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Skin-derived fibroblasts from long-lived species are resistant to some, but not all, lethal stresses and to the mitochondrial inhibitor rotenone

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Summary

Fibroblast cell lines were developed from skin biopsies of eight species of wild-trapped rodents, one species of bat, and a group of genetically heterogeneous laboratory mice. Each cell line was tested *in vitro* for their resistance to six varieties of lethal stress, as well as for resistance to the nonlethal metabolic effects of the mitochondrial inhibitor rotenone and of culture at very low glucose levels. Standard linear regression of species-specific lifespan against each species mean stress resistance showed that longevity was associated with resistance to death induced by cadmium and hydrogen peroxide, as well as with resistance to rotenone inhibition. A multilevel regression method supported these associations, and suggested a similar association for resistance to heat stress. Regressions for resistance to cadmium, peroxide, heat, and rotenone remained significant after various statistical adjustments for body weight. In contrast, cells from longer-lived species did not show significantly greater resistance to ultraviolet light, paraquat, or the DNA alkylating agent methylmethanesulfonate. There was a strong correlation between species longevity and resistance to the metabolic effects of low-glucose medium among the rodent cell lines, but this test did not distinguish mice and rats from the much longer-lived little brown bat. These results are consistent with the idea that evolution of long-lived species may require development of cellular resistance to several forms of lethal injury, and provide justification for evaluation of similar properties in a much wider range of mammals and bird species.

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Introduction

Single gene mutations that extend lifespan in the nematode worm *Caenorhabditis elegans* often render the worms resistant to multiple forms of lethal injury, including heat, heavy metals, ultraviolet (UV) irradiation, and oxidizing agents including hydrogen peroxide (H_2O_2) and the free radical generator paraquat (Larsen, 1993; Lithgow *et al*., 1994; Johnson *et al*., 1996). These observations have suggested that the mutations may prolong lifespan because they render some or all cells within the worm resistant to various forms of damage from intrinsic or extrinsic agents. A combination of genetic and biochemical analyses has suggested that in these mutants diminished signals from a receptor for insulin-like signals act downstream, via the FOXO family DNA-binding protein DAF-16, to induce a wide range of effector proteins that collectively protect against multiple forms of cellular injury (Guarente & Kenyon, 2000; Murphy *et al*., 2003).

Following these hints, we have previously evaluated the properties of fibroblast cells, derived from biopsies of adult skin, from various stocks of long-lived mutant mice (Murakami *et al*., 2003; Salmon *et al*., 2005). The Snell dwarf mouse is homozygous for a loss-of-function mutant of the *Pit1* gene (Camper *et al*., 1990), which controls development of the embryonic anterior pituitary. As a consequence Snell dwarf mice have very low, or undetectable, circulating levels of growth hormone and its mediator insulin-like growth factor-1 (IGF-I), thyroid-stimulating hormone and its mediators T3 and T4, and prolactin (Bartke *et al*., 2001). This set of hormonal changes leads to a reduction in young adult body weight of about 70%, and to an approximately 40% extension of lifespan (Flurkey *et al*., 2001). We have shown previously that cultured fibroblasts from adult Snell dwarf mice are resistant to death induced by heat, H_2O_2, cadmium, paraquat, and UV, as well as to the DNA alkylating agent methyl methanesulfonate (MMS) (Murakami *et al*., 2003; Salmon *et al*., 2005). Fibroblasts from the Ames dwarf mouse, in which a similar pituitary abnormality leads to dwarfism and extended longevity (Brown-Borg *et al*., 1996), were also found to be resistant to cadmium, UV, and peroxide (Salmon *et al*., 2005). In addition, we evaluated cells from growth hormone receptor knock-out (GHR-KO) mice, which resemble the Ames and Snell dwarf mice in their

increased lifespan and in their low levels of circulating IGF-I, but which differ from the Ames and Snell mice in their abnormally high growth hormone levels and relatively normal levels of prolactin and the thyroid hormones (Coschigano *et al.*, 2000). We found (Salmon *et al.*, 2005) that fibroblasts from the GHR-KO mice were resistant to UV, peroxide, and paraquat (although not to cadmium), suggesting that development of stress-resistant fibroblasts in these mice may to a great extent represent the effects of diminished circulating IGF-I. In these studies stress tests were conducted on cells grown *in vitro* for several weeks and through many rounds of cell division, suggesting that resistance to lethal injury represents an epigenetic change, induced *in vivo* but remaining as a stable cellular property after cell population expansion in serum-containing growth medium. Resistance is absent, or much reduced, in cell lines developed from Snell dwarf mice at ages of 7 days or less (Salmon *et al.*, 2005), showing that the resistance does not depend on the presence of the *Pit1*^{dw} allele itself, but rather on developmental events that occur in the hormone-deficient mice subsequent to the first week of life.

More recently, we have found (Leiser *et al.*, 2006) that fibroblasts from Snell dwarf mice show a second property of interest: they are relatively resistant, compared to cells from littermate controls, to the metabolic effects induced by the mitochondrial inhibitor rotenone or by culture in medium containing very low glucose levels. The assay involves testing the ability of cells to reduce an extracellular electron acceptor, the water-soluble tetrazolium dye WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate). Transferring cultures of control fibroblasts to medium containing either rotenone or drastically lower glucose levels leads to a decline in WST-1 reduction within 15–30 min. Diminished WST-1 reduction occurs without a corresponding decline in cell viability, and neither rotenone nor low glucose medium leads to a decline in reduction of an intracellular electron acceptor, the tetrazolium dye MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The effects of low glucose or of rotenone are fully reversible, in that return to control medium after as much as 24 h promptly leads to full resumption of cell growth and WST-1 reduction. Interestingly, when skin-derived fibroblast from nonmutant, genetically heterogeneous mice were evaluated, those cell lines most resistant to the metabolic effects of low glucose were also significantly more resistant to the lethal effects of cadmium and H_2O_2, suggesting that resistance to both lethal and metabolic agents might be partially modulated by common factors. While it is unlikely that stress resistance of skin cells plays an important causal role in the extended longevity of Snell dwarf mice, these data suggest a model in which hormonal abnormalities in these mice might induce both metabolic alterations and stress resistance in multiple cell types, some of which do contribute to the unusual resistance of these animals to multiple forms of late-life illnesses.

Although single-gene mutations and selective breeding can seldom increase mammalian lifespan by more than 40% within a species (Miller & Austad, 2006), natural selection has produced differences in longevity, among species, of much greater magnitude. Within the mammals, for example, members of some species typically survive only 2–3 years under optimal conditions, whereas others typically produce individuals that can attain ages of 50 years old or more (Comfort, 1979; Carey & Judge, 2000). Even within a single mammalian order, different species can share radically different maximal lifespans. The shortest-lived rodents, for example, have maximal lifespans of less than 4 years, while other species have recorded lifespans of 18 years (porcupine) or 24 years (beaver) in the wild (S. Austad, personal communication).

To see if skin-derived fibroblast cells from long-lived mammals might resemble cells from dwarf mice in their resistance to multiple lethal and nonlethal agents, we developed a panel of cells from multiple adult donors of eight species of wild-trapped rodents: mouse, rat, red squirrel, white-footed mouse, deer mouse, fox squirrel, porcupine, and beaver (Table 1). In our study we also included cell lines from a stock of laboratory-raised (DC) mice whose genes derive from four commonly used domesticated inbred lines, because of previous observations that wild-derived mice differed from laboratory-adapted mouse stocks in size,

Table 1 Summary of samples used in this study

Species	Common name	Lifespan estimate (years)*	Body weight (g)	Number of samples
Mus musculus	House mouse (laboratory)	2.7	41	10
Mus musculus	House mouse (wild)	3.4	21	9
Rattus norvegicus	Norway rat	5	200	6
Tamiasciurus hudsonicus	Red squirrel	7	200	9
Peromyscus leucopus	White-footed mouse	8.2	23	7
Peromyscus maniculatus	Deer mouse	8.3	21	5
Sciurus niger	Fox squirrel	13	800	9
Erethizon dorsatum	North American porcupine	18	8600	8
Castor canadensis	North American beaver	24	20250	1
Myotis lucifugus	Little brown bat	34	10	8

*With the exception of house mice, the lifespan estimate is the maximum recorded for each species (S. Austad, personal communication). For house mice, the lifespan estimate is the mean lifespan of the longest lived 10% of naturally aging populations maintained in our laboratory under standard husbandry conditions.

hormone levels, maturation rate, and longevity (Miller *et al*., 2002). Lastly, we included multiple cell lines from a species of bat (the little brown bat, *Myotis lucifugus*), which is much longer lived than rodents of similar body weight. Our data suggest that longevity, in this collection of species, is associated with cellular resistance to the lethal effects of cadmium, H_2O_2, and heat, as well as to the metabolic effects of mitochondrial inhibition.

Results

Previous work from our laboratory has shown that primary fibroblast cultures developed from the skin of adult dwarf mice are resistant to at least six forms of lethal injury: heat, cadmium, H_2O_2, UV light, paraquat, and the DNA alkylating agent MMS. In addition, cells from dwarf mice show a metabolic abnormality: they are relatively refractory, compared to cells from littermate controls, in response to culture conditions that inhibit the reduction of extracellular electron acceptors, such as growth in medium that is low in glucose or which contains nonlethal levels of the mitochondrial inhibitor rotenone. Because mammals, including the rodents that are the particular focus of this study, have evolved lifespans that vary over at least an order of magnitude, we wished to determine if fibroblasts from a range of different species show a similar pattern of resistance to lethal stresses and metabolic inhibitors. To investigate this question, we assayed cell lines developed from a convenience sample of mammalian species shown in Table 1.

Several features of the experimental design and analysis deserve explicit justification. Two kinds of mice (*Mus musculus*) were used in this study, and treated separately for analysis. Laboratory mice were the product of a four-way cross among commonly used inbred strains; these animals were bred in the laboratory and housed in vivarium conditions until euthanized for study. A second set of samples was derived from wild-trapped mice. Because long-term domestication and inbreeding lead to dramatic changes in behavior, developmental rate, body size, and longevity (Miller *et al*., 1999, 2000, 2002), we thought it of interest to include samples from both kinds of mice in our study for comparison. All other samples were from wild-trapped animals. The age of these animals is not known, although by size each donor was judged to be young adult. We did not consider the sex of the donor at any point in the analysis; all of the DC laboratory mice were males, but the sex of the wild-captured animals was not consistently recorded. All of the wild-trapped animals were trapped within 400 km of Ann Arbor, MI, USA, thus providing some uniformity of adaptation to specific climate conditions. Lastly, we included in our study set one species of bat in addition to the eight species of rodents, because of previous work (Austad & Fischer, 1991; Austad, 2005) suggesting that bats were particularly long lived compared to nonflying eutherians of similar body size.

The design was thus compatible with several analytical strategies addressing a set of related and overlapping issues, including comparisons among all species; among rodents only; among wild-trapped rodents only; or between pairs of species of particular interest. We also considered, and will discuss below, analytical methods that adjust in various ways for phylogenetic relationships or for interspecies differences in body weight.

Figure 1 presents, for each of the six lethal stresses tested, scatterplots of mean LD_{50} values against the lifespan estimate for each of the nine kinds of animals listed in Table 1 (i.e. treating laboratory mice and wild-trapped mice separately). The bars show standard errors of the mean for each LD_{50} value, except for beaver (for which only a single individual donor was tested). The regression values (Pearson R^2, and associated P value) refer to a standard linear-regression model in which each species contributed a single, average value for its LD_{50}, with no adjustment for phylogeny, body weight, or number of individuals tested per species. The results of this analysis suggest that for this group of samples maximum lifespan is positively correlated with fibroblast cell line resistance to cadmium and H_2O_2. Regressions for MMS and heat are not significant, but yield $P < 0.08$ in each case, suggesting a trend for association between lifespan and resistance to these two stressors. There is no indication for any relationship between lifespan and resistance to UV light or to paraquat.

Skin-derived fibroblasts from Snell dwarf mice are also resistant to the metabolic effects of the mitochondrial inhibitor rotenone and of low-glucose culture media (Leiser *et al*. 2006). Although neither low-glucose medium nor rotenone leads to cell death in the conditions used, both lead rapidly (within 15 min) to a reversible inhibition of the ability of mouse fibroblasts to reduce extracellular electron acceptors such as the tetrazolium dye WST-1. Interestingly, a test of cell lines from nonmutant genetically heterogeneous laboratory mice showed that those individual mice whose cells were most resistant to the lethal effects of cadmium and H_2O_2 were also most resistant to the metabolic inhibition caused by low-glucose conditions, suggesting that common cellular factors might contribute to resistance to both lethal and nonlethal agents. Because cells from long-lived species were relatively resistant to cadmium and H_2O_2, we tested these cells for resistance to low-glucose medium and rotenone as well. Results are shown in Fig. 2, and show that cells from long-lived species are relatively resistant to the inhibitory effects of rotenone ($R^2 = 0.58$, $P < 0.02$). The data from the low-glucose tests were ambiguous: when all species were included, $R^2 = 0.32$, with $P = 0.09$; when only rodents were evaluated, $R^2 = 0.7$, $P < 0.005$.

Secondary analyses

We also conducted a series of secondary analyses. Some were conducted to evaluate specific contrasts of interest, and some of these were motivated by a desire to test our conclusions for robustness to the arbitrary design decisions, such as the inclusion of a nonrodent species and the decision to treat laboratory mice and wild-trapped mice separately. Other analyses were undertaken to evaluate alternate regression approaches that are favored by some, but not all, experts in interspecies contrasts.

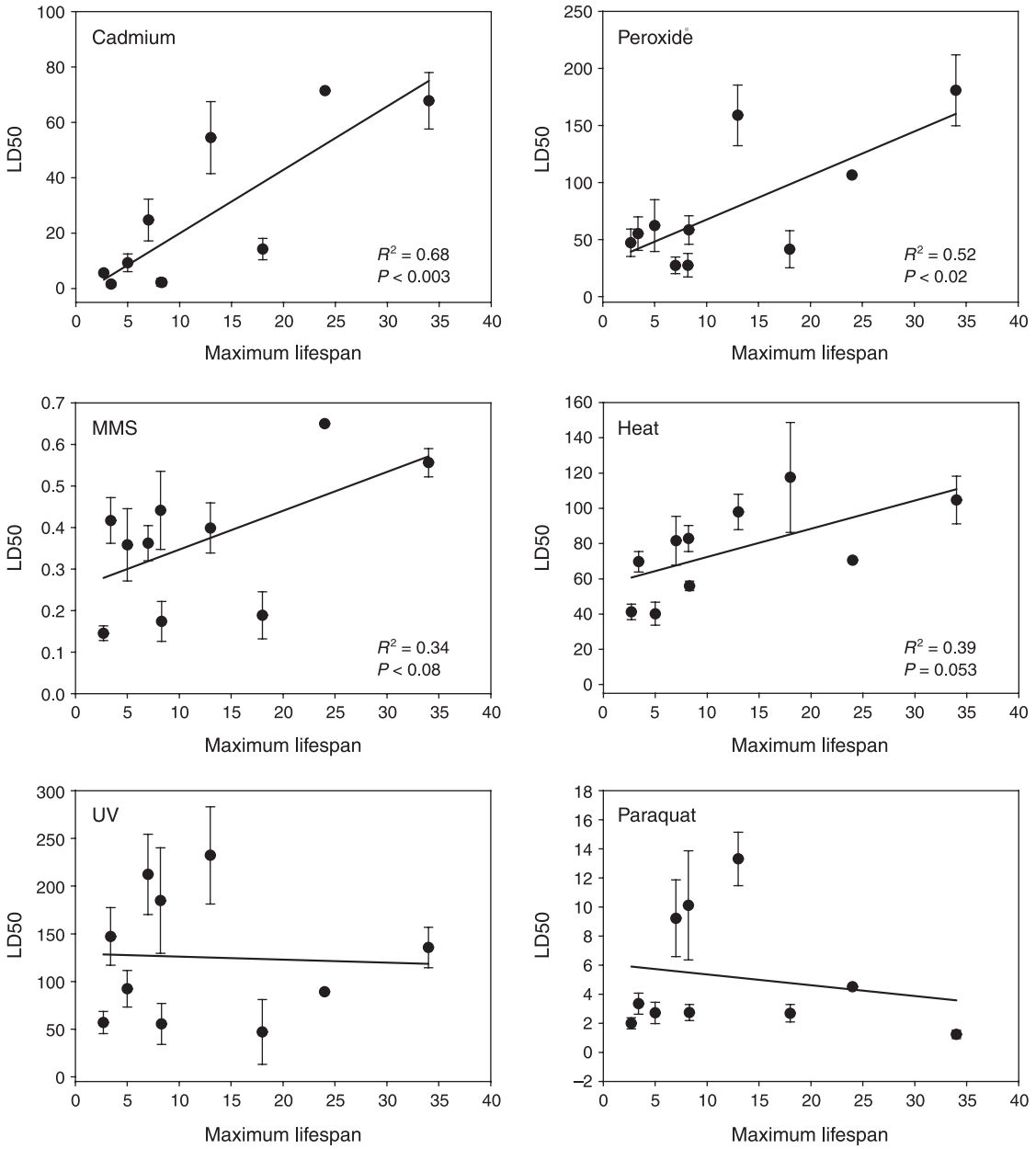

Fig. 1 Each scatterplot shows an association between species maximum lifespan and mean LD_{50} value for each of 10 species (treating laboratory mice and wild-trapped mice as separate species for reasons explained in the text). From left to right, points represent laboratory mouse, wild-trapped mouse, rat, red squirrel, white-footed mouse, deer mouse, fox squirrel, porcupine, beaver, and little brown bat. Formal species names and number of independent samples are given in Table 1. Error bars show standard errors of the mean. The line shows the outcome of a least squares regression. Pearson R^2 and P values (quoted only where $P < 0.1$) reflect standard linear regression of maximum lifespan against mean LD_{50} values for the set of nine species, as in the first column of Table 2. Units for LD_{50} are in μM (cadmium and H_2O_2), mM (MMS and paraquat), J m^{-2} (UV light) or min at 42 °C (heat).

Table 2 presents the results of three different regression analyses of a data set containing all of the tested species (including laboratory and wild mice as separate entries, and including the bat species). The first column shows standard linear regression results, regressing the mean LD_{50} (or ED_{50} for nonlethal stresses glucose and rotenone) value for each species against the estimate of maximum lifespan for each species. These calculations were presented above in the context of Fig. 1.

The second column shows a different approach, in which each individual donor was included in a two-level regression, similar to the 'nested regression' models sometimes used in other contexts. The calculation separated overall variation into 'between-species' and 'within-species' components, in a linear mixed-effects model, and made the assumption that the sample of species was randomly selected from a larger population of species from which the data might have been obtained. The tabulated P value represents the significance of the 'across-

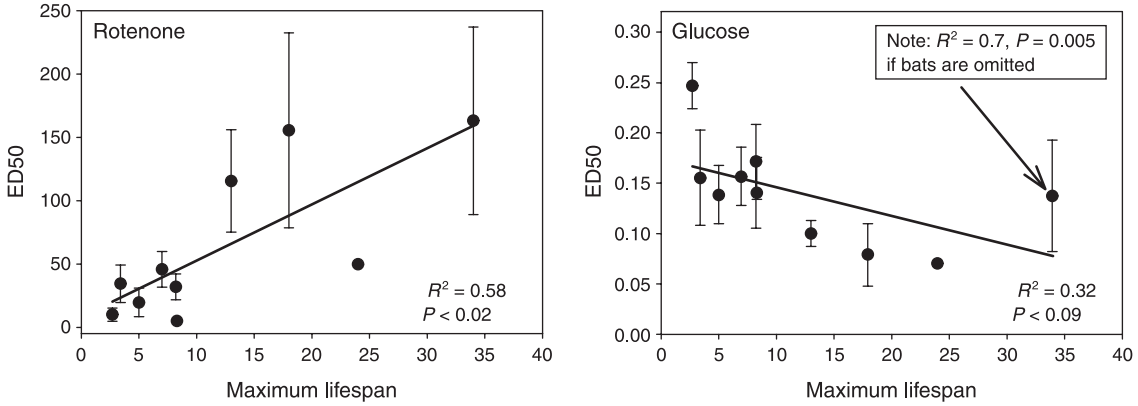

Fig. 2 As in Fig. 1, except that the vertical axis shows mean ED_{50} values, i.e. the dose of rotenone or glucose that led to a 50% reduction in WST-1 reduction compared to cultures in control medium, for each of 10 species (treating laboratory mice and wild-trapped mice as separate species for reasons explained in the text). Note that increased resistance to rotenone leads to higher ED_{50} levels, but that increased resistance to withdrawal of glucose is reflected by lower ED_{50} values, i.e. a requirement for more extreme removal of glucose to achieve equivalent metabolic inhibition. Units for ED_{50} are μM (for rotenone) and mg mL^{-1} (glucose).

Table 2 Comparison of regression methods for association between species lifespan and eight tests of cell culture properties

	Simple linear regression	Multilevel regression	Phylogenetically independent contrasts
All species (with laboratory and wild mice)			
Cadmium	R^2 = 0.68, P = 0.003	P = 0.0004	R^2 = 0.57, P = 0.01
H_2O_2	R^2 = 0.52, P = 0.018	P = 0.015	R^2 = 0.43, P = 0.04
MMS	R^2 = 0.34, P = 0.08	P = 0.18	R^2 = 0.20, P = 0.19
Heat	R^2 = 0.39, P = 0.053	P = 0.02	R^2 = 0.10, P = 0.37
UV	R^2 = 0.002, P = 0.90	P = 0.69	R^2 = 0.02, P = 0.66
Paraquat	R^2 = 0.03, P = 0.69	P = 0.43	R^2 = 0.07, P = 0.45
Rotenone	R^2 = 0.58, P = 0.011	P = 0.045	R^2 = 0.33, P = 0.08
Glucose	R^2 = 0.32, P = 0.087	P = 0.27	R^2 = 0.09, P = 0.41

Table 3 Regression analyses relating lifespan to stress resistance or metabolic inhibition for wild-trapped rodents only

	Simple linear regression	Multilevel regression	Phylogenetically independent contrasts
Wild-trapped rodents			
Cadmium	R^2 = 0.57, P = 0.031	P = 0.107	R^2 = 0.57, P = 0.029
H_2O_2	R^2 = 0.18, P = 0.30	P = 0.37	R^2 = 0.26, P = 0.196
MMS	R^2 = 0.11, P = 0.41	P = 0.29	R^2 = 0.37, P = 0.37
Heat	R^2 = 0.21, P = 0.26	P < 0.0001	R^2 = 0.07, P = 0.53
UV	R^2 = 0.095, P = 0.46	P = 0.53	R^2 = 0.09, P = 0.47
Paraquat	R^2 = 0.003, P = 0.97	P = 0.91	R^2 = 0.007, P = 0.99
Rotenone	R^2 = 0.29, P = 0.17	P < 0.0001	R^2 = 0.18, P = 0.29
Glucose	R^2 = 0.81, P = 0.002	P = 0.0002	R^2 = 0.72, P = 0.008

species' term in the regression model. This analysis, like the standard linear regression, suggests significant effects for cadmium, peroxide, and rotenone across this group of species; in addition, the regression for heat stress, for which P = 0.053 in the standard regression, is significant in the two-level regression at P = 0.02.

There is controversy (discussed below) about the appropriateness of including information about phylogenetic relatedness for evaluation of interspecies effects in small data sets of this kind. Nevertheless, we used a popular method (Felsenstein, 1985; Garland *et al*., 1992) for evaluating phylogenetically independent contrasts within this data set, and the results are shown in the last column of Table 2. The association of cadmium and peroxide resistance with species longevity remains significant after adjustment in this way; the rotenone association is no longer significant (P = 0.08).

The analyses shown in Figs 1 and 2 and in Table 2 make use of information from all of the cell lines we tested, including cells from laboratory mice and from a bat species. We have also performed regression analyses using a subset of the data limited to wild-trapped rodents only (with both bats and laboratory mice excluded). Table 3 shows regressions for this truncated data set. Among this set of species there is a significant association between longevity and glucose resistance, for each of the three regression methods. In this smaller data set, however, some of the associations seen in Table 2 are no longer statistically significant, and testing the robustness of our conclusions clearly will require additional information from a wider range of species.

Some authorities (Speakman, 2005) have suggested that regressions of longevity on cellular parameters often reflect the well-documented trend towards longer lifespans among larger mammals (Austad & Fischer, 1991), and have recommended that regression analyses be adjusted for species differences in body weight. Table 4 shows the outcome of two such adjusted regression studies. In each case the dependent variable is not lifespan *per se*, but the residual of lifespan against species-specific body weight. These residuals were calculated by Dr. João Pedro de Magalhaes, using an extensive data base of lifespan and body weight values for 1334 mammalian species (de Magalhaes *et al*., 2005). The first data column shows regressions in which LD_{50} (or ED_{50}, for glucose and rotenone) are regressed against this weight-adjusted lifespan residual. There are significant associations for cadmium, peroxide, heat, and

Table 4 Regression analyses for residuals – body weight adjustment

	LD_{50} × lifespan residual	LD_{50} residual × lifespan residual
Cadmium	$R^2 = 0.43$, $P = 0.04$	$R^2 = 0.55$, $P = 0.01$
H_2O_2	$R^2 = 0.46$, $P = 0.031$	$R^2 = 0.52$, $P = 0.02$
MMS	$R^2 = 0.16$, $P = 0.26$	$R^2 = 0.14$, $P = 0.28$
Heat	$R^2 = 0.49$, $P = 0.025$	$R^2 = 0.57$, $P = 0.01$
UV	$R^2 = 0.02$, $P = 0.69$	$R^2 = 0.03$, $P = 0.65$
Paraquat	$R^2 = 0.001$, $P = 0.93$	$R^2 = 0.01$, $P = 0.83$
Rotenone	$R^2 = 0.56$, $P = 0.013$	$R^2 = 0.78$, $P = 0.001$
Glucose	$R^2 = 0.12$, $P = 0.32$	$R^2 = 0.23$, $P = 0.16$

rotenone in this set of models. The second column shows a similar approach, in which the residual of lifespan (regressed against body weight) is compared to the residual of the LD_{50} value, itself regressed against body weight for those species for which we have stress resistance data. Here, too, significant associations are seen for cadmium, peroxide, heat, and rotenone.

Lastly, we used subsets of our data to consider a small number of specific pairwise comparisons of particular biogerontological interest. The first two columns of Table 5 evaluate whether laboratory mice differ from wild-trapped mice in the resistance of their cells to the agents under consideration. Cells from wild-trapped mice are significantly more resistant to MMS, heat, and UV light, and significantly more resistant to the effects of low-glucose medium. There is no evidence of preferential resistance to peroxide or paraquat. Cells from wild-trapped mice were, unexpectedly, dramatically and significantly more sensitive to cadmium toxicity than were cells from laboratory mice. Cells from wild-trapped mice were 3.4-fold more resistant to rotenone-mediated inhibition, but there was a great deal of scatter among individual animals, and the difference was not statistically significant ($P < 0.07$).

There is also considerable interest in the question of why flying mammals, such as bats, are typically so much longer lived than nonflying animals of similar size (Austad & Fischer, 1991; Austad, 2005). We therefore include in Table 5 a comparison of cells from the little brown bat to cells from (wild-trapped) mice and rats for each of the agents studied. Bat cells are significantly more resistant than mouse or rat cells to cadmium, H_2O_2, and heat, among the lethal stresses, and also significantly more resistant to the nonlethal mitochondrial inhibitor rotenone. The difference in resistance to MMS reaches statistical significance for rat cells ($P = 0.03$), but not for mouse cells ($P = 0.06$). Unexpectedly, cells from this bat species are significantly more sensitive to paraquat than cells from wild-trapped mice. There are no differences between bats and either of these two rodents in resistance to UV or to low glucose medium under our conditions.

We are willing to provide the complete data set to other investigators who wish to evaluate other specific comparisons of interest or to employ other methods of analysis.

Discussion

Primary dermal fibroblast cell lines isolated from eight species of rodents (house mice, deer mice, white-footed mice, Norway rats, red squirrels, fox squirrels, beavers and porcupines), and a single species of bat (little brown bat) were used to examine the relationship between lifespan and resistance to many kinds of cell injury. Previously, we have shown that cell lines from long-lived Ames dwarf (*df/df),* Snell dwarf (*dw/dw*), and GHR-KO mice each exhibit increased resistance to multiple cytotoxic agents *in vitro* relative to cell lines derived from nonmutant controls (Murakami *et al*., 2003; Salmon *et al*., 2005). These findings led us to hypothesize that differences in stress resistance of adult dermal fibroblast cells might serve as an indicator to cellular properties involved in disease resistance and longevity in intact organisms, even though it is unlikely that the dermal fibroblasts themselves play an important role in lifespan determination.

Expert opinion is divided on the question of how best to evaluate hypotheses about distribution of biochemical and cellular phenotypes across a range of species that differ in typical or maximal longevity. Some have argued (Speakman, 2005) that such analyses are invalid unless the traits are first adjusted for body weight, to avoid reporting relationships that merely represent correlations among traits known to depend heavily

Table 5 Comparison of cells from wild mice, laboratory mice, rats, and bats

Treatment	Laboratory mice	Wild mice	Rat	Bat	p(t) – Laboratory vs. wild mice	p(t) – Bat vs. wild mice	p(t) – Bat vs. rat
Cadmium	5.7 ± 1.1	1.6 ± 1.0	9.3 ± 3.2	68 ± 10	0.00	0.00	0.00
H_2O_2	47 ± 12	55 ± 15	62 ± 23	181 ± 31	0.81	0.00	0.01
MMS	0.15 ± 0.02	0.42 ± 0.06	0.36 ± 0.09	0.56 ± 0.03	0.00	0.06	0.03
Heat	41.2 ± 4.4	69.7 ± 5.8	40.1 ± 6.5	105 ± 13	0.00	0.02	0.00
UV	56.9 ± 11.5	147.1 ± 30.2	92 ± 19	136 ± 21	0.03	0.84	0.17
Paraquat	2.0 ± 0.4	3.4 ± 0.7	2.7 ± 0.7	1.24 ± 0.29	0.26	0.03	0.08
Rotenone	10.0 ± 5.2	34.5 ± 14.8	19.6 ± 11.3	163 ± 74	0.07	0.02	0.01
Glucose	0.247 ± 0.02	0.156 ± 0.05	0.14 ± 0.03	0.14 ± 0.06	0.03	0.61	0.52

Values are mean LD_{50} ED_{50} values ± SEM for $N = 9$ laboratory mice, $N = 10$ wild-trapped mice, and $N = 7$ or 8 bats. Significance levels show two-sided *t*-tests; because the distributions were non-normal, values were log transformed prior to the *t*-test calculations. Units are as indicated in the legend to Figs 1 and 2. Note that high values represent increased resistance, except for glucose, in which a low value indicates resistance to glucose withdrawal.

on body size and its associated thermogenic metabolic demands. This notion, while important to consider, would eliminate from consideration nearly all cellular properties that lead to delayed aging in mammals, because large species of mammals are, in general, longer lived than small species. Some experts maintain that cross-species comparisons require adjustment for the degree of phylogenetic independence among the species evaluated, while other authorities include such adjustments only when there is a demonstrable and statistically significant effect of phylogenetic relatedness in the data set itself (Abouheif, 1999; Freckleton *et al*., 2002; Blomberg *et al*., 2003). Assessments of the strength of this 'phylogenetic signal' are, however, difficult to make with data sets containing fewer than about 20 species. Neither is there a consensus about how to analyze data sets in which estimates from each species vary in their precision, in our case because some species are represented by multiple individuals, but sometimes by only one specimen. Because of these uncertainties, we have presented several different analyses of the same data set so that proponents of each approach can evaluate the strength and weaknesses of the evidence available to us. Fortunately, most of the conclusions we reach are robust to the analytical approach; we point out exceptions where these seem of interest.

Considering the entire set of species, long lifespan is associated with relative resistance to cadmium, H_2O_2, and the nonlethal inhibitor rotenone. There are also suggestions of a similar relationship for heat ($P = 0.053$) and MMS ($P = 0.08$). Although to be conservative we used two-tailed *P* values as our significance criteria, we note that in each case the direction of the regression is consistent with our original working hypothesis, i.e. that cells from longer-lived species would be more resistant to the lethal stresses and nonlethal metabolic inhibitors, as are fibroblasts from long-lived mutant Snell dwarf mice. When multilevel regression is used to take account of differences among the species in number of individuals tested (and differences in variance within a species), the associations for cadmium, H_2O_2, and rotenone remain significant, and in addition heat stress has a significant association at $P = 0.02$. When the data are evaluated using the method of phylogenetically independent contrasts, the cadmium and H_2O_2 effects remain significant, despite the loss of statistical power in this approach; the rotenone association remains suggestive at $P = 0.08$. Thus, despite the small number of species and individuals examined, we see an association for cadmium and H_2O_2 that is robust across analytical methods, and have varying degrees of support for parallel hypotheses for rotenone, heat, and perhaps MMS.

In addition to these regressions of cellular traits against lifespan, we also performed a regression based on the residual of lifespan adjusted for body weight, and a regression in which both the cellular trait and lifespan were body-weight adjusted (see Table 4). Both approaches revealed significant associations for cadmium, H_2O_2, heat, and rotenone. It thus seems unlikely, in this data set, that the differences among species in resistance to these four conditions reflect variation with body size alone.

The main data set includes data from one nonrodent species (the little brown bat), and also from a group of laboratory-adapted mice. The decision to include both laboratory-adapted mice as well as those derived from recently trapped wild progenitors was motivated by the finding that wild-derived mice live longer, are smaller, mature more slowly, and have lower levels of several hormones compared to typical laboratory mice (Miller *et al*., 2002). To see if our principal findings depended on the inclusion of bats and laboratory-adapted mice, we evaluated a reduced data set consisting of wild-trapped rodents only. In this truncated data set we see a strong correlation for resistance to low-glucose media, with $R^2 = 0.7$ ($P = 0.005$) using standard regression, and similar, significant associations with the other two analytical methods. Indeed, among the wild-trapped rodents, the association between longevity and glucose effect is stronger than the associations with any of the tested lethal agents. The relationship of cadmium to lifespan was also supported in this subset of the data, and the multilevel regression indicated associations for heat and rotenone as well.

It is important to note that although WST-1 is used in tests of both lethal stresses (cadmium, heat, H_2O_2) and in the evaluation of responses to low glucose, the decline in WST-1 reduction under low glucose conditions is not accompanied by cell death. The lower level of WST-1 reduction seen in low glucose conditions seems likely to reflect alterations in metabolic pathways needed to produce the reducing equivalents for WST-1 cleavage at the plasma membrane (Berridge *et al*., 1996; Herst *et al*., 2004). Fibroblasts remain fully viable even 48 h after initiation of glucose deprivation, with full recovery of WST-1 metabolic activity 1 h after return to regular growth medium with a glucose concentration of 4 mg mL^{-1}. In addition, we found that glucose withdrawal for a period of 1 h or 24 h had no effect on the levels of DNA synthesis in mouse fibroblasts as measured by radiolabelled thymidine incorporation (not shown). Similarly, the effects of rotenone on WST-1 signal inhibition are fully reversible at the doses used. For example, when cells are exposed to 5 μM rotenone for 4 h and then allowed to recover in rotenone-free medium overnight, cell viability as measured by WST-1 reduction returns to a level greater than 80% of that seen in untreated cells.

In a study of fibroblasts from individual genetically heterogeneous laboratory mice (Leiser *et al*., 2006), we found a significant correlations between resistance to low glucose and resistance to death induced by H_2O_2 ($R = -0.58$, $P < 0.001$) and between glucose and cadmium resistance ($R = -0.53$, $P = 0.006$), but not between glucose and the other lethal agents tested. Resistance to lethal injury induced by cadmium or H_2O_2, as well as resistance to the inhibitory effects of low glucose and of rotenone are also seen in cell lines from Snell dwarf mice (Leiser *et al*., 2006). We thus speculate that the biochemical alterations that mitigate the effects of rotenone or glucose withdrawal on cellular metabolism overlap closely with those that impart resistance to cadmium and H_2O_2, whether these differences are caused by the hormonal milieu of dwarf mice, or instead are the result of coevolution during the course of divergence among mammals of aging rate and longevity.

It is clear that our culture conditions do not fully reproduce the environment of fibroblasts in the skin of intact animals. Our standard stress assays (for the agents shown in Fig. 1) incorporate a 24-h period of incubation in serum-free medium prior to exposure to stress, because we have found that the presence of fetal calf serum increases resistance to these agents by 3-fold to 20-fold, thereby obscuring the differences in sensitivity between dwarf and control cells (Murakami *et al*., 2003). We do not know whether stress resistance would vary among cells from different species if these assays were carried out in the presence of fetal calf serum. Serum contains growth factors to which cells in normal (nonwounded) skin are not ordinarily exposed, but it is also likely that serum-free cultures lack factors, present in normal plasma or tissue fluids, that might modulate cell viability in the original intact tissue. It will be of interest, in follow-up studies, to see if cells in the skin of dwarf mice or of long-lived mammalian species are comparatively resistant to damage induced by agents such as UV light, heat, or toxic levels of oxidizing chemicals.

Overall, our data are consistent with other studies demonstrating an association between mammalian lifespan and cellular physiology *in vitro*. The closest correspondence is to the study of Kapahi *et al*. (1999), which demonstrated that resistance of fibroblasts to oxidative stressors was positively correlated with mammalian longevity across several taxonomic orders. Kapahi evaluated cells from hamster, rat, marmoset, rabbit, sheep, pig, cow and human, and showed significant associations with maximal lifespan for H_2O_2 and paraquat in addition to sodium arsenite, *tert*-butyl hydroperoxide, and sodium hydroxide. Likewise, several other reports, usually involving fewer species, have demonstrated associations involving oxidative stress, antioxidant capacity and maximum lifespan (Tolmasoff *et al*., 1980; Sohal *et al*., 1990; Ogburn *et al*., 1998, 2001). Hydrogen peroxide and cadmium are both known to induce cytotoxicity via reactive oxygen species (Stohs & Bagchi, 1995). Thus, in our study an enhanced resistance to these two stressors in the long-lived species implies that the evolution of long-lived species requires augmentation of cellular pathways that regulate reactive oxygen species (ROS)-induced damage. Unexpectedly, however, there was no relationship between lifespan and resistance to the toxic effects of paraquat, even though paraquat is a well known oxidative stressor, and one to which fibroblasts from Snell dwarf mice and other long-lived mutants show *in vitro* resistance. This suggests that there is a division in the pathways involved in mediating the resistance to particular types of oxidative stress, i.e. paraquat-induced production of superoxide at intracellular sites (Bus *et al*., 1976; Halliwell & Gutteridge, 1989) as contrasted to damage induced rapidly by contact with extracellular H_2O_2, and that production of long-lived species of rodents may not require alteration of the defenses tested by paraquat in culture.

We find no evidence for an association between longevity and resistance to UV light in any of the data sets or with any of the analytical methods used. Other groups have suggested that the repair of UV-induced DNA lesions is correlated with longevity across mammals (Hart & Setlow, 1974; Francis *et al*., 1981; Treton & Courtois, 1990), and also within a single order, the primates (Hall *et al*., 1984). Within the rodents, however, the data are sparse and ambiguous (Cortopassi & Wang, 1996), and some studies have found no evidence for a relationship between lifespan and UV resistance among species of mammals (Kato *et al*., 1980) or nonmammalian vertebrates (Woodhead *et al*., 1980). Furthermore, rodent cells are unusual in that the repair of UV-induced DNA damage does not correlate with cell survival (reviewed in Hanawalt, 2001). Thus, despite our negative evidence in the UV data set, we think it would be informative to explore differences among these and other mammalian species in the capacity of fibroblasts to repair UV or MMS-induced DNA lesions.

Our study design has some features that weaken the generality of our conclusions. For the wild-trapped animals, we do not know the exact donor age, although body size and conformation suggested that the animals were young adults. We did not control the time of year at which samples were taken, except that all individuals were captured between May and October. We did not control for donor sex or reproductive status, although we note that our studies of laboratory mice have given no indication of gender effects on fibroblast stress resistance (A. B. Salmon, unpublished). We did, however, control two variables known to influence properties of cultured fibroblasts: (i) each biopsy was taken from the same place (lower abdomen), and (ii) cells were tested after the same number of population doublings, and after one cycle of cryopreservation.

Species-specific phenotypes reflect the evolutionary selection pressures unique to each species, as well as the constraints imposed by a shared evolutionary history with other members of a phylogenetic lineage (Garland *et al*., 1992, 2005; Austad, 2005). We sought to diminish the confounding effect of evolutionary history by focusing most of our attention on a single order of mammals, the rodents, unlike other studies in which familiar pet or domestic species, from multiple mammalian orders (or sometimes even distinct classes of vertebrates) were evaluated. We also included data on the little brown bat, a member of the order Chiroptera. Bats are comparable in size to house mice, but are exceptionally long lived for their size, even after accounting for effects of hibernation (Wilkinson & South, 2002). Our working hypothesis was that cells from bats would be more resistant to each agent than cells from mice or rats, and we see in Table 5 that this idea was confirmed by data on cadmium, H_2O_2, MMS (significant for rats, $P = 0.06$ for the contrast with mice), heat, and rotenone. Unexpectedly, bat cells seem to be more sensitive to paraquat than cells from mice ($P = 0.03$) and, perhaps, rats ($P = 0.08$), another indication that sensitivity to paraquat and to H_2O_2 may be governed by different cellular defense mechanisms. We have only evaluated cells from one bat species, and the hypothesis that in general bat cells are more resistant to these agents than cells of rodents will require further work. Similarly, it would be of interest to determine if species-specific longevity was correlated with cellular resistance to these stresses among the different chiropteran species.

The contrast between laboratory and wild-derived mice, also shown in Table 5, gives some indication about the speed with which differences in fibroblast stress resistance can be produced by evolutionary pressures. The common laboratory stocks from which the DC mice are derived emerged from wild populations only in the last 100–200 years (Silver, 1995), and during that time have adapted to selective pressures of laboratory vivaria by changes in body size, litter size, hormone levels, activity patterns, maturation rates, and multiple behavioral traits. We can be confident that many of these traits are determined by genetic differences between laboratory and wild mice, because the traits remain distinct in the second-generation offspring of wild-captured mice, whose lives, like those of their parents, have been spent entirely in the laboratory environment (Miller *et al*., 1999, 2000, 2002). Fibroblasts from wild mice are significantly more resistant than those of laboratory (DC) mice in responses to MMS, heat, UV, and low-glucose media, resembling cells from long-lived mutant mice in each of these respects, and show a similar trend ($P = 0.07$) towards rotenone resistance as well. These data suggest that resistance to some of these agents can evolve within a few hundreds of years, and may not require the millions of years that typically separate distinct species. Curiously, wild mouse cells are not more resistant than laboratory mouse cells to cadmium or to H_2O_2, the two agents for which the evidence for interspecies correlation is strongest. In fact, cadmium resistance is dramatically and significantly higher in cells from laboratory mice than in cells from wild-trapped mice; we speculate that this may reflect selection, under laboratory conditions, for resistance to heavy metals that may be a more significant reproductive hazard in the laboratory than in most natural settings. The differences in the spectrum of stress agents that discriminate long- from short-lived species, and those which discriminate the short-lived laboratory stock from wild mice may reflect the timescale over which evolutionary pressures act to produce speciation, elements of the mouse genome that are more or less susceptible to rapid selection, and the peculiarities of the laboratory environment.

Our findings are also limited in scope in two critical dimensions: we have examined only a single cell source (the skin of adults), and most of our data come from a single order of mammals. Meeting the first concern will require development of new, carefully validated methods to study stress resistance of other cell types, such as vascular endothelial cells, lymphocytes, neurons, muscle cells, etc. Developing methods to culture other differentiated cell types from multiple species would require a good deal of effort. Long-term, transformed cells are often convenient for serial studies, but studies of primary, early passage cells (as in our own study) helps to avoid the artifacts of *in vitro* selection for cell resistance to stress. It now seems clear that the oxygen concentrations (20%) used for typical *in vitro* cell cultures can induce mutations and growth abnormalities in mouse cells that do not occur when the cells are grown at the lower oxygen concentrations (e.g. 3%) characteristic of most postcapillary beds in intact animals (Busuttil *et al*., 2003; Parrinello *et al*., 2003; Maynard & Miller, 2006). Attempts to study resistance of specific cell types in intact animals also pose serious technical obstacles, beyond the scope of this discussion.

The second weakness – the lack of species diversity in our collection – may be somewhat easier to fix. It would be of interest to determine whether the pattern of associations noted here between longevity and stress resistance can be replicated in studies that involve a much wider range of rodents. It would, we think, be of even greater interest to see if the pattern of stress resistance we have documented would also be seen among a set of bird species with differential longevity, or among species of primates, or bats. There may be a multiplicity of pathways that enable evolutionary adaptation to niches that support slow aging, or there may be only a few ways to make a long-lived mammal. Learning how nature creates a long-lived species is a key problem in biogerontology. Our data suggest that studies of the pathways that mediate interspecies differences in fibroblasts stress resistance may help us to deconstruct this aspect of evolutionary cell biology.

Experimental procedures

Primary and secondary cell cultures and establishment of cryopreserved cell lines

Wild rodents and bats were caught opportunistically by Marc Steinke of Michigan Wildlife Removal and by Phil Myers of the University of Michigan in an area extending approximately 400 km north and 80 km south of Ann Arbor, MI, USA. After field euthanization, abdominal skin areas were sterilized with 70% ethanol wipes and biopsies of at least 5 mm by 5 mm in area were obtained and placed in complete media (CM) made of Dulbecco's modified Eagle medium (DMEM, high-glucose variant, Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 20% heat-inactivated fetal bovine serum, antibiotics (100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin; Sigma, St. Louis, MO, USA) and 0.25 μg mL^{-1} of fungizone (Biowhittaker-Cambrex Life Sciences, Walkersville, MD, USA) on ice and shipped overnight to our laboratory. For laboratory-raised (DC) mice, animals were humanely euthanized by approved protocols, the abdomen was washed with 70% ethanol, and abdominal skin biopsies of at least 5 mm by 5 mm in area were obtained and placed in CM.

Fibroblast cultures from all biopsies were isolated as we have previously reported (Murakami *et al*., 2003; Salmon *et al*., 2005). Cells were harvested by trypsinization for subculturing after the initial cultures had reached at least 90% confluence. Because some initial cultures grew more rapidly than others, the initial subculturing (Passage 0) took place between 6 and 9 days of growth after initial seeding. There were no significant differences among species in the mean interval between seeding and the first subculture (not shown). Cell cultures were then fed at day 3 (replacing two-thirds of the medium), and subcultured at day 7 at a density of 7.5×10^5 in tissue culture flasks of 75 cm^2 surface area to produce Passage 1 cultures. Seven days later, when cells were again confluent, they were harvested and cryopreserved at 10^6 cells per mL in a solution containing CM

supplemented with an additional 5% fetal bovine serum and fresh dimethylsulfoxide (DMSO, Hybri-MAX, Sigma) at a final concentration of 10%. The cells were then stored in liquid N_2 for up to 18 months before assessment of stress resistance. Cell aliquots were thawed rapidly using 10 volumes prewarmed (37 °C) CM, then centrifuged 5 min at 200 ***g***. The cell pellet was resuspended in approximately 6 mL CM and a small volume was used for viability assessment using trypan blue staining. Cells were then cultured at a density of 1×10^4 live cells per cm^2 of flask surface area into tissue culture flasks of 75 cm^2 surface area. These passage 2 cells were fed on day 3 by replacement of two-thirds of the medium, and subcultured on day 7 at a density of 1×10^5 cells per cm^2 flask surface area into tissue culture flasks (passage 3) of 175 cm^2 surface area.

Assessment of fibroblast resistance to lethal stress

For each single set of stress assay experiments, representatives from multiple species (at least six) were assayed in parallel to minimize the effects of day to day variation. Passage 3 cultures were used to assess resistance to cadmium, paraquat, UV, and MMS, as well as for creating passage 4 cells. Passage 4 cells were then used to assay resistance to H_2O_2 and heat and for the rotenone and glucose resistance assays. Each test procedure began by culturing the cells at a density of 3×10^4 cells in 100 μL CM in 96-well microtiter plates in complete medium for 24 h, followed by a period of 24 h in medium lacking serum but containing 2% bovine serum albumin (BSA, Sigma) with antibiotics and fungizone at the same concentration as CM. This preculture in serum-free medium was introduced because of our evidence that serum greatly increases stress resistance in mouse cell cultures, obscuring differences in cell stress resistance that can be demonstrated after serum has been removed (Murakami *et al*., 2003). Tests for resistance to cadmium, peroxide, paraquat, UV, and MMS were performed as previously reported (Murakami *et al*., 2003; Salmon *et al*., 2005). For assessment of heat resistance, cells were washed, DMEM prewarmed to 42 °C was added to each well, and the plates were then sealed with parafilm and placed on a 42 °C heat block for a range of times. After treatment, cells were washed with 37 °C 1× PBS, and incubated with 37 °C DMEM supplemented with 2% BSA, antibiotics and fungizone, and survival was measured 18 h later using conversion of the extracellular tetrazolium dye WST-1 to its colored formazan product as in (Murakami *et al*., 2003). All incubations (except for heat) were at 37 °C in a humidified incubator with 5% CO_2 in air.

Assessment of cell metabolism in low-glucose medium

Cells harvested and grown to the fourth passage as above were plated at a density of 3×10^5 per mL in DMEM with 20% fetal bovine serum with antibiotics and fungizone. After an 18-h overnight incubation, cells were washed twice with 37 °C 1× PBS and incubated in DMEM containing a range of glucose concentrations. These concentrations were made using media containing glucose-free DMEM, supplemented with 20% heat-inactivated dialyzed fetal bovine serum, antibiotics, fungizone, and supplemental glucose (Sigma). CM was used as a control. After a 1-h incubation in these media, WST-1 was added, and the extent of its conversion to formazan evaluated 3 h later as a measure of metabolic activity. It is important to note that although WST-1 is used in tests of both lethal stresses (cadmium, heat, H_2O_2) and in the evaluation of responses to low glucose, the decline in WST-1 reduction under low glucose conditions is not accompanied by cell death (Leiser *et al*., 2006).

Inhibition of cell metabolism by rotenone

Cells harvested and grown to the fourth passage as above were plated at a density of 3×10^5 per mL in DMEM with 20% fetal bovine serum with antibiotics and fungizone. After an 18-h overnight incubation, cells were washed once with 37 °C 1× PBS, and incubated in CM with a range of doses of the mitochondrial inhibitor rotenone (Sigma) dissolved in DMSO, or with DMSO only as a control. After a 1-h incubation in these rotenone doses, WST-1 was added and the extent of its conversion to formazan evaluated 3 h later. As in glucose assays, the conversion of WST-1 in rotenone assays is not accompanied by cell death (Leiser *et al*. 2006).

Calculation of LD_{50} and ED_{50} values, and statistical analysis

For calculation of the resistance of each cell line to chemical stressors, at each dose of chemical stressor, mean survival was calculated for triplicate wells for each cell line. The LD_{50}, i.e. dose of stress agent that led to survival of 50% of the cells, was then calculated using Probit analysis as implemented in NCSS software (NCSS, Kaysville, UT, USA). For this analysis, extremely low doses of stress agents that caused no cell death in fibroblasts, as measured by WST-1 assay, were censored from all data sets. ED_{50} values for glucose withdrawal and rotenone treatments were calculated in a similar manner to estimate the level of glucose or rotenone associated with a 50% reduction in cellular metabolic activity.

Estimates of species lifespan and body weight

Mammalian Species Accounts, published by the American Society of Mammalogists, were used as the source of body weight and estimated maximum lifespan: *Castor canadensis* (no. 120 in the Mammalian Species Accounts), *Erethizon dorsatum* (no. 29), *Myotis lucifugus* (no. 142), *Peromyscus leucopus* (no. 247), *Sciurus niger* (no. 479), *Tamiasciurus hudsonicus* (no. 586). In instances where there were no body weight or lifespan data recorded, or if there was no species account available, these data were provided by Dr. Steven Austad (personal communication). Lifespan and body weight data for the stock of laboratory-raised DC mice are from a laboratory population maintained by us under standard husbandry conditions (Miller

et al., 2002). Lifespan and body weight data for the animals tested in this study are presented in Table 1.

Standard linear regression

Initially, simple linear regression was used to examine the relationship between the reported species maximum lifespan and the mean LD_{50} for each of the eight stressors. A single lifespan estimate was used for each species.

Multilevel regression

In this analysis we assumed that the species were randomly selected from the entire spectrum of extant species, and consequently treat these data as an example of clustered observations. To evaluate the relationship between LD_{50} and species lifespan, we used a mixed effects/hierarchical model, more specifically a linear mixed effects model with a random intercept describing variation between species. We also evaluated, in parallel, linear and quadratic fixed effects associated with species-specific lifespan, and models allowing heterogeneity of the residual variance, but these models did not improve the fit to the data.

Phylogenetically independent contrasts

Phylogenetically independent contrasts were constructed using the PDTREE module in the Phenotypic Diversity Analysis Programs software package available from Dr. Theodore Garland, University of California, Riverside, CA, USA (Garland *et al*., 1993, 1999; Garland & Ives, 2000). The phylogenetic relationships used to construct the contrasts were compiled using published phylogenies derived from mitochondrial and nuclear gene sequence data (Adkins *et al*., 2003; DeBry & Sagel, 2001; DeBry & Seshadri, 2001; Lundrigan *et al*., 2002; Montgelard *et al*., 2002; Amrine-Madsen *et al*., 2003; Jansa & Weksler, 2004; Steppan *et al*., 2004a,b, 2005). A copy of the resulting phylogenetic tree is available from the authors upon request.

Regression on body weight residuals

Two sets of models were constructed to determine whether the relationship between lifespan and stress resistance might simply reflect the well-known relationship between increased lifespan with increasing body size (Austad, 2005). The first set of models was constructed using simple linear regression to examine the relationship between the stress resistance estimator (i.e. the mean LD_{50} or ED_{50} for each stressor) and the residual of lifespan against species-specific body weight. These residuals were calculated by Dr. João Pedro de Magalhaes using an allometric equation scaling the estimated maximum lifespan to body weight. The second set of models also used simple linear regression, but in this case the relationship between the residuals of each stress resistance measure (after the adjustment for body weight) and the residuals of the estimated maximum lifespan (also after the adjustment for body weight) was determined.

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