in species that are not particularly tolerant to oxidative or other stressors (Zemlyak et al., 2009). Taken together, these data indicate that intracellular antioxidant enzymes can effectively mediate cellular stress resistance.

Several authors (e.g., Hermes-Lima and Zenteno-Savín, 2002; Carey et al., 2003) have suggested that upregulation of intracellular antioxidant enzymes during estivation and hibernation protects against stress-related cellular injury. Indeed, there is some evidence for this in estivating pulmonate land snails (Hermes-Lima and Storey, 1995; Ramos-Vasconcelos and Hermes-Lima, 2003). Conversely, I found no major role for either intracellular SOD isoform, nor for GPx and glutathione reductase (GR) in augmenting oxidative stress resistance in heart and brain tissues of hibernating 13-lined ground squirrels (Spermophilus tridecemlineatus; Chapter 6), which is inconsistent with the above hypothesis for a role of intracellular antioxidant enzymes to confer enhanced stress resistance during periods of estivation and hibernation of increased risk of oxidative damage during.

To further characterize the changes that occur to the antioxidant and oxidative stress profile during metabolic depression, I have investigated antioxidant capacity in estivating P. dolloi. I focused the study on heart and brain, tissues that are highly sensitive to oxidative damage in most animal species. Measurements of antioxidant enzyme levels and/or activities were performed in lungfish that had been: (1) estivating for 60d, (2) food deprived for 60d, or (3) active and fed over this period. Interestingly I found that estivation evoked a marked upregulation of the major antioxidant enzymes in
\textit{P. dolloi}, in addition there was little evidence for the accumulation of oxidative damage during estivation.

\textbf{7.2 Experimental Procedures}

\textbf{7.2.1 Materials}

Chemicals were purchased from Bioshop (Burlington, ON, Canada) and Sigma-Aldrich (Oakville, ON, Canada; including Fluka and Caledon). BioRad protein dye was purchased from BioRad Laboratories (Hercules, CA, USA). Prestained broad range protein marker was obtained from BioLabs (New England, MA, USA). Memcode reversible protein stain kit was from Pierce Biotechnology (Rockland, IL, USA). The CuZnSOD antibody was purchased from Abcam (Cambridge, MA, USA) and the antibody to MnSOD was purchased from Stressgen Biotechnologies (Assay Designs, Ann Arbor, MI, USA). Antibodies to 4-hydroxynonenal and nitrotyrosine were obtained from R and D Systems (Minneapolis, MN, USA). Infrared dye-conjugated secondary antibodies were purchased from Rockland Immunochemicals (Gilbertsville, PA, USA). Reagents for the protein carbonyl assay were purchased from Cayman Chemicals (Ann Arbor, MI, USA).

\textbf{7.2.2 Animals}

African lungfish (\textit{P. dolloi}) were obtained from a fish supplier in Singapore and shipped to the University of Guelph Hagan Aqualab (Guelph, ON, Canada) where they were kept as previously described. Briefly, fish were kept in freshwater at 25\degree C under 12L:12D artificial photoperiods and fed a diet of frozen blood worms (\textit{Chironomidae} larva) and trout chow pellets (Martin Feeds, Elmira, ON, Canada) for 12 months prior to the commencement of the experiment. A complete description of the experimental setup.
is in Frick et al. (2008a,b). Briefly, lungfish (58.8 ± 4.7 g) were divided into three treatment groups (N=10 for each treatment); 1) control, held in water and fed every other day a diet of frozen blood worms (*Chironomidae* larva) and trout chow pellets, 2) estivated, and 3) fasted, held in water without food for the duration of the experiment (60d). Estivation was stimulated following the method described by Delaney et al., (1974). Briefly, lungfish were placed in a muslin sack with the head oriented toward the top of the sack. The sack was suspended inside a container filled with water. The water was drained slowly (1 cm/day) from the container until the sack became completely dry (5-7 days). Lungfish were considered estivating once they formed a complete cocoon. They lived in the absence of water for the duration of the experiment (60d).

### 7.2.3 Tissue collection and homogenization

Tissue was collected as described previously in Frick et al. (2008a,b). Briefly, fish were killed by a blow to the head following which heart and brain were excised, flash-frozen in liquid nitrogen and stored at -80°C. Heart and brain tissue from control, estivating and fasted lungfish were homogenized in homogenization buffer (10 mM KH$_2$PO$_4$ (pH 7.4), 20 mM EDTA, 30 mM KCl, 0.1% Triton X-100, 10% glycerol). Homogenization was performed at 4°C with a PowerGen 125 homogenizer (Fisher Scientific, Ottawa, ON, Canada) on full speed for 10 s for 3 cycles with 10 s rests between each cycle. Samples were centrifuged for 10 min at 500 g (4°C). Protein concentrations of the supernatants were measured using the Bradford technique with a BioRad protein kit. Homogenates were stored at -80°C.
7.2.4 Western blots

i) Superoxide dismutases - Heart homogenate (30 μg protein) and brain homogenate (20 μg protein) were separated by SDS-PAGE and electrotransferred to a PVDF membrane. Membranes were stained with Memcode reversible protein stain to ensure equal and uniform transfer. Membranes were incubated in blocking solution (5% skim milk in 1X PBS) for 45 min. Following blocking, membranes were incubated with anti-CuZnSOD (1:1000) or anti-MnSOD (1:1000) overnight in blocking buffer (5% skim milk in 1X PBS-T) at 4°C. Following several washes in blocking buffer, membranes were incubated with anti-rabbit secondary antibodies for one hour at room temperature. Membranes were washed in PBS and visualized using the Odyssey infrared imaging system from LI-COR Biosciences (NE, USA). Protein level quantification was done using an internal standard and the Odyssey imaging system software, version 1.0.

ii) Oxidative damage - Heart and brain homogenates (80 μg) were resolved and treated as above. Following blocking, membranes were incubated with anti-4-hydroxynonenal (1:500) or anti-nitrotyrosine (1:500) overnight in blocking buffer at 4°C. Following washing, membranes were incubated with anti-mouse secondary antibody for one hour at room temperature. 4-hydroxynonenal BSA (10 μg; Cedarlane Laboratories; Burlington, ON, Canada) or nitrotyrosine BSA (50 ng; Cayman Chemicals; Ann Arbor, MI, USA) were included as positive oxidative damage controls. Prestained broad range protein markers (BioLabs; NE, USA) were also resolved on each gel. For 4-hydroxynonenal, the single most prominent band was ~62 kDa, and this signal was quantified. For nitrotyrosine a single prominent band of ~43 kDa was quantified. Quantification involved the use of a single standard loaded at different concentrations to establish a standard curve from which values were determined by interpolation. Membranes were
visualized using the Odyssey infrared imaging system from LI-COR Biosciences (NE, USA) and protein quantification used the Odyssey imaging system software, version 1.0.

7.2.5 In-gel superoxide dismutase assay
The activities of MnSOD and CuZnSOD were measured as in Chapter 3.

7.2.6 Other enzyme assays
All other enzyme assays were measured as in Chapter 3.

7.2.7 Protein Carbonyl assay
Protein homogenates were treated with either 2,4-dinitrophenylhydrazine (DNPH) or 2.5M hydrochloric acid as a control for 1 h in the dark. 20% TCA was added to each solution, centrifuged at 10000 g for 10 min at 4°C and the supernatant was discarded. This step was repeated with 10% TCA. The pellet was washed twice with an ethanol/ethyl acetate mixture, resuspended and centrifuged at 10000 g for 10 min at 4°C. After the final wash, the pellet was resuspended in guanidine hydrochloride and centrifuged at 10000 g for 10 min at 4°C. Absorbance of the supernatant was measured in a 96-well plate at a wavelength of 375 nm using a Bio-Tek Power Wave plate reader spectrophotometer.

7.2.8 Statistical analysis
Within-tissue comparisons between protein levels and/or activities between control, estivating, and fasted lungfish were completed with ANOVA using Systat v.12. Post-hoc comparisons between means were done by Sidak’s significant differences method. Data is presented as means ± standard error of the mean (SEM). A p-value of <0.05 was considered significant.

7.3. Results
For immunodetection of MnSOD and CuZnSOD, membranes were stained with Memcode to ensure equal loading and transfer (not shown). MnSOD and CuZnSOD subunits were immunodetected in both heart and brain tissues of the control, estivating and fasted lungfish (Fig. 7.1 shows brain). MnSOD protein levels in heart tissue did not differ between control, estivating and fasted animals.

Fig. 7.1: Representative Western blot showing MnSOD and CuZnSOD levels in brain tissue from control, fasted and estivating lungfish.

However, MnSOD is localized exclusively to the mitochondrial matrix. Frick et al. (2008b) found that the activities of two other mitochondrial enzymes, citrate synthase and cytochrome c oxidase (CCO) are reduced in estivating P. dolloi, which suggests that mitochondrial volume density of heart tissue is reduced in estivators. Standardization of brain MnSOD to brain CS activity indicates that MnSOD/CS is elevated in estivators (Fig. 7.2b). However, it is not known whether CS and CCO are accurate proxies of mitochondrial volume density in estivating lungfish, particularly as the activities of several other mitochondrial enzymes are unchanged during estivation in P. dolloi (Frick et al., 2008b). In brain tissue, MnSOD protein levels were elevated more than three-fold in estivators compared to either control or fasted animals (Fig. 7.2a), and no differences
in CS activity were seen between control, food deprived and estivating lungfish (Fig. 7.2c shows brain). CuZnSOD protein levels, were not different between experimental groups in heart tissue but were elevated in brain tissue of estivators (Fig. 7.2d).

Fig. 7.2: MnSOD and CuZnSOD protein levels in heart and brain tissue of lungfish. (a) MnSOD protein levels in heart and brain tissues of control, estivating and fasted lungfish. (b) Brain MnSOD activity corrected for mitochondrial abundance (citrate synthase activity). (c) Citrate synthase activity for heart and brain tissues of control, estivating and fasted lungfish. (d) CuZnSOD protein levels in heart and brain tissues of control, estivating and fasted lungfish. Open bars = control; hatched bars = estivating, filled bars = fasted. All values are means ± SEM of duplicate measurements made on 7-10 individual animals. * = significantly different (p < 0.05).

MnSOD and CuZnSOD protein levels typically parallel the respective enzyme activities, since neither isoform has been reported to be physiologically regulated by post-translational modification. However, MnSOD can be inactivated by high levels of
reactive nitrogen species (RNS) (Bayir et al., 2007; Demicheli et al., 2007) and CuZnSOD by high levels of RNS or reactive oxygen species (ROS) (Hodgson and Fridovich, 1975; Alvarez et al., 2004), so that protein levels could misrepresent in vivo SOD activities. I thus attempted to measure the activities of both SODs. Native-gel electrophoresis was used to separate MnSOD and CuZnSOD, and KCN sensitivity was used to distinguish the two enzymes, as this compound inhibits CuZnSOD but not MnSOD (Fig. 7.3a shows heart). Unfortunately, CuZnSOD did not resolve as a discrete band under a variety of conditions and thus CuZnSOD activity could not be quantified accurately (Fig. 7.3a). MnSOD activity, however, was easily quantifiable, and was elevated in estivators compared to both control and fasted animals (Fig. 7.3b).

Fig. 7.3: MnSOD protein activities in heart and brain of lungfish. (a) Representative gel showing the identification of MnSOD and a diffuse CuZnSOD signal. Gel also shows SOD standards (bovine heart CuZnSOD) and inhibition of CuZnSOD with KCN. (b) MnSOD protein activity in heart and brain tissues of control, estivating and fasted lungfish. Open bars = control; hatched bars = estivating, filled bars = fasted. All values are means ± SEM of single measurements on 7-10 individual animals. * = significantly different (p < 0.05) and † = significantly different (p < 0.01).
Interestingly, the elevation of MnSOD activity in estivators was not as great as the
elevation of MnSOD protein level, which may suggest some inactivation of the protein
during estivation.

\[ \text{H}_2\text{O}_2 \text{ detoxification was assessed by measuring the activities of CAT and the two} \]
\[ \text{enzymes that make up the glutathione cycle; GPx and GR. CAT activity was} \]
\[ \text{significantly higher in heart and brain tissues of estivating lungfish compared to both} \]
\[ \text{control and fasted animals (Fig 7Aa). GPx activity was significantly elevated in heart} \]
\[ \text{and brain tissue during estivation (Fig. 7Ab). In the latter tissue, starvation also induced} \]
\[ \text{an elevation of GPx relative to control animals. GR activity appeared to be elevated in} \]
\[ \text{heart tissue of estivating lungfish compared to control and fasted animals, however this} \]
\[ \text{did not reach statistical significance. Conversely, GR activity was significantly higher in} \]
\[ \text{brain tissue isolated from estivating lungfish compared to control and fasted animals (Fig} \]
\[ 7.4c).} \]

Upregulation of antioxidants in heart and brain tissues during estivation may be
secondary to the accrual of oxidative damage while in the hypometabolic state or it may
represent an adaptive defence against oxidative damage that could occur during arousal.
Therefore the levels of several biomarkers of oxidative and nitrative damage in heart and
brain tissues were measured: protein nitrotyrosine, protein carbonyls and 4-
hydroxynonenal (HNE) adducts.
Fig. 7.4: Catalase, glutathione peroxidase and glutathione reductase protein activities measured in heart and brain of lungfish. (a) Catalase; (b) glutathione peroxidase; and (c) glutathione reductase in heart and brain of control, estivating and fasted lungfish. Open bars = control; hatched bars = estivating, filled bars = fasted. All values are means ± SEM of duplicate measurements made on 6-10 individual animals. * = significantly different (p < 0.05) and ‡ = significantly different (p < 0.01).

Nitrotyrosine adducts were elevated in brain tissue of estivators compared to control and fasted lungfish (Fig. 7.5a). In contrast, protein carbonyl levels were not higher in estivators compared to control and fasted lungfish, though there was a marginal, non-significant trend in this direction in both heart and brain tissues (Fig. 7.5b). 4-hydroxynonenal, a highly reactive aldehyde formed during the peroxidation of polyunsaturated fatty acids reacts non-enzymatically with histidine, cysteine, and lysine to form stable protein adducts, that can be immunodetected. There was no statistically significant increase in 4-hydroxynonenal protein adducts in heart or brain tissues of estivators (Fig. 7.5c).
Fig. 7.5: Levels of oxidative damage in heart and brain tissues of control, estivating and fasted lungfish. (a) Relative nitrotyrosine adducts in heart and brain of control, estivating and fasted lungfish. Values are means ± SEM of duplicate measurements made on 4-10 individual animals. * = significantly different (p < 0.05). (b) Protein carbonyl damage measured in the heart and brain of control, estivating and fasted lungfish. Values are means ± SEM of duplicate measurements made on 4-5 individual animals. (c) Relative 4-hydroxynonenol adducts in heart and brain of control, estivating and fasted lungfish. Values are means ± SEM of a single measurement made on 4-10 individual animals.

7.4 Discussion

Enhanced systemic and/or cellular stress resistance has been described in multiple animal species entering quiescent states such as diapause (Honda and Honda, 2002), hibernation (e.g. Dave et al., 2006; Christian et al., 2008), and estivation (Smith, 1930; Smith, 1931). Several authors (e.g. Hermes-Lima and Zenteno-Savín, 2002; Carey et al., 2003) have suggested that this cellular resistance is mediated in part by the upregulation of antioxidant enzymes, possibly to ameliorate macromolecular damage from ROS.
production during arousal from torpor. I tested the hypothesis that the African lungfish *P. dolloi* adaptively enhances antioxidant capacity during estivation. I found that most of the intracellular antioxidant enzyme levels and/or activities were indeed significantly upregulated in brain and, to a lesser extent heart tissue. These increases in antioxidant activities would be expected to greatly enhance the cellular stress resistance of estivating lungfish, based on studies in which the overexpression of individual antioxidant enzymes has conferred substantial protection against various physiological stressors (see, for example, Murakami et al., 1997; Solaini and Harris, 2005; Shan et al., 2007; Zemlyak et al., 2009). This would be expected to make *P. dolloi* more tolerant of stresses associated with estivation, such as transient hypoxia, hyperthermia and dehydration.

Antioxidant capacity has been measured in other estivators. Most of these studies have focused upon terrestrial snails, and I am aware of just two studies of vertebrate species. The results of these invertebrate and vertebrate studies are quite varied, however, with no strong pattern of change in antioxidant enzyme capacities during estivation (summarized in Table 7.1). It is obvious that changes in the activity of any individual antioxidant enzyme during estivation are highly dependent upon species, tissue and season. For example, total cellular superoxide dismutase activity in snail hepatopancreas is increased during estivation by 25% in *Otala lactea*, reduced 39% in *Helix aspersa* when the experiment is done during the summer, and unchanged in *H. aspersa* when the experiment is performed during the winter. From these data it is difficult to conclude that estivating snails adaptively upregulate SOD activity to enhance their tolerance to the stress of estivation and arousal. Some differences in measured SOD activity may be due to methods used to quantify the enzymes. Measurements in crude
tissue homogenates will be influenced by MnSOD, CuZnSOD and possibly other SOD enzymes as well as small molecule antioxidants (see Chapter 6). In contrast, separate measurement of MnSOD and CuZnSOD using electrophoretic separation, in combination with immunodetection of the individual enzymes, is likely to provide more information regarding physiological adaptations with respect to these enzymes (Chapter 6).

A similar absence of an identifiable pattern is evident for other antioxidant enzymes, and this variability in the response to estivation is apparent also in vertebrate species (Table 7.1). For example, CAT, GPx and GR activities are each observed to either increase or decrease during estivation, depending upon the species, tissue, or the season during which the experiment is performed. In contrast, the data for *P. dolloi* indicate a relatively substantial, broad and global upregulation of antioxidant enzymes during estivation. This is certainly consistent with the hypothesis of adaptive upregulation of defences against ROS; however, given the broad range of results reported in the literature it is not possible to conclude that this is typical of all estivators. Differences between findings may be dependent upon how measurements were made (e.g. total SOD activity versus individual MnSOD and CuZnSOD quantification), the duration of estivation (which varies from ~15d to 180d), or they may simply reflect differences in how particular species adapt to the metabolic stress of estivation. Antioxidant enzymes represent only one facet of the cellular adaptive response that can be recruited in the adaptation to environmental stress (Gracey and Cossins, 2003; Kültz, 2003).

Several biomarkers of lipid and protein oxidative and nitrative damage were measured to determine whether estivation is associated with an accumulation of such damage in cellular macromolecules. There was little evidence of oxidative damage in
heart or brain tissue of estivators, compared to active or food deprived fish. There was, however, a significant increase in protein nitrotyrosine in brain tissue of estivators. Nitrotyrosine is produced from tyrosine nitration that can be caused by peroxynitrite, a product of the reaction of superoxide with nitric oxide. It is thus interesting to note that nitric oxide synthase (NOS) is upregulated during estivation in some tissues of *P. dolloi* (Amelio et al., 2009). Although no data has been reported for brain, it is possible that increased NOS activity in this tissue leads to elevated NO levels and increased peroxynitrite production. Indeed, the upregulation of SODs would provide a means of minimizing peroxynitrite formation in this case.

Two other markers of oxidative damage in lipids (HNE) and protein (carbonyls) were not elevated during estivation in *P. dolloi*. It is difficult to compare my data to those from other studies of estivation, because these latter results range almost as widely as those published for antioxidant status. Two studies have reported elevated lipid peroxidation during estivation in tissues of toads and terrestrial snails (Grundy and Storey, 1998; Ramos-Vasconcelos and Hermes-Lima, 2003). However, a third study has reported no change in lipid peroxidation in tissues of estivating snails (Ramos-Vasconcelos et al., 2005). Similarly, one study has reported elevated levels of protein carbonylation in some tissues during estivation (Ramos-Vasconcelos and Hermes-Lima, 2003), while another found no difference (Ramos-Vasconcelos et al., 2005). Taken together, it is difficult to draw broad conclusions from the published data, given the apparent dependence of the results upon such variables as species, tissue, duration of estivation, and season of sampling. I found only limited evidence that *P. dolloi* experienced oxidative stress during estivation: protein carbonyls may have shown a
general trend of elevation, but this was not statistically significant. Only the elevation of nitrotyrosine in brain tissue of estivators was significant. However, perhaps the substantial upregulation of antioxidant capacity in this species facilitates the avoidance of oxidative macromolecular damage during estivation.

Although the results from this study support the hypothesis that intracellular antioxidant enzymes are upregulated during estivation to protect against oxidative stress, the absence of consistency between studies limits the strength of this conclusion. If I extend the comparison to hibernation, the variability between results is possibly even greater. *H. pomatia* exhibits a dramatic upregulation (up to 350%) of antioxidant enzymes in several tissues, similar in magnitude and breadth to that observed in estivating *P. dolloi*, during winter torpor (Nowakowska et al., 2009). In contrast, I found that intracellular antioxidant enzymes were not globally upregulated in liver, heart and brain tissues of hibernating 13-lined ground squirrels (*Spermophilus tridecemlineatus*; Chapter 6). Also, the levels of these enzymes in tissues of *S. tridecemlineatus* are comparable to those in mammalian species that do not hibernate (Chapter 3), indicating that antioxidant enzyme levels are not constitutively high in hibernating species. In hibernating Arctic ground squirrels (*S. parryii*) cellular stress was absent during arousal (Ma et al., 2005). On the other hand, oxidative damage occurs in intestinal tissue during hibernation of *S. tridecemlineatus* (Carey et al., 2000). Taken together these results suggest simply that the occurrence of oxidative stress during quiescence and/or mechanisms of adaptation to it are highly species and tissue specific. Oxidative stress resistance is often a component of ‘multiplex’ stress resistance (Miller, 2009) that does not appear to be mediated simply by upregulation of antioxidant enzyme levels. Rather,
the mechanisms by which multiplex stress resistance is achieved are likely to include a wide range of molecular adaptations (see, for example, Kültz, 2003), with specific adaptations differing between species, tissue and physiological state.
Table 7.1: Changes in antioxidant expression and/or activity during estivation.

<table>
<thead>
<tr>
<th>Species</th>
<th>Estivation Duration</th>
<th>Tissue</th>
<th>Superoxide Dismutase</th>
<th>Catalase</th>
<th>Glutathione Peroxidase</th>
<th>Glutathione Reductase</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invertebrates (snails)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Octala lactea</em></td>
<td>30d</td>
<td>hepatopancreas</td>
<td>+25%*</td>
<td>-10%</td>
<td>+150%*</td>
<td>-25%</td>
<td>Hermes-Lima &amp; Storey 1995</td>
</tr>
<tr>
<td></td>
<td></td>
<td>foot muscle</td>
<td>+60%*</td>
<td>+90%*</td>
<td>-10%</td>
<td>-5%</td>
<td></td>
</tr>
<tr>
<td><em>Helix aspersa</em></td>
<td>20d</td>
<td>hepatopancreas</td>
<td>-16%</td>
<td>NC</td>
<td>+491%*</td>
<td>+29%</td>
<td>Ramos-Vasconcelos &amp; Hermes-Lima 2003</td>
</tr>
<tr>
<td>(winter)</td>
<td></td>
<td>foot muscle</td>
<td>-9%</td>
<td>+48%*</td>
<td>+298%*</td>
<td>+25%</td>
<td></td>
</tr>
<tr>
<td><em>H. aspersa</em></td>
<td>20d</td>
<td>hepatopancreas</td>
<td>-39%*</td>
<td>-32%*</td>
<td>+368%*</td>
<td>-16%</td>
<td>Ramos-Vasconcelos et al. 2005</td>
</tr>
<tr>
<td>(summer)</td>
<td></td>
<td>foot muscle</td>
<td>-14%</td>
<td>-40%*</td>
<td>+32%</td>
<td>+13%</td>
<td></td>
</tr>
<tr>
<td><em>Biomphalaria tenagophila</em></td>
<td>15d</td>
<td>hepatopancreas</td>
<td>-42%*</td>
<td>-10%</td>
<td>+14%*</td>
<td>+24%</td>
<td>Ferreira et al. 2003</td>
</tr>
<tr>
<td>Vertebrates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Protopterus dolloi</em></td>
<td>60d</td>
<td>heart</td>
<td>NC (MnSOD)</td>
<td>+85%*</td>
<td>+20%*</td>
<td>+300%*</td>
<td>Present study</td>
</tr>
<tr>
<td>(African lungfish)</td>
<td></td>
<td>brain</td>
<td>NC (CuZnSOD)</td>
<td>+300%*</td>
<td>+120%*</td>
<td>+225%*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+50%* (MnSOD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Scaphiopus couchii</em></td>
<td>60d</td>
<td>liver</td>
<td>+100%*</td>
<td>-35%*</td>
<td>-53%*</td>
<td>-65%*</td>
<td>Grundy &amp; Storey 1998</td>
</tr>
<tr>
<td>(spade foot toad)</td>
<td></td>
<td>heart</td>
<td>-55%*</td>
<td>-30%*</td>
<td>NC</td>
<td>-33%*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>kidney</td>
<td>-30%*</td>
<td>+55%*</td>
<td>+100%*</td>
<td>-40%*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>muscle</td>
<td>+30%*</td>
<td>+7%</td>
<td>+25%</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td><em>Cyclorana alboguttata</em></td>
<td>180d</td>
<td>muscle</td>
<td>-70%*</td>
<td>+32%</td>
<td>-33%</td>
<td></td>
<td>Hudson et al. 2006</td>
</tr>
<tr>
<td>(green-striped burrowing frog)</td>
<td></td>
<td>(mRNA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*” = statistically significant difference (P < 0.05). NC = no change. n.d. = not determined.
Chapter 8. General discussion

Cellular stress resistance appears to be a key correlate of lifespan. This correlation in fact, holds true in both inter-specific (Kapahi et al., 1999) and intra-specific (Salmon et al. 2005; Labinskyy et al. 2006; Harper et al. 2007) contexts as well as within both whole animal (Bokov et al., 2009) and cellular levels (Kapahi et al., 1999; Salmon et al. 2005; Labinskyy et al. 2006; Harper et al. 2007). This suggests that longer-lived species and/or strains have enhanced protection due to improved maintenance of cellular homeostasis. Stress resistance and lifespan appear to be influenced by the IIS pathway (reviewed by Longo and Fabrizio, 2002), specifically through the activity of FOXO transcription factors that regulate the expression of genes whose protein products are associated with enhanced stress resistance, including intracellular antioxidant enzymes and DNA damage response genes (reviewed by Carter and Brunet, 2007). Reduced IIS has been associated with increased cellular stress resistance in a variety of contexts, including 1) multi-species comparisons of mammals, in which increased body mass and/or MLSP correlate negatively with plasma IGF-1 levels (Chapter 2) and positivity with cellular stress resistance (Kapahi et al., 1999); 2) various intra-species models of increased longevity, including Snell dwarf mice (Murakami et al., 2003; Salmon et al., 2005; Maynard and Miller, 2006); and 3) hibernating mammals (Dave et al., 2006; Christian et al., 2008).

IIS and antioxidant enzymes in stress resistance and longevity

As outlined above, a potential mechanism for enhancing cellular stress resistance and increasing longevity may be increasing intracellular antioxidant enzyme capacities. To evaluate this, intracellular antioxidant enzyme protein levels and/or activities were
determined in animal species, strains or physiological states associated with enhanced stress resistance and increased longevity. These measurements were made in: 1) dermal fibroblasts propagated in vitro; 2) brain tissue; 3) heart tissue; and 4) liver tissue. Interestingly, there was limited evidence that any of the five major intracellular antioxidant enzymes play a role in conferring stress resistance and/or longevity. Positive correlations were observed between brain MnSOD and CAT activity with MLSP of 14 vertebrate endotherms, as well as the enhanced response of GPx and CAT activity in dermal fibroblasts isolated from Snell dwarf mice following exposure to oxidative stress. Antioxidant enzyme activities were broadly upregulated during estivation in the lungfish. Taken together, however, these associations represented exceptions rather than rules, as in the vast majority of instances there was little or no association between cellular stress resistance and antioxidant enzyme capacity.

Numerous mouse strains with a genetically disrupted IIS pathway have shown connections between reduced IIS and upregulation of MnSOD activity (Baba et al., 2005; Yamamoto et al., 2005). In addition, MnSOD protein level is increased during CR, via FOXO3a deacetylation (Wang et al., 2007). In contrast, the three IIS models that were used in the above studies (Chapter 3; 5; and 6) did not reveal a broad upregulation of MnSOD protein and/or activity in all tissues. The discrepancy between studies may arise from the fact that, in some instances MnSOD ‘activity’ has actually been inferred from mRNA levels, which may not reflect MnSOD protein level or activity (Gygi et al., 1999; Kops et al., 2002; Nemoto and Finkel, 2002). In fact mammalian MnSOD protein synthesis can be translationally regulated via MnSOD mRNA binding protein (MnSOD-BP; Knirsch and Clerch, 2001). In addition increased MnSOD protein level or activity
may not reflect enhanced protection but rather be simply the result of increased mitochondrial biogenesis. FOXO transcription factors also activate the expression of peroxisome proliferator-activated receptor (PPARγ) coactivator 1α (PGC-1α) (Corton and Brown-Borg, 2005), which regulates mitochondrial and peroxisomal biogenesis (Schrader and Yoon, 2007). Thus, synthesis of a broad range of mitochondrial proteins may be upregulated under conditions in which MnSOD induction has been reported, such that MnSOD levels per mitochondrion do not change.

There are numerous other intracellular antioxidant enzymes that were not investigated in this thesis, but are known to be regulated by the activity of FOXO transcription factors, and any of these could play a more prominent role in stress resistance and longevity. For example loss of peroxiredoxin 1, which is regulated by FOXO transcription factors, reduces lifespan in mice (Neumann et al., 2003; Chiribau et al., 2008; Lee et al., 2009). Similarly, evidence also suggests a regulatory role of FOXOs for thioredoxin 1 (de Candia et al., 2008) which may influence murine lifespan (Mitsui et al., 2002).

**DNA repair in stress resistance and longevity**

Enzymes involved in the repair of oxidative DNA damage act as a second line of defense and therefore may also potentially underlie cellular stress resistance and animal lifespan. FOXO transcription factors upregulate the expression of the DNA response protein GADD45 (Tran et al., 2002), which has been shown to have roles in both nucleotide excision repair (NER) and base excision repair (BER; Maeda et al 2005; Jung et al., 2007). This suggests that BER may have a role in enhancing stress resistance and longevity. The activities of APE and polβ, two rate-limiting BER enzymes was
measured within intra-specific and inter-specific contexts. APE and polβ activities were elevated in Snell dwarf tissue compared to normal littermates, suggesting a potential role in the enhanced cellular stress resistance and longevity of these mice. Interestingly, when dermal fibroblasts from Snell normal and dwarf mice were cultured under basal conditions no difference in APE and polβ activity was observed between Snell and normal mice. However, when the dermal fibroblasts were exposed to exogenous oxidants APE and polβ activity were elevated within Snell dwarf mouse dermal fibroblasts compared to normal littermates. Taken together these results are supportive of the hypothesis that BER plays a role in increasing cellular stress resistance in the long-lived Snell dwarf mice.

In contrast, however, neither APE activity nor polβ activity correlated with species MLSP in an inter-specific context. In fact, polβ activity was inversely correlated with species body mass. Lower BER activity in larger animals may result from lower mitochondrial abundance and reduced intracellular ROS production observed in larger animals (Porter and Brand, 1995; Ku et al., 1993) which is supported by lower levels of oxidative damage in nuclear DNA is larger animals (Barja and Herrero, 2000). In addition the activities of protein repair and/or maintenance enzymes are also reduced in larger animals (Salway et al., 2010). polβ activity however may also correlate negatively with species body mass due to the lower level of mitotic cells in larger animals. Interestingly, plasma IGF-1 levels (Chapter 2) and telomerase activity (Seluanov et al., 2007) which are associated with cellular replication are also reduced in larger animals.

In addition to BER there are several other DNA repair pathways that may also have a role in conferring stress resistance and animal lifespan. FOXO transcription
factors have been shown to regulate the expression of Gadd45 and damage-specific DNA binding protein (DBB) (Tran et al., 2002; Ramaswamy et al., 2002). Mice deficient in NER repair enzymes show signs of premature aging (de Boer et al., 2002). Similarly, deficiencies of NER enzymes are linked to human premature aging syndromes such as Cockayne syndrome (Lehmann, 2003). Therefore, further work is required to determine if NER or other DNA repair pathways play a more prominent role than BER in conferring stress resistance and longevity in animals.

Are all cells considered equal?

While there were instances in which antioxidant and BER enzyme activities were associated with increased stress resistance and/or longevity, my data clearly indicate that this is not necessarily required. These results suggest the need to probe further into the molecular physiology of stress resistance and longevity to discover their mechanistic basis. For example, the majority of the enzyme measurements were taken from brain, heart and liver tissue homogenates which are primarily composed of post-mitotic cell types. This approach could have masked possible differences in specific types of cells, particularly tissue resident stem cells, which constitute a small minority of the overall population of cells in most highly oxidative tissues. Stem cell maintenance has recently been hypothesized as a regulator of animal aging and longevity. Stem cells themselves "grow old" and no longer appear to function in their role of cellular replacement (reviewed by Mantel and Broxmeyer, 2008). In particular the accumulation of DNA damage within stem cells has been associated with stem cell failure and aging (reviewed by Kenyon and Gerson, 2007). Therefore protection against oxidative stress, perhaps via
enhanced intracellular antioxidant and BER capacities, may be revealed through the examination of isolated tissue-resident stem cell populations.

Molecular mechanisms of stress resistance in the Snell dwarf mouse

Cellular stress resistance is observed in virtually all long-lived mutant mouse models, including Snell dwarf mice (Murakami et al., 2003; Salmon et al., 2005; Maynard and Miller, 2006), and one goal of this thesis was to identify molecular mechanisms that may contribute to this phenomenon. It did not appear that enhanced ROS detoxification plays a significant role in either fibroblasts or whole tissues of Snell dwarf mice, given the general absence of antioxidant enzyme upregulation under basal conditions (Chapter 3; 5). However, polβ activity was higher in Snell brain and heart tissue compared to normal littermates, and there was evidence that fibroblasts were better able to upregulate BER enzymes following exposure to exogenous stressors. This suggests a potential role for BER in mediating the stress resistance of Snell dwarf mouse cells. There appears also to be a role for NER in enhancing stress resistance and longevity in Snell dwarf mice (Salmon et al., 2009). Recently, Leiser and Miller (2010) reported elevated levels of Nrf2 and several Nrf2-regulated genes in Snell dwarf fibroblasts, suggesting an altered stress response. These results are interesting in the context of the rapid induction of certain antioxidant and BER enzymes identified in Chapter 5. Perhaps Snell dwarf mouse cells are able to respond more rapidly to stresses.

Molecular mechanisms of stress resistance in long-lived vertebrate species

Cellular oxidative stress resistance correlates positively with animal lifespan in an inter-specific context (Kapahi et al., 1999), and the plasma IGF-1 results reported in
Chapter 2 are consistent with this observation, as reduced IGF-1 signaling should be associated with increased stress resistance based on our current understanding. However, the molecular mechanisms underlying this phenomenon remain unclear. Salway et al. (2010) found that many protein repair and recycling activities are negatively correlated with species body mass, similar to the negative correlation of polβ observed here (Chapter 4). In addition, there was little evidence that intracellular antioxidant enzyme activities are positively correlated with body mass or lifespan (Chapter 3). Taken together, these results are not consistent with an upregulation of the stress resistance proteome in longer lived species. It will be necessary to consider additional molecular traits in the future to identify the contributing molecular mechanisms.

Molecular mechanisms of stress resistance in hibernation and estivation

Cellular stress resistance is generally characteristic of quiescence. In C. elegans dauers this appears to be mediated by a downregulation of IIS. Oxidative stress resistance and reduced IIS are similarly observed during torpor in hibernating mammals (Dave et al., 2006; Christian et al., 2008). Interestingly though, antioxidant enzyme activities were not broadly upregulated during torpor in brain, heart and liver of 13-lined ground squirrels (compared to active squirrels). Again, stress resistance associated with tissues of hibernators may arise from other mechanisms, including extracellular antioxidant enzymes (Drew et al., 1999, Ohta et al., 2006). Nrf2 mRNA and protein levels are elevated during hibernation in 13-lined ground squirrels, suggesting that Nrf2 signaling may mediate some of the phenotype. Nrf2 target genes include thioredoxin and heme-oxygenase-1, the latter which functions in the production of the antioxidant biliverdin (Morin et al., 2008). Other protective mechanisms may contribute to the stress
resistant phenotype of hibernators. For example, glucose-regulated protein 78 (GRP78), a chaperone involved in the unfolded protein response increases in brain tissue of torpid 13-lined ground squirrels (Mamady and Storey, 2006).

Few studies have addressed stress resistance during estivation, though lungfish in this state tolerate heat and dessicative stress. In contrast to hibernation in the 13-lined ground squirrel (Chapter 6), estivation in the African lungfish was accompanied by a pronounced trend toward elevating activities of antioxidant enzymes in brain and heart tissue (Chapter 7). Thus, there is no evidence that hibernation and estivation involve similar molecular adaptations conferring stress resistance.

Conclusion

Overall, while there were instances in which enhanced antioxidant and BER enzyme activities were associated with increased stress resistance and/or longevity, this was typically not the case, indicating that other mechanisms must be involved. These data suggest the need to re-examine and refine the ‘oxidative stress’ hypotheses of longevity by probing further into the molecular physiology of longevity to discover its mechanistic basis.
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196


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