Reduced free-radical production and extreme longevity in the little brown bat (*Myotis lucifugus*) versus two non-flying mammals

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Abstract

The extended longevity of bats, despite their high metabolic rate, may provide insight to patterns and mechanisms of aging. Here I test predictions of the free radical or oxidative stress theory of aging as an explanation for differences in lifespan between the little brown bat, *Myotis lucifugus* (maximum lifespan potential MLSP = 34 years), the short-tailed shrew, *Blarina brevicauda* (MLSP = 2 years), and the white-footed mouse, *Peromyscus leucopus* (MLSP = 8 years) by comparing whole-organism oxygen consumption, hydrogen peroxide production, and superoxide dismutase activity in heart, kidney, and brain tissue. Mitochondria from *M. lucifugus* produced half to one-third the amount of hydrogen peroxide per unit of oxygen consumed compared to mitochondria from *B. brevicauda* and *P. leucopus*, respectively. Superoxide dismutase (SOD) activity did not differ among the three species. These results are similar to those found for birds, which like bats have high metabolic rates and extended longevities, and provide support for the free radical theory of aging as an at least partial explanation for the extreme longevity of bats.
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Keywords: Bats; *Blarina brevicauda*; Free radical theory; Longevity; *Myotis lucifugus*; Oxidative stress; *Peromyscus leucopus*

1. Introduction

1.1. Bats and aging

Research on aging and differences in longevity among species has been fueled by recent advances in gerontology and cellular biology and by the link between aging and diseases such as Parkinson's and Alzheimer's (Graff et al., 1999). Most eukaryotic organisms age; exceptions include some yeasts and metazoans that reproduce by paratomy (Bell, 1984; Osiewacz, 2002). Determining differences in aging patterns among species through comparative studies, and finding mechanisms underlying these patterns in long-lived organisms is key to understanding the aging process. We have known for a long time that bats are exceptionally long-lived mammals. Many bat longevity records date back to 1965–1975 (Tuttle and Stevenson, 1982) and were obtained by recapture of tagged, wild individuals. However,

to date few studies have focused on explaining the extended longevity of these animals (e.g. Bouliere, 1958; Wilkinson and South, 2002) and no studies have attempted to test recent physiological theories of aging in bats.

Several theories have been developed addressing ultimate and proximate causes of aging (for a review see Kirkwood and Austad, 2000; Austad, 2001). In general, mammalian longevities are inversely correlated with body size and metabolic rate (rate of living theory, Rubner, 1908; Pearl, 1928). Bats, however, are an exception. On average, bats live three times longer than other mammals with similar basal metabolic rates (Austad and Fischer, 1991). As an extension of the rate of living theory, it has been proposed that the long lifespan of bats is attributable to hibernation (Bouliere, 1958), when bats reduce body temperature to just above ambient temperature and slow down metabolism (Davis, 1970). Wilkinson and South (2002) found that hibernating bats tend to live longer than species that do not enter hibernation. However, hibernation cannot be the sole determinant of bat longevity considering non-flying mammals that hibernate show a lifespan predicted by their metabolic rates and many non-hibernating bats live longer than expected

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based on their metabolic rate (Tuttle and Stevenson, 1982).

1.2. The free radical theory of aging

A proximal theory of aging that has generated considerable interest is the free radical or oxidative stress theory (Harman, 1956), which proposes that aging is the result of cellular damage caused by free radical by-products of aerobic respiration. Mitochondria are responsible for the production of ATP in cells and in this process they also generate free radicals or reactive oxygen species, such as the superoxide ion and hydrogen peroxide. These chemicals are highly reactive and can damage a variety of cellular structures including nuclear DNA (Goyns, 2002) and cytoplasmic proteins and membranes (Sohal and Dubey, 1994; De Grey, 2002a,b), and interfere with lysosomal activity (Brunk and Terman, 2002). Mitochondria are the main sites of free radical generation in the cell and as such, they are also the chief targets of oxidative damage (Beckman and Ames, 1998). Damage to a mitochondrion accumulates over time making it less efficient at producing ATP and increasing the release of free radicals, resulting in even more damage. Eventually, the mitochondria of a cell cannot meet cellular energetic needs resulting in mitochondrial and/or cellular death (Harman, 1981; Rose et al., 2002). Under equal conditions, a high metabolic rate would be expected to lead to high free radical production. Thus, the free radical theory provides a potential mechanism for the correlation between metabolic rate and lifespan described by the rate of living theory.

The free radical theory of aging predicts that species with greater longevity mitigate free radical damage by producing fewer free radicals or by eliminating free radicals with greater efficacy. This theory has received support from a variety of studies. Ross (2000) found that age-related increases in free radical levels were lower in long-lived lines of fruit flies (*Drosophila melanogaster*) than in short-lived lines. Birds, which are long-lived and have high metabolic rates, produce fewer free radicals than mammals with similar metabolic rates and shorter lifespan (Herrero and Barja, 1998). Ku et al. (1993) found a negative correlation between free radical production and longevity of five mammal species ranging in size from a rat (*Rattus rattus*) to a cow (*Bos taurus*). Similar results have been found in other studies on fruit flies, birds, nematodes, amphibians, fish, and fungi (Larsen, 1993; Vanfletern, 1993; Barja et al., 1994a,b; Beckman and Ames, 1998; Harshman and Haberer, 2000; Osiewacz, 2002).

1.3. Free radical production in mammals

To date, work correlating free radical production to longevity in mammals (Ku et al., 1993; Sohal et al., 1990, 1993, 1994, 1995) has been done on species with longevities typical of their metabolic rates and body sizes. Therefore, it is difficult to tease apart whether the relationship reported between free radical production and longevity is a direct correlation or if it is due to the correlation in these animals between specific metabolic rate and longevity. Research on birds, however, indicates that in organisms with high metabolic rates and long lifespan, free radical production is a better predictor of longevity than metabolic rate. Based on these results, it is possible that free radical production is not always directly proportional to metabolic rate and free radical production is the important determinant of longevity (Loft et al., 1994; Pérez-Campo et al., 1998; Barja, 1999; Van Voorhies, 2001). Bats are metabolically similar to birds—endothermic animals with high metabolic rates and long lifespan. Measuring free radical production in bats may provide additional support to the importance of free radicals in determining longevity and the notion that metabolic rate and free radical production do not always covary linearly.

According to the free radical theory of aging, bats are long-lived because they reduce accumulation of free radical damage to their mitochondrial membranes, proteins, and DNA by producing fewer free radicals than other mammals of similar metabolic rate but shorter lifespan. Additionally, bats might mitigate damage by having increased antioxidant defenses. Some, but not all, studies have found that induction of endogenous antioxidants and treatment with exogenous antioxidants increases the mean lifespan of invertebrates (Allen et al., 1983; Miquel, 1983; Sohal et al., 1984) and vertebrates (Miquel, 1983; López-Torres et al., 1993a,b). Superoxide dismutase (SOD) is an induced enzyme that scavenges the superoxide ion and is a first line of defense against oxidative damage in the mitochondrion (Gralla, 1997; Touati, 1997). An increase in SOD activity to scavenge free radicals may result in decreased oxidative damage.

To assess the role of free radical production and SOD activity in determining bat longevity, I compared whole-organism oxygen consumption (an indirect measure of metabolic rate), free radical production, and SOD activity in kidney, heart and brain tissues from little brown bats (*Myotis lucifugus*), white-footed mice (*Peromyscus leucopus*), and short-tailed shrews (*Blarina brevicauda*). I chose these three tissues because they have been used in previous studies on free radical production and because heart and brain cells are post-mitotic whereas kidney cells are constantly replaced. The 8 g bat, *M. lucifugus*, has a record longevity of 34 years (Davis and Hitchcock, 1995) in the wild. Based on data from Wilkinson and South (2002), the longevity quotient of *M. lucifugus* (Austad and Fischer, 1991), which is the ratio of the actual longevity of a species and the longevity predicted based on body size alone, indicates that this bat lives 8.1 times longer than expected. Like most shrews, *B. brevicauda* (18–30 g) has a high metabolic rate and lives only 1–2 years in captivity. Survival is lower in the wild (Pearson, 1945). The longevity quotient of *B. brevicauda* is 0.5 or half the longevity predicted by its body size. I chose to include *P. leucopus* (15–25 g) in the study as an intermediate species with a long lifespan relative to other

rodent species (8 years in captivity; Sohal et al., 1993). The longevity quotient for *P. leucopus* is 1.5 or 50% longer than predicted based on body size. The longevity values I used in this study are the longest lifespan recorded for each species. I assumed this value to be equivalent to the maximum lifespan potential (MLSP) of a species. MLSP is the maximum lifespan of an organism in the absence of extrinsic mortality, which should be correlated with free radical production. While the MLSP for *B. brevicauda* and *P. leucopus* are from captive studies, which implies the absence of extrinsic mortality, the MLSP for *M. lucifugus* came from a wild population. Nonetheless, bats are exposed to few extrinsic mortality factors (such as predation) because of their nocturnal habits and ability to fly (Wilkinson and South, 2002).

Based on the free radical theory of aging, I predicted that despite a high metabolic rate *M. lucifugus* has lower free radical production than *P. leucopus* and *B. brevicauda*, and that free radical production of all three animals is inversely related to maximum lifespan. I also predicted that *M. lucifugus* has higher SOD activity than *P. leucopus* and *B. brevicauda*, and this increased activity contributes to their extended longevity.

2. Materials and methods

2.1. Collection of study animals

I collected five adult female little brown bats (*M. lucifugus*) from a nursery colony inhabiting the James J. Hill barn in North Oaks, Minnesota, (45°05.8′N, 93°06.2′W) from 8:00 to 10:00 h on 17 July 2002. Individuals chosen had visible teats and were lactating (evaluated by manual palpitation of teats) but had no young physically attached to them, indicating that they had given birth at least once, and therefore were at least 1 year old. I captured five adult female *P. leucopus* in a wooded knoll in Cedar Creek Natural History Area, Minnesota, (45°26.1′N, 93°13.2′W) from 23 August to 2 September 2001 and four female and three male adult (determined post-humously) *B. brevicauda* along a railroad crossing in Glencoe, Minnesota, (44°45.2′N, 94°12.5′W) during 15–18 August 2002. All female *P. leucopus* had visible teats but were not lactating. I used standard Sherman traps baited with rolled oats and peanut butter and set overnight to capture *P. leucopus* and *B. brevicauda*.

2.2. Oxygen consumption measures

Upon capture, I placed animals in individual dark cloth bags in which they remained until tissues were harvested. These bags were left undisturbed and moved only when necessary, to reduce the amount of handling stress to the animal. I measured resting metabolic rates of all animals between 12:00 and 16:00 h on the day of capture, when the animals were post-absorptive (Kronfeld-Schor et al., 2000). I placed an individual animal, still within its cloth bag, in a closed chamber (1 gallon paint can) with a carbon dioxide absorbant (Malcosorb, Fisher Scientific, Springfield, NJ). The animals were allowed to acclimatize to the chamber for 20 min before measuring oxygen consumption. Attached to the air-tight chamber was an S-shaped glass tube, a section of which contained water, and a graduated syringe containing oxygen. The consumption of oxygen inside the chamber caused the meniscus of the water to travel in the glass tube. I constantly added oxygen from the syringe to the chamber returning the water meniscus to its original location to replace the oxygen consumed by the animal. I recorded the oxygen consumed every 2 min to ensure a constant rate of oxygen consumption and continued this process for 1 h. To ensure stable temperature during the oxygen consumption measures, the chambers were submerged in water at 22 °C (room temperature) and all results were adjusted to 25 °C and 1 atm pressure.

2.3. Tissue harvest and hydrogen peroxide production measures

The evening (1700–2000 h) after measuring oxygen consumption, I sacrificed the animals through anesthesia with isoflurane and cervical dislocation. All animals were sacrificed within 2 h after sunset, a time at which all three species would be active. I harvested brain, heart, and kidney tissues and stored them in mitochondrial isolation buffer (0.3 M sucrose, 0.01 M EGTA, 0.005 M MOPS, 0.005 M H_2PO_4, 0.1% BSA, pH 7.4) at 4–8 °C. Because free radical flux is linked to metabolic rate, production of hydrogen peroxide could change as quickly as oxygen consumption and I cannot affirm that the measured oxygen consumption of an individual was the same at the time of tissue harvest. However, to make interspecific comparisons of free radical production it was more important to harvest the tissues at a time when all animals would normally be active, and thus circadian rhythms did not affect results.

I measured hydrogen peroxide production of mitochondria in tissues as a proxy for free radical production. The measurement of hydrogen peroxide production by mitochondria is correlated with damage caused by reactive oxygen species to the inner mitochondrial membrane (Sohal and Dubey, 1994), and this damage is positively correlated with free radical or reactive-oxygen species production. Live, intact mitochondria were extracted from the harvested tissues by gently pounding the tissues in a chilled mortar. The mitochondria in the resulting homogenate were isolated by differential centrifugation in mitochondrial isolation buffer at $700 \times g$ for 10 min and then $10,000 \times g$ for 10 min. Using the protocol of Hyslop and Sklar (1984), the oxidation of *para*-hydroxyphenylacetate (PHPA) during the reduction of hydrogen peroxide by horseradish peroxidase provides a quantitative measure of hydrogen peroxide production by mitochondria. I conducted this assay at 22 °C and determined the rate of generation by monitoring the increase in fluorescence by oxidized PHPA at an excitation maximum

of 360 nm and an emission maximum of 436 nm with a G.K. Turner Model 111 fluorometer.

2.4. Superoxide dismutase activity measures

I measured SOD activity in tissue homogenates using the NBT–BCS assay (Spitz and Oberley, 1989). This assay monitors the reduction of nitro-blue-tetrazolium to blue formazan by a superoxide ion flux at 560 nm and 22 °C in a Beckman Genesys 5 spectrophotometer. SOD in sample tissues inhibits the reaction and the percent inhibition is correlated to the activity of SOD in the sample. To standardize results, I divided hydrogen peroxide production and SOD activity by the total protein content of the isolated mitochondria and the homogenates, respectively. I measured protein content using a total protein measuring kit based on the Hartley–Lowery assay (Cat. No. A-610, Sigma–Aldrich, Saint Louis, MO). Protein concentration is correlated with a change of dye color, which I measured in a Genesys 5 spectrophotometer at 595 nm.

2.5. Statistical analyses and mitochondrial inefficiency measure

I used a one-way ANOVA with a critical value of 0.05 followed by a two-way Tukey–Kramer multiple comparison (JMP statistical package, SAS Institute Inc., Cary, NC) which controls the simultaneous confidence level at 95%, to test for differences in hydrogen peroxide production and SOD activity among the three species. Normality of the data was tested using a quantile–quantile plot (JMP statistical package, SAS Institute Inc., Cary, NC) and no transformations of the data were necessary. Additionally, I calculated the mitochondrial inefficiency of each tissue for each species. This value is a measure of the amount of free radicals produced in a tissue in relation to the amount of oxygen consumed by the individual—in other words, the free radical production in 1 h divided by the amount of oxygen consumed in 1 h per gram of body mass. To assess the relationship between mitochondrial inefficiency (a continuous variable) with MLSP (a categorical variable), I conducted a logistic regression (JMP statistical package, SAS Institute Inc., Cary, NC).

3. Results

3.1. Oxygen consumption

Because there were no significant differences in oxygen consumption, free radical production, and SOD activity between the two sexes in *B. brevicauda*, I pooled the data for all analyses. The oxygen consumption (ml O_2/(g h)) measures I obtained for each species were within the range of those recorded in the literature (Neal and Lustick, 1973; Buckner, 1964; Glazier, 1985; Kurta and Kunz, 1988), though the measures obtained for *M. lucifugus* were on average higher than those obtained by Kurta and Kunz (1988) for solitary, lactating female *M. lucifugus*. Oxygen consumption differed significantly among the tested species. *B. brevicauda* and *M. lucifugus* consumed similar amounts of oxygen per gram of body mass and approximately four times more than that consumed by *P. leucopus* ($P = 0.0001$; Table 1).

3.2. Free radical production and mitochondrial inefficiency

The average hydrogen peroxide production in all three tissues of *B. brevicauda* was at least twice as high as in tissues from *M. lucifugus* and *P. leucopus* (brain: $F_{2,14} = 183.2$; heart: $F_{2,14} = 11.9$; kidney: $F_{2,14} = 31.5$; $P = 0.001$ for all comparisons; Table 1). While hydrogen peroxide production was higher in all three tissues of *M. lucifugus* relative to tissues from *P. leucopus*, Tukey–Kramer multiple comparisons indicated that these differences were statistically significant ($P < 0.05$) only in the brain tissues. The mitochondrial inefficiency measures calculated for all three tissues of *M. lucifugus* were lower than those calculated for *B. brevicauda* and *P. leucopus*, though significantly so only in brain and kidney tissues (brain: $F_{2,14} = 13.16$, $P = 0.0006$; heart: $F_{2,14} = 3.03$, $P = 0.081$; kidney: $F_{2,14} = 6.78$, $P = 0.0087$; Fig. 1).

3.3. Superoxide dismutase activity

I present only SOD activity measured in heart and brain tissues because kidney homogenates produced results with extreme variation, likely due to differences in the histology of cortex and medulla tissues, which I did not separate prior

Table 1
Oxygen consumption, hydrogen peroxide production, and SOD activity measured for *B. brevicauda* ($N = 7$), *M. lucifugus* ($N = 5$), and *P. leucopus* ($N = 5$)

	Oxygen consumption (ml O_2/(g h))	Hydrogen peroxide production (nmol H_2O_2/min µg protein)			SOD activity (U SOD/mg protein)	
		Brain	Heart	Kidney	Brain	Heart
B. brevicauda (18–30 g)	4.19 ± 0.36[a]	201.97 ± 5.2[a]	119.07 ± 13.2[a]	116.97 ± 8.1[a]	37.46 ± 13.1[a]	47.42 ± 9.5[a]
M. lucifugus (7–10 g)	4.83 ± 0.30[a]	85.07 ± 6.2[b]	50.63 ± 15.6[b]	47.91 ± 9.6[b]	38.23 ± 7.7[a]	94.87 ± 28.6[a]
P. leucopus (15–25 g)	1.19 ± 0.05[b]	60.25 ± 6.2[c]	24.76 ± 15.6[b]	22.93 ± 9.6[b]	29.25 ± 5.7[a]	45.32 ± 15.2[a]
Probability > *F*	0.0001	<0.0001	0.001	<0.0001	0.8291	0.1831

Values are means of sample plus standard error calculated from a pooled estimate of error variance. Bottom row are statistical significance of *F*-test comparing the three species. Superscript letters indicate statistically significant differences between species based on a Tukey–Kramer multiple comparison.
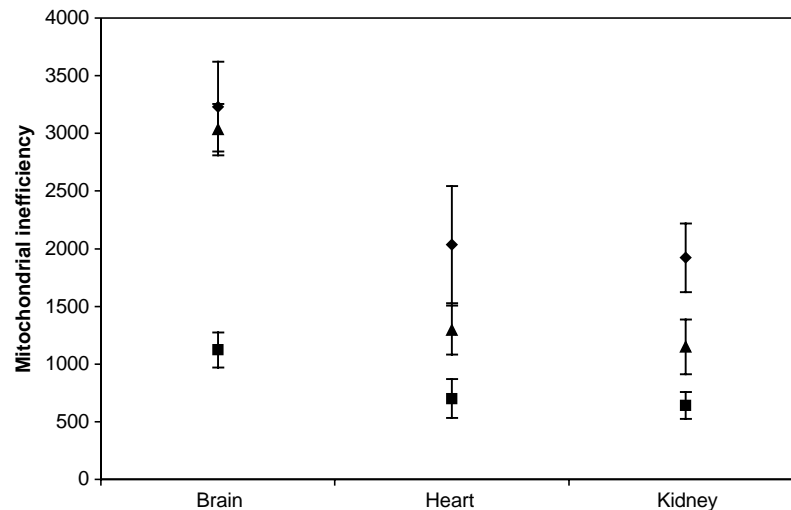
Fig. 1. Mean mitochondrial inefficiency (nmol H_2O_2 per μg protein/ml O_2 per g body mass) over 1 h for brain, heart, and kidney tissues of *B. brevicauda* (◆), *M. lucifugus* (■), and *P. leucopus* (▲). Bars indicate standard error of the mean.

to preparing the homogenate. There were no significant differences in SOD activity of brain and heart tissue among the three tested species (brain: $F_{2,13} = 0.19$, $P = 0.83$; heart: $F_{2,11} = 1.99$, $P = 0.18$; Table 1).

4. Discussion

4.1. Mitochondrial inefficiency

As predicted by the free radical theory of aging, *B. brevicauda*, which has the shortest lifespan of the three tested species, had the highest level of hydrogen peroxide production. Contrary to my prediction, *M. lucifugus* did not have the lowest level of hydrogen peroxide production. Although the difference was significant only in brain tissue, *M. lucifugus* produced higher levels of hydrogen peroxide than *P. leucopus* in all three tissues. However, when one takes into account the amount of oxygen consumed by these two species, *M. lucifugus* actually produces less hydrogen peroxide per unit of oxygen consumed. Over 90% of the oxygen consumed by an endotherm is used by mitochondria, and thus consumption is correlated with ATP production (Richter and Schweizer, 1997). Under the free radical theory of aging, a mitochondrion that produces large amounts of ATP while producing low amounts of free radicals is considered an efficient mitochondrion, as it meets cellular energetic demands with a reduced risk of oxidative damage. In 1 h, the five *P. leucopus* I tested consumed an average of 1.19 ml O_2/g body mass. During that hour, brain tissue of those animals produced an average of 3615 nmol H_2O_2/μg mitochondrial protein. In other words, *P. leucopus* produced 3038 units of H_2O_2 per unit (ml O_2/(g h)) of O_2 consumed. I refer to this ratio as the mitochondrial inefficiency of an organism. *M. lucifugus*, on the other hand, consumed on average 4.83 ml

O_2/g body mass in 1 h and produced 5104 nmol H_2O_2/μg brain mitochondrial protein during that time. This results in a mitochondrial inefficiency of 1057 units of H_2O_2 per unit of O_2 consumed. Thus, taking into account oxygen consumption as a proxy for ATP production, brain tissue from *M. lucifugus* produced approximately one-third the amount of hydrogen peroxide produced by brain tissue from *P. leucopus* for every unit of oxygen consumed (Fig. 1). Comparing *M. lucifugus* to *B. brevicauda*, mitochondrial inefficiency values for all three tissues from *M. lucifugus* were approximately one-third of the values for *B. brevicauda* (Fig. 1). Overall, *M. lucifugus* had reduced mitochondrial inefficiency (lower values indicating reduced free radical production per unit of oxygen consumed) in all three tissues relative to both *B. brevicauda* and *P. leucopus* (Fig. 1), although the differences were statistically significant only in brain and kidney tissues. According to the Tukey–Kramer multiple comparisons, these differences are due to *M. lucifugus* having significantly lower mitochondrial inefficiency values than *B. brevicauda* and *P. leucopus* in brain tissue and significantly lower mitochondrial inefficiency values than *B. brevicauda* in kidney tissue. These data provide evidence in support of the free radical theory of aging as at least a partial explanation for the extended maximal longevity of *M. lucifugus*.

Pérez-Campo et al. (1998) argue that free radical production levels should not be divided by metabolic rates or oxygen consumption prior to assessing correlation with maximum lifespan. When measuring free radical production in species with longevities typical of their metabolic rate, it is possible that the relationship between longevity and free radical production is simply a result of differences in metabolic rate, which correlates with lifespan. In other words, lower metabolic rate leads to lower free radical production, which in turn is negatively correlated with longevity as a consequence of the correlation between metabolic rate and longevity. However, for animals in which metabolic rate

is not correlated with longevity as predicted by the rate of living theory, as is the case of *M. lucifugus*, high respiratory activity obscures the relatively low production of free radicals when making comparisons between species of unequal metabolic rates. I believe in this case oxygen consumption must be considered as I have shown above.

The low hydrogen peroxide production in *M. lucifugus* relative to *B. brevicauda* despite similarly high metabolic rates lends support to the contention that free radical production is not always proportional to metabolic rate. It appears that bats, like birds, have mitochondria that are able to produce the high quantities of ATP necessary for flight functions in these animals, while producing relatively low amounts of free radicals.

I did not measure body temperature of the study subjects. Considering that lowering body temperature by 1 °C decreases oxygen consumption by 11% in vertebrates (Wood, 1995), this may have confounded results. All subjects were active and immediately responded to touch. Because I conducted this study during the summer, I assumed that body temperature in *B. brevicauda* and *P. leucopus* were relatively constant throughout the day because neither is likely to enter torpor during the summer. *B. brevicauda* never enters torpor (Dawson and Olson, 1987) and based on monitoring of daily metabolic patterns, *P. leucopus* does not appear to enter torpor during the summer (Randolph, 1980). *M. lucifugus* on the other hand, were collected during the day when they are most likely in torpor. This entails a decrease in body temperature to ambient temperature (Davis, 1970), which is accompanied by a decrease in oxygen consumption. In other words, the oxygen consumption I measured in the sample of *M. lucifugus* is potentially an underestimate of oxygen consumption at dusk, when tissues were harvested to measure hydrogen peroxide production and SOD activity. Slightly higher oxygen consumption in *M. lucifugus* would have resulted in even lower values for mitochondrial inefficiency, strengthening the observation of lower free radical production in this species.

Conversely, the *M. lucifugus* used in this study were all lactating females and this may have caused elevated metabolic rates associated with the energetic expense of lactation. While the whole-body oxygen consumption measures obtained for these five animals were within the range of values recorded in the literature, they were on average higher than the mass-specific oxygen consumption measures obtained by Kurta and Kunz (1988) from solitary, lactating females (4.83 ± 0.3 versus 3.81 ± 0.31 ml $O_2/(g\,h)$). If I recalculate the mitochondrial inefficiency values for *M. lucifugus* using the average oxygen consumption from Kurta and Kunz (1988), the values increase but are still lower than the mitochondrial inefficiency of the three tissues from *B. brevicauda* and *P. leucopus*. Additionally, the statistically significant differences among the three species (in brain and kidney tissues) remain. It is interesting to note that the oxygen consumption measures for lactating female *M. lucifugus* obtained in this study

are lower than the oxygen consumption measures obtained by Kurta and Kunz (1988) for solitary male *M. lucifugus* (5.48 ± 0.18 ml $O_2/(g\,h)$).

Another caveat to the interpretation of these data is the fact that oxygen consumption was not measured in the isolated tissues and mitochondria used to measure H_2O_2 production and SOD activity. Kidney, heart, and brain tissues likely have differing energetic needs. Thus, tissue and mitochondrial oxygen consumption may differ from whole-organism oxygen intake as well as among the tissues. Future studies will include measures of tissue and organelle metabolism.

4.2. Superoxide dismutase activity

Contrary to my prediction, SOD was not higher in brain or heart tissue of *M. lucifugus* relative to the two non-flying mammals (Table 1). An alternative hypothesis would be that SOD activity is lowest in *M. lucifugus* because its mitochondria produce the lowest amounts of free radicals per unit oxygen consumed. However, data from this study do not support this hypothesis as there were no significant differences in SOD activity among the three species. The lack of correlation between SOD activity and MLSP corroborate observations by Pérez-Campo et al. (1998) that while manipulations of antioxidants (including SOD) increase *mean* lifespan, antioxidant levels are not correlated or are negatively correlated with *maximum* lifespan in a variety of mammals. Originally, the data used by Pérez-Campo et al. were presented as positively correlated with maximum lifespan (Tomalsoff et al., 1980; Cutler, 1984, 1986), but only after measured antioxidant levels were divided by basal metabolic rate of the whole animal. Pérez-Campo et al. (1998) argue that the correlation was the result of dividing antioxidant levels by basal metabolic rate, which correlates positively with maximum lifespan in the animals tested. It is interesting that when comparing distantly related species (Pérez-Campo et al., 1998; this study), SOD activity does not correlate with MLSP. Yet, when comparing two more closely related species (*Mus musculus* and *P. leucopus*; Sohal et al., 1993) SOD activity is higher in the longer-lived organism. These observations raise questions about the role of antioxidant up-regulation at different times in the evolution of long-lived organisms and what selective pressures may have selected for increased or decreased investment in antioxidant defenses (Beckman and Ames, 1998).

Superoxide dismutase activity does not appear to contribute to maximum lifespan extension in *M. lucifugus*. This makes sense as it is likely less costly for a bat to reduce free radical production than to maintain a heightened and expensive defense system, which can never be 100% efficient (Barja, 1999). However, SOD is not the only free radical scavenger present in an organism but rather, the defense system is a coordinated plethora of antioxidants including enzymatic scavengers such as SOD and catalase, exogenous molecules such as vitamins, hydrophilic radical scavengers such as ascorbate and glutathione, and other molecules

responsible for maintaining a reducing environment in the cell. Additionally, the nature of this defense system differs among species and among tissues within an individual (Beckman and Ames, 1998). Because cells function in a set reducing environment (Van Voorhies, 2001), changes in the levels of any of these antioxidant defenses would necessarily affect all other antioxidants and many of these antioxidants are inducible (Beckman and Ames, 1998). A full understanding of the role of antioxidants in determining the extended longevity of *M. lucifugus* requires measures of several antioxidants, isolated from various tissues and at various points in the lifetime of the animal.

4.3. Mechanisms for reduced free radical production in M. lucifugus

It has been proposed that low efficiency of oxidative phosphorylation can lead to extended longevity through the overexpression of antioxidant genes in response to increased free radical production (Esposito et al., 1999; Kirchman et al., 1999). Although measures of antioxidant activity in *M. lucifugus* other than SOD are necessary to fully assess this potential mechanism, my data indicate that mitochondria in *M. lucifugus* are instead simply producing low levels of free radicals per unit of oxygen consumed compared to *B. brevicauda* and *P. leucopus*. This is particularly intriguing considering the very high metabolic rates observed in bats during periods of high activity (i.e. during flight). However, mitochondrial free radical production does not necessarily increase in state-III-respiration relative to state-IV-respiration, despite higher oxygen consumption (Boveris and Chance, 1973; Richter and Schweizer, 1997). State-IV-respiration is characterized by steady mitochondrial oxygen consumption when ADP substrate is limited (a state of rest) and state-III-respiration is characterized by an increase in mitochondrial oxygen consumption when ADP substrate is not limited (an active state). While typically mitochondria in tissues are in respiratory states intermediate to states III and IV (Barja, 1999), we can learn about how respiratory state influences free radical production by measuring hydrogen peroxide production in mitochondria transitioning between states IV and III. Studies on heart and brain mitochondria from parakeets (*Melopsittacus undulates*), canaries (*Serinus canarius*), and laboratory mice (*Mus musculus*, OF1 strain from Iffa-Creddo, Lyon, France) did not show increases in oxygen radical production proportional to the increase in tissue oxygen consumption associated with the transition from state IV to III (Herrero and Barja, 1997, 1998). This phenomenon might serve to prevent overwhelming antioxidant defenses during increased respiratory activity and might also explain why life-long exercise does not decrease MLSP or mean lifespan in humans (Lee et al., 1995) or rodents (Holloszy et al., 1985).

The reduced free radical production in state III may be in part due to the less reduced state of electron carriers of the electron transport chain in state III, as less reduced electron carriers are less likely to leak electrons to oxygen, and thus produce reactive species (Barja, 1999). An alternative but related explanation may be that mitochondria in a cell use different mechanisms for the production of ATP during exercise. Cairns et al. (1998) point out that heart and brain tissue, which have high energetic demands, produce additional ATP needed through the TCA cycle pathway. With this pathway, electrons from $FADH_2$ enter the electron transport chain at complex II (instead of complex I) and the proton extrusion that generates the membrane potential needed for ATP synthesis takes place at only complexes III and IV. This pathway, while thermodynamically less efficient because it requires more electrons and more oxygen, produces a fast flux of ATP. Because electrons enter the electron transport chain at complex II, complex I, which is a principle generator of free radicals in the electron transport chain (Richter and Schweizer, 1997; Barja, 1999), does not become as reduced in this pathway. The lower state of reduction would reduce the amount of electrons it leaks to oxygen. A similar result has been reported for the fungus *Podospora anserina* (Osiewacz, 2002). Mutant strains of this fungus use an alternative pathway in the electron transport chain because their mutation impairs function of complex IV. This alternative pathway transfers electrons to oxygen to make water upstream from complex III, preventing the generation of reactive oxygen species at that complex. Indeed, generation of reactive oxygen species is lower in these mutants than in wild-type strains (Dufour et al., 2000) and the mutants exhibit lifespan increases of about 60%.

4.4. Longevity likelihood based on mitochondrial inefficiency measures

How well does mitochondrial inefficiency predict the likelihood of an organism to have an MLSP of 2, 8 or 34 years? Logistic regression models between MLSP and mitochondrial inefficiency of each tissue type tested had statistically significant fit (brain: $P < 0.0003$, heart: $P < 0.015$, kidney: $P < 0.0036$; Fig. 2A–C). Interpreting the model results from the brain tissue, the log odds ratio indicates that at a mitochondrial inefficiency of 1200 units of H_2O_2 per unit of O_2 consumed, the likelihood of living 2 years is 5%, of living 8 years is 5%, and of living 34 years is 90% (Fig. 2A). Whereas with a mitochondrial inefficiency of 3000 units of H_2O_2 per unit of O_2 consumed, the likelihood of living 2 years increases to 55%, of living 8 years increases to 45%, and of living 34 years decreases to 0%. In fact, in the logistic regression models for all three tissues, mitochondrial inefficiencies above 2700 units of H_2O_2 per unit of O_2 consumed result in a 0% probability of living 34 years.

Inclusion of data from other studies and species into these models is not possible. To begin with, methods used often differ among studies. Secondly, all studies to date have used domesticated or laboratory-reared animals as study subjects, except perhaps the *P. leucopus* used by Sohal et al. (1993), though they do not provide information on the origin of these
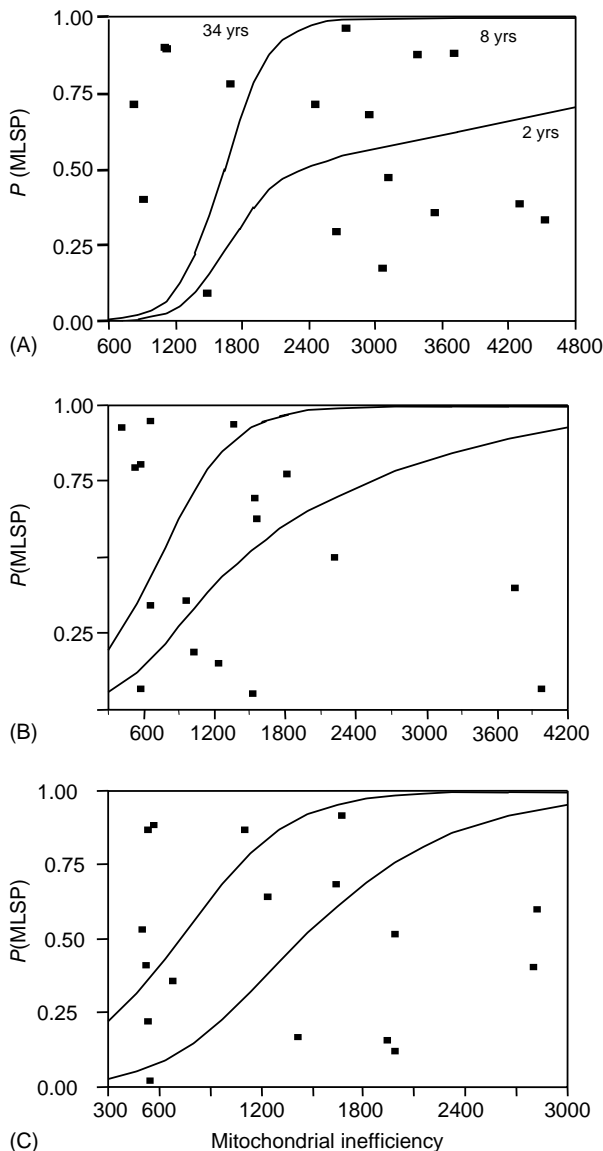
significantly lower than in the wild population tested for this study (25.7 ± 4.07 versus 85.1 ± 3.17 nmol H_2O_2/min µg mitochondrial protein, Student's t-test, $t = 3.7$, $P < 0.0001$; Brunet-Rossinni, unpublished data). The lower hydrogen peroxide production in the captive population may be due to decreased activity, since this population was restricted to a small cage for three months and ate mealworms out of a dish instead of having to forage for food. Lastly, even if subjects tested in other studies were from wild populations, environmental and dietary differences between populations are likely to affect metabolism and free radical production.

M. lucifugus produced fewer free radicals per unit of oxygen consumed than *B. brevicauda* and *P. leucopus*, while SOD activity did not differ between these three species. Like in other studies, these data lend support to the free radical theory of aging as at least a partial explanation for differences in lifespan among species. Because hydrogen peroxide production does not appear to correlate directly with metabolic rate in *M. lucifugus*, knowledge about the free radical production in *M. lucifugus* at different times of the year and during different activities is required for a more rigorous assessment of their lifetime production of free radicals. However, I present the first assessment of oxygen radical generation in bat mitochondria and I believe that more in-depth studies on variation in metabolic rates, free radical production and antioxidant capacity in bats can provide substantial insight to the mechanisms and patterns of aging.

Acknowledgements

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Fig. 2. Logistic probability plot for the logistic regression model fit to mitochondrial inefficiency (nmol H_2O_2 per µg protein/ml O_2 per g body mass) of brain (A), heart (B), and kidney (C) tissue and MLSP of *M. lucifugus*, *B. brevicauda*, and *P. leucopus* ($P < 0.0003$). Markers for the data are drawn at their x-coordinate (mitochondrial inefficiency), with the y-position jittered randomly within the probability space corresponding to the MLSP category (2, 8 or 34 years). The curves represent probability clines for each category of MLSP. At any given mitochondrial inefficiency value, the corresponding y-coordinate on the bottom curve represents the probability of having an MLSP of 2 years. The difference between the y-coordinates on the second curve and the bottom curve represents the probability of having an MLSP of 8 years, and the remaining distance on the y-axis corresponds to the probability of having an MLSP of 34 years.

animals. The subjects of my study were all wild-caught and there are likely to be differences in the metabolism and free radical production of captive versus wild populations (Van Voorhies, 2001). In fact, hydrogen peroxide production by brain mitochondria from five adult *M. lucifugus* kept in captivity from the end of May to mid-August 2001, was

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