Pergamon

Free Radical Biology & Medicine, Vol. 19, No. 4, pp. 499–504, 1995

0891-5849/95 $9.50 + .00

0891-5849(95)00037-2

Brief Communication

MITOCHONDRIAL SUPEROXIDE AND HYDROGEN PEROXIDE GENERATION, PROTEIN OXIDATIVE DAMAGE, AND LONGEVITY IN DIFFERENT SPECIES OF FLIES

RAJINDAR S. SOHAL, BARBARA H. SOHAL, and WILLIAM C. ORR
Department of Biological Sciences, Southern Methodist University, Dallas, TX, USA

(*Received* 4 *November* 1994; *Revised* 30 *January* 1995; *Accepted* 16 *February* 1995)

Abstract—The objective of this study was to further elucidate the role of oxidative stress in the aging process by determining whether or not the rates of mitochondrial superoxide anion radical and hydrogen peroxide (H_2O_2) production, the activity of cytochrome *c* oxidase, and the concentration of protein carbonyls are correlated with the life span potential of different species. A comparison was made among five different species of dipteran flies, namely, *Drosophila melanogaster* (fruit fly), *Musca domestica* (house fly), *Sarcophaga bullata* (flesh fly), *Calliphora vicina* (blow fly) and *Phaenecia sericata* (a species of blow flies), which range more than 2-fold in their life span potentials. The average life span potential of these species was found to be inversely correlated with the rates of mitochondrial superoxide and H_2O_2 production and with the level of protein carbonyls, and to be directly related to the activity of cytochrome *c* oxidase. The significance of these findings in context of the validity of the oxidative stress hypothesis of aging is discussed. It is inferred that longer life span potential in these insect species is associated with relatively low levels of oxidant generation and oxidative molecular damage. These results accord with our previous findings on different mammalian species.

Keywords—Free radicals, Aging, Cytochrome oxidase, Insects, Life spans, Mitochondria, Oxidative damage, Reactive oxygen species

INTRODUCTION

A current hypothesis concerning the underlying causes of the aging process implicates oxidative molecular damage as a contributing factor.[1–5] Such damage is believed to arise from an imbalance between the metabolically generated reactive active oxygen species (ROS) and the antioxidative defenses. The detection of oxidatively modified molecules, such as protein carbonyls and 8-hydroxydeoxyguanosine, considered to be products of protein[3,4] and DNA oxidations,[5] respectively, in healthy organisms has provided support for the idea that tissues are under a certain level of oxidative stress even under normal physiological conditions.

The objective of this study was to test the predictions of the hypothesis that oxidative stress is a causal factor in the aging process. It can be reasoned that if this hypothesis were valid, the life span potentials of closely related species would be inversely related to the level of oxidative stress. Because oxidative stress is dependent upon the imbalance between two factors, namely, pro-oxidants and antioxidants, it may be predicted that life span potential would be inversely related to the former and directly related to the latter.

A previous study in this laboratory on a group of mammalian species, with a similar metabolic potential, indicated that life span potential (MLSP) was inversely related to rates of mitochondrial superoxide anion radical and hydrogen peroxide (H_2O_2) generation.[6] Conversely, no clear-cut relationship was discernable between MLSP and the overall level of antioxidative defenses, comprised of activities of superoxide dismutase, catalase, and glutathione peroxidase, and concentration of glutathione.[7]

One of the currently unresolved controversies in gerontology is whether or not the fundamental causes of the aging process are universal, transcending phylogenetic boundaries.[8] For example, is senescence in insects and mammals due to the involvement of at least some common mechanisms?

This research was supported by a grant (RO1 AG7657-06) from the National Institutes of Health—National Institute on Aging, Bethesda, MD.

Address correspondence to: Rajindar S. Sohal, Department of Biological Sciences, Southern Methodist University, Dallas, TX 75275, USA.

In context of this rationale, the present study was conducted on five closely related species of (dipteran) flies to determine whether the variations in their average life span potential (ALSP) were indeed inversely correlated with rates of mitochondrial superoxide anion radical and H_2O_2 generation as found previously in the mammalian species.[9] An explanation of the variations in mitochondrial oxidant generation was sought by the comparison of cytochrome *c* oxidase activity in different species. In addition, the relationship between ALSP and molecular oxidative damage was examined by comparing the concentration of protein carbonyls, which are believed to be specific products of protein oxidation.[3,4] Results suggest a similarity between mammals and insects in these oxidative stress-related factors.

MATERIALS AND METHODS

Animals

After emergence from the pupae, adult flies were segregated by sex and kept at 25°C and 50% relative humidity. Excepting *Drosophila,* they were fed on sucrose and water; *Drosophila* were fed on the standard mixture of sterilized cornmeal-sugar-(inactive) yeast-agar. Previous studies on the effect of diet on life span of adult dipteran flies in this laboratory and elsewhere have indicated that flies live longest on sucrose-alone diet as compared to a more complex protein- or fat-containing diet.[10] Only male flies were used in this study.

Measurement of mitochondrial superoxide anion radical and H_2O_2 production

Mitochondria were isolated from the thoracic flight muscles of flies of specific ages by a modification of the method by Wood and Nordin,[11] as described previously.[12] Briefly, the thoraces were gently crushed in buffer A containing 154 mM KCl and 1 mM EGTA (pH 7.0) with a mortar and pestle, avoiding grinding action, which can cause physical damage to mitochondria. The resulting mash was filtered through eight layers of muslin and the filtrate was centrifuged at 150 $\times$ *g* for 3 min; the supernatant was centrifuged at 3000 $\times$ *g* for 8 min and the pellet was resuspended in the specified buffer.

Rate of superoxide anion radical generation was measured in submitochondrial particles (smps) as superoxide dismutase (SOD)-inhibitable reduction of acetylated ferricytochrome *c*, as described previously.[9] Both the system and the reference cuvette contained 30–50 μg smp protein, 0.1 M potassium phosphate buffer (pH 7.4), 7.8 μM acetylated ferricytochrome *c*, 0.6 μM antimycin A, and 20 mM α-glycerophosphate; the reference cuvette contained in addition 120 units of SOD/ml.

The rate of H_2O_2 released by isolated mitochondria was measured fluorometrically by the method of Hyslop and Sklar,[13] which is based on the coupled oxidation of *p*-hydroxyphenylacetic acid (PHPA) and reduction of H_2O_2 by horseradish peroxidase, as described previously.[14] The reaction mixture consisted of buffer (154 mM KCl, 5 mM phosphate, 3 mM $MgCl_2$, and 0.1 mM EGTA, pH 7.4), mitochondria (10–30 μg protein), 1.1 mM PHPA, 1.3 units of horseradish peroxidase/ml (Sigma Type VI), and 7 mM α-glycerophosphate. Intact insect mitochondria are rather impermeable to succinate but readily use α-glycerophosphate. The rate of H_2O_2 generation was measured as an increase in fluorescence at an excitation maximum of 320 nm and emission maximum of 400 nm using a Perkin Elmer LS-5 spectrofluorometer. Known concentrations of H_2O_2 were used to construct a standard curve.

Measurement of cytochrome c oxidase activity and protein carbonyl content

Activity of cytochrome *c* oxidase was measured in the sonicated mitochondria by the method described by Birch-Machin et al.[15] (without potassium hexacyanoferrate). The reaction mixture consisted of 20 mM phosphate (pH 7.0), 3 mM cytochrome *c* (II), 30 mM dodecyl-*b*-*d*-maltoside and 0.1–0.2 μg mitochondrial protein. The rate of oxidation of cytochrome *c* (II) was measured at 550 nm with 580 nm being the reference wavelength, using an extinction coefficient of $\Delta^{\epsilon\ \text{red-ox}} = 18.7\ \text{mM}^{-1}\ \text{cm}^{-1}$.

Protein carbonyl content was measured in the whole body homogenates of the flies by the procedure of Levine et al.[16] using dinitrophenylhydrazine (DNPH), as described previously.[17]

Table 1. Average Life Span Potential of Different Species of Dipteran Flies

Species	Common Name	Average Life Span (Days)	*N*
Phaenecia sericata	Blow fly	29.5 ± 2.8	33
Calliphora vicina	Blow fly	29.7 ± 2.2	32
Sarcophaga bullata	Flesh fly	49.0 + 3.2	36
Musca domestica	House fly	53.5 ± 1.4	105
Drosophila melanogaster	Fruit fly	65.5 ± 1.8	80

Values are mean ± SEM. Excepting *Drosophila,* flies were confined individually in ~150 ml glass jars; *Drosophila* were kept in 8-dram shell vials.

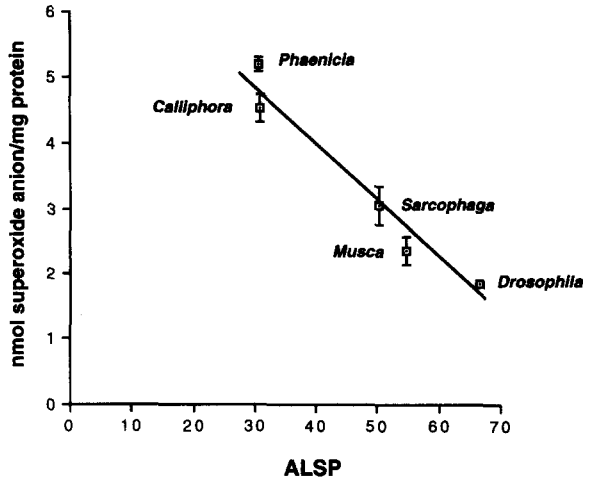

Fig. 1. Relationship between the rate of mitochondrial superoxide anion radical generation and the average life span potential (ALSP) in five different species of dipteran flies. The rate of superoxide generation was measured in submitochondrial particles from flight muscles as SOD-inhibitable reduction of acetylated ferricytochrome *c* using α-glycerophosphate as a substrate and antimycin A as a respiratory inhibitor. Values are average ±SD of four to six determinations.

RESULTS

Determination of ALSP

Determination of ALSP in flying insects requires careful procedural standardization because insect longevity is strongly influenced by the environmental conditions affecting the level of physical activity. Factors such as population density, sexual aggressiveness, and the size of the housing containers, have all been shown to have a significant effect on life span.[18] To avoid potential complications, it was decided to determine the average life span of flies that were singly confined in 150 ml glass jars, except for *Drosophila,* which were kept in the usual 8 dram shell vials. Maximum life span was not used because in some cases it did not correspond to the average survival period, as an occasional fly would sometimes inordinately linger on in a doddered condition.

The average life spans of the different species of flies, listed in Table 1, ranged between 29 and 65 days, forming three distinct groups with different ALSPs. For example, ALSP of *Phaenicia* and *Calliphora* was relatively similar but shorter than the other species, whereas the ALSP of *Musca* and *Sarcophaga* was quite similar but shorter than in *Drosophila.* For the purpose of this study, namely, to determine whether ALSP was correlated with the rates of oxidant generation, it was deemed highly desirable that the group of species to be compared include not only those with dissimilar but also those with similar ALSPs.

Relationship between ALSP and rates of mitochondrial superoxide and H_2O_2 production

Studies on both mammalian[19,20] and insect[21,22] mitochondria, using specific substrates and inhibitors, have revealed that ubisemiquinone/cytochrome *b* region is the primary site of superoxide anion radical generation. The rate of superoxide anion radical generation, measured in smps from 7-day-old flies, varied about 3-fold among the different species and was found to be inversely correlated with the ALSP of different species ($r = .95$; $p < .004$) (Fig. 1).

A comparison of the rate of H_2O_2 release by mitochondria from 13- to 14-day-old flies of different species is presented in Figure 2. Again, the ALSP of the different species was found to be inversely correlated with the rate of mitochondrial H_2O_2 release ($r = 0.87$; $p < .058$). It should be explained that the reason for the use of flies of this age is that because the rate of mitochondrial H_2O_2 release increases during aging, it was reasoned that the differences among different species would be more readily detectable at a relatively older age. It may also be pointed out that under the conditions employed here, the measured rate of superoxide anion radical generation would reflect the potential rate, whereas that of H_2O_2 release would better approximate physiological conditions because no respiratory inhibitors were used in the latter and measurements were made under State 4 respiratory conditions (i.e., excess substrate but no exogenous ADP).

Comparison of cytochrome c oxidase activity

Cytochrome *c* oxidase activity, measured in 7-day old flies, was compared among different species be-

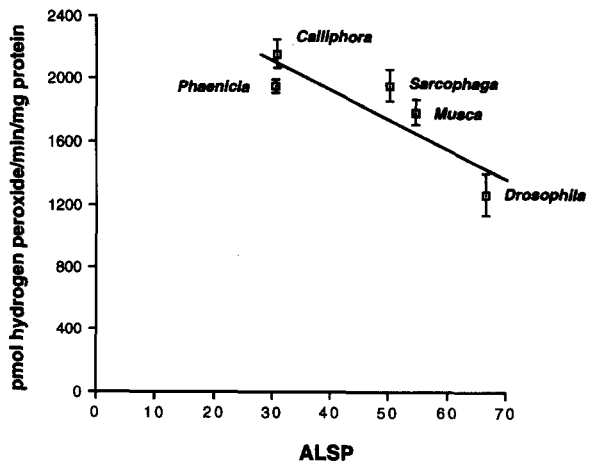

Fig. 2. Relationship between the rate of H_2O_2 release by mitochondria from thoracic flight muscles and average life span potential (ALSP) of five different species of flies. Rates of H_2O_2 release were measured in isolated mitochondria, obtained from 13- to 14-day-old flies, as an increase in fluorescence due to oxidation of PHPA and the coupled reduction of H_2O_2 by horseradish peroxidase. Results are average ±SD of three to six measurements.

cause the rate of mitochondrial H_2O_2 release was previously found by us to be inversely related to experimental variations in cytochrome *c* oxidase activity.[21] Cytochrome *c* oxidase activity exhibited an approximately 5-fold variation in activity among different species and was directly correlated with the ALSP of the species ($r = .97$; $p < .006$) (Fig. 3).

Comparison of protein carbonyl content

A comparison of the protein carbonyl content in the whole body homogenates of 15-day-old flies of different species, presented in Figure 4, indicated an approximately 3-fold variation among the different species exhibiting an inverse relationship with ALSP of the different species ($r = .92$; $p < .02$).

DISCUSSION

The different species of flies used in this study are phylogenetically closely related. They not only belong to the same Class (Insecta) and Order (Diptera) but also the same Suborder (Cyclorrhapha). Furthermore, the metabolic potential of these flies is quite similar, being around 25 kcal/g body weight.[23-25] Metabolic potential is defined as the total amount of energy consumed per unit mass during the entire adult life span. Thus, for such closely related species, the more than 2-fold variation in the ALSP can be considered to be of sufficient magnitude for correlational studies. Furthermore, two pair of species, namely, *Calliphora* and *Phaenecia*, and *Musca* and *Sarcophaga*, had quite comparable life spans and also exhibited relatively

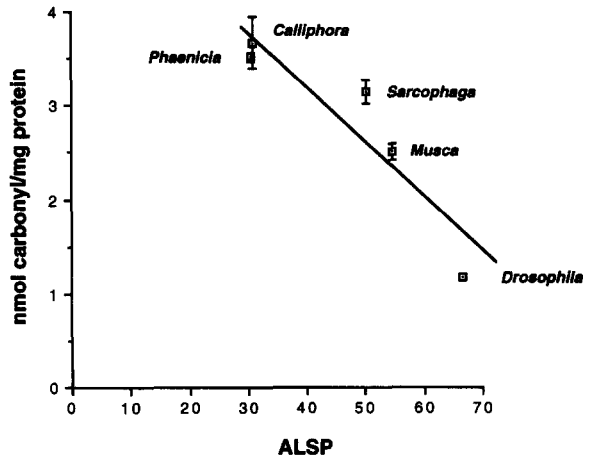

Fig. 3. Relationship between the activity of cytochrome *c* oxidase in mitochondria from flight muscles and ALSP of five different species of dipteran flies. Activity was measured in sonicated mitochondria by monitoring the oxidation of cytochrome *c* (II). Results are average ±SD of three to six determinations.

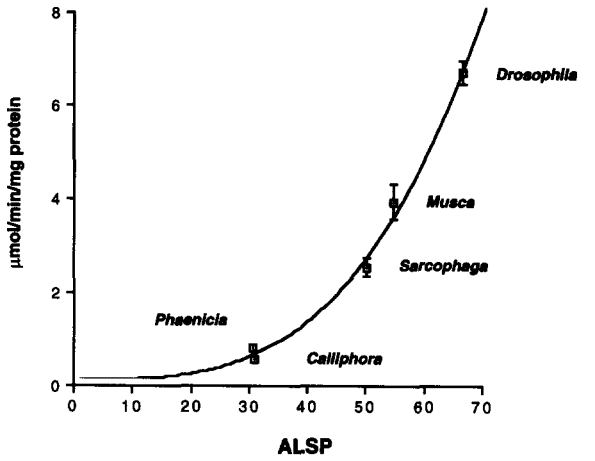

Fig. 4. Correlation between protein carbonyl content in the whole body homogenates and average life span potential (ALSP) of five different species of dipteran flies. Carbonyl content was measured by the DNPH method of Levine et al.[15] Values are average ±SD of four to six determinations.

similar biochemical characteristics that were examined in this study.

Under identical assay conditions, the rates of mitochondrial superoxide anion radical and H_2O_2 generation were found to be inversely related to the ALSP. Because superoxide anion radical is produced by the autoxidation of certain components of the respiratory chain,[26] the observed 3-fold variation in the rate of superoxide anion radical generation among the different species suggests the existence of as yet unknown structural differences in the electron transport chain. Although the nature of such differences is presently unclear, one possibility, on the basis of a previous study in this laboratory,[21] may be the variation in the activity of cytochrome *c* oxidase. Cytochrome *c* oxidase activity was previously observed to sharply decline and the rate of mitochondrial H_2O_2 production to increase during the latter part of the life span in the housefly. An experimentally induced partial inhibition of cytochrome *c* oxidase activity by low concentrations of KCN was found to sharply enhance the rate of mitochondrial H_2O_2 release. The mechanism by which cytochrome *c* oxidase activity would affect the generation of H_2O_2, or its stoichiometric precursor superoxide anion, might involve the redox status of the electron carriers. For example, the highest rates of mitochondrial H_2O_2 generation are observed in State 4 respiration conditions or when the respiratory chain is blocked by inhibitors such as antimycin A or myxothiazol; that is, under conditions where the upstream electron carriers are in a highly reduced state, consequently permitting their autoxidation.[19-21,26]

Results of this study, indicating that the rates of

mitochondrial ROS generation are inversely correlated with ALSP, are in agreement with those obtained previously in a similar comparison among different mammalian species.[9] Thus, the interspecies variations in the life spans within two very widely divergent phylogenetic groups (i.e., the mammals and the insects), are correlated with the same characteristic, namely, the rate of mitochondrial superoxide and H_2O_2 generation. Furthermore, results of other previous studies in this laboratory have indicated that the rates of mitochondrial oxidant generation also correspond to intraspecies differences in the life span. For example, the rates of mitochondrial superoxide anion radical and H_2O_2 generation were found to be inversely related to the life expectancy of housefly cohorts.[12,27] Flies exhibiting postponed senescence phenotype and those whose life spans had been experimentally extended, by lowering the rate of metabolism, were also found to have relatively lower rates of mitochondrial superoxide anion radical and H_2O_2 generation, when compared at the same age.

Further support for the view that the rates of mitochondrial superoxide anion radical and H_2O_2 generation may play a role in the aging process is provided by studies indicating that such rates increase as a function of age in mammals[28-30] as well as in insects.[14]

The mechanism by which elevated rates of mitochondrial superoxide and H_2O_2 generation may be linked to the aging process may by hypothesized to involve the corresponding enhancement of molecular oxidative damage. For example, studies by Stadtman and coworkers[3,4] have shown that Fenton-type reactions of H_2O_2 with transition metals can induce protein carbonyl modifications. Furthermore, concentration of protein carbonyls has been found to increase with age in a number of model systems[3] and is associated with the intraspecies variations in the life span of houseflies.[17] Further corroboration for the view that this phenomenon may be involved in the aging process is provided by the results of the present study showing, for the first time, that the life span potential of different species is inversely related to the residual level of protein oxidative damage, measured at a comparable age. Altogether, results of this study support the hypothesis that mitochondrial ROS generation may be one of the causal factors in the aging process.

REFERENCES

1. Harman, D. Aging: A theory based on free radical and radiation chemistry. *J. Gerontol.* **11**:298–300; 1956.
2. Sohal, R. S. The free radical hypothesis of aging: An appraisal of the current status. *Aging Clin. Exp. Res.* **5**:3–17; 1993.
3. Stadtman, E. R. Protein oxidation and aging. *Science* **257**:1220–1224; 1992.
4. Stadtman, E. R.; Starke-Reed, P. E.; Oliver, C. N.; Carney, J. M.; Flyod, R. A. Protein modifications in aging. In: Emerit, I.; Chance, B., eds. *Free radicals and aging.* Basel: Birkhauser; 1992:64–72.
5. Ames, B. N. Endogenous oxidative DNA damage, aging and cancer. *Free Radic. Res. Commun.* **7**:121–128; 1989.
6. Ku, H.-H.; Sohal, R. S. Comparison of mitochondrial prooxidant generation and antioxidant defenses between rat and pigeon: Possible basis of variation in longevity and metabolic potential. *Mech. Ageing Dev.* **72**:67–76; 1993.
7. Sohal, R. S.; Sohal, B. H.; Brunk, U. T. Relationship between antioxidant defenses and longevity in different mammalian species. *Mech. Ageing Dev.* **53**:217–227; 1990.
8. Finch, C. E. *Longevity, Senescence and the Genome.* Chicago: University of Chicago Press; 1990.
9. Ku, H.-H.; Brunk, U. T.; Sohal, R. S. Relationship between mitochondrial superoxide and hydrogen peroxide production and longevity of mammalian species. *Free Radic. Biol. Med.* **15**:621–627; 1993.
10. Ragland, S. S. *Aging rate in relation to physical activity, metabolic rate, and free radical production in the housefly, Musca domestica.* Ph.D. Dissertation, Southern Methodist University: Dallas; 1984.
11. Wood, F. E.; Nordin, J. H. Temperature effects on mitochondrial respiration of *Protophormia terranovae* and *Musca domestica. Insect Biochem.* **10**:95–99; 1980.
12. Sohal, R. S. Hydrogen peroxide production by mitochondria may be a biomarker of aging. *Mech. Ageing Dev.* **60**:189–198; 1991.
13. Hyslop, P. A.; Sklar, L. A. A quantitative fluorimetric assay for the determination of oxidant production by polymorphonuclear leukocytes: Its use in the simultaneous fluorimetric assay of cellular activation processes. *Anal. Biochem.* **141**:280–286; 1984.
14. Sohal, R. S.; Sohal, B. H. Hydrogen peroxide release by mitochondria increases during aging. *Mech. Ageing Dev.* **57**:187–202; 1991.
15. Birch-Machin, M.; Jackson, S.; Kler, R. S.; Turnbull, D. M. Study of skeletal muscle mitochondria dysfunction. In: Lash, L. H.; Jones, D. P., eds. *Methods in toxicology.* Vol. 2. New York: Academic Press; 1993:51–69.
16. Levine, R. L.; Garland, D.; Oliver, C. N.; Amici, A.; Climent, I.; Lenz, A. G.; Ahn, B. W.; Shaltiel, S.; Stadtman, E. Determination of carbonyl content in oxidatively modified proteins. *Meth. Enzymol.* **186**:464–478; 1990.
17. Sohal, R. S.; Agarwal, S.; Dubey, A.; Orr, W. C. Protein oxidative damage is associated with life expectancy of houseflies. *Proc. Natl. Acad. Sci. USA* **90**:7255–7259; 1993.
18. Ragland, S. S.; Sohal, R. S. Mating behavior, physical activity and aging in the housefly, *Musca domestica. Exp. Geront.* **8**:135–145; 1973.
19. Turrens, J. F.; Freeman, B. A.; Levitt, J. G.; Crapo, J. D. The effect of hyperoxia on superoxide production by lung submitochondrial particles. *Arch. Biochem. Biophys.* **217**:401–410; 1982.
20. Turrens, J. F.; Freeman, B. A.; Crapo, J. D. Hyperoxia increases H_2O_2 release by lung mitochondria and microsomes. *Arch. Biochem. Biophys.* **217**:411–421; 1982.
21. Sohal, R. S. Aging, cytochrome oxidase activity and hydrogen peroxide release by mitochondria. *Free Radic. Biol. Med.* **14**:583–588; 1993.
22. Bolter, C. J.; Chefurka, W. Extramitochondrial release of hydrogen peroxide from insect and mouse liver mitochondria using the respiratory inhibitors phosphine, myxothiazol and antimycin and spectral analysis of inhibited cytochromes. *Arch. Biochem. Biophys.* **278**:65–72; 1990.
23. Tribe, M. A. Age-related changes in the respiratory physiology of flight muscle tissue from the blowfly, *Calliphora erthrocephala. Exp. Gerontol.* **2**:113–121; 1966.
24. Miquel, J.; Lundren, P. R.; Bensch, K. G.; Atlan, H. Effects of temperature on the life span, vitality and fine structure of *Drosophila melanogaster. Mech. Ageing Dev.* **5**:347–370; 1976.

25. Sohal, R. S. The rate of living theory: A contemporary interpretation. In: Collatz, K. G.; Sohal, R. S., eds. *Insect aging.* Heidelberg: Springer-Verlag; 1986:23–44.
26. Chance, B.; Sies, H.; Boveris, A. Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* **59:**527–603; 1979.
27. Farmer, K. J.; Sohal, R. S. Relationship between superoxide anion radical generation and aging in the housefly, *Musca domestica. Free Radic. Biol. Med.* **7:**23–29; 1989.
28. Nohl, H.; Hegner, D. Do mitochondria produce oxygen radicals *in vivo? Eur. J. Biochem.* **82:**563–567; 1978.
29. Sohal, R. S.; Arnold, L.; Orr, W. C. Effect of age on superoxide dismutase, catalase, glutathione reductase, inorganic peroxides, TBA-reactive material, GSH/GSSG, NADPH/$NADP^+$ and NADH/NAD^+ in *Drosophila melanogaster. Mech. Ageing Dev.* **56:**223–235; 1990.
30. Sohal, R. S.; Ku, H.-H.; Agarwal, S.; Forster, M. J.; Lal, H. Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse. *Mech. Ageing Dev.* **74:**121–133; 1994.

ABBREVIATIONS

ALSP—average life span potential
MLSP—maximum life span potential
PHPA—*p*-hydroxyphenylacetic acid
SOD—superoxide dismutase
smps—submitochondrial particles