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Protein oxidative damage is associated with life expectancy of houseflies

(aging/oxygen free radicals/protein oxidation/oxidative stress)

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ABSTRACT **The objective of this study was to test some of the predictions of the oxidative-stress hypothesis of aging, which postulates that aging is causally associated with the molecular damage inflicted by reactive oxygen species. Protein carbonyl content was used as an index of molecular oxidative modifications. The carbonyl content was found to be associated with the physiological age or life expectancy of flies rather than with their chronological age. Exposure of flies to sublethal hyperoxia (100% oxygen) irreversibly enhanced the carbonyl content of the flies and decreased their rate of oxygen consumption. Results of this study indicate that protein carbonyl content may be a biomarker of aging and support the general concept that oxidative stress may be a causal factor in the aging process.**

Oxygen free radicals have been widely postulated to play a causal role in the aging process (1). It is assumed that antioxidative and reparative abilities of cells are insufficient, leading to the gradual accumulation of oxidative molecular damage and the loss of homeostatic efficiency in association with the aging process. This hypothesis has gained support from the observations that the concentration of a variety of molecular products of free radical reactions accumulate with age (2–4). Nevertheless, the role of oxidative damage in the aging process remains speculative because its association with actual mortality has not been specifically established.

The objective of this study was to test some of the predictions of the oxidative-stress hypothesis of aging. If the hypothesis were valid, oxidative damage would be predicted to be associated with the rate of physiological rather than chronological aging. Physiological age is defined here as "nearness to death" with 1.0 being the end-point. Furthermore, the variations in longevity observed among individuals belonging to the same cohort group should be expected to correspond to the magnitude of manifested oxidative damage. Another prediction of the hypothesis would be that experimental oxidative stress should have an additive or accelerating effect on the aging of the treated organisms.

Studies by Stadtman, Oliver, and associates (5–9) have demonstrated that cellular proteins undergo extensive oxidative modifications, manifested as carbonyl derivates, as a consequence of aging in several model systems. Carbonyl content may thus be a marker of *in vivo* tissue oxidative damage. Results of this study indicate that the concentration of the protein carbonyl groups is associated with the life expectancy of flies.

MATERIALS AND METHODS

Animals. Eggs were obtained from a stock of houseflies, originally procured from the Department of Zoology, University of Cambridge (U.K.) in 1980 and continuously bred in this laboratory. Eggs were placed in moist CSMA (Chemical Specialties Manufacturers' Association) fly larval medium. After emergence from the pupae, adult flies were segregated by sex and kept at 25°C and 50% relative humidity. Flies were fed on sucrose and water, which promote a longer life-span than a diet containing fats or proteins. Only male flies were used for experimental studies.

Experimental Variations in Physical Activity and Life-Span of Flies. Previous studies in this laboratory have shown that the life-span and the aging rate of the flies are inversely related to the rate of metabolism, which can easily be manipulated by variations in the level of physical activity (10–12). The level of physical activity was altered by confining flies within containers of different sizes. To maintain relatively high levels of physical activity (HA), flies were kept in 1-ft^3 cages, where they were able to fly. To lower the level of physical activity (LA), flies were individually confined in 150-ml glass urine-specimen jars, fitted with a cardboard maze, where the flies could walk but were unable to fly, due to restriction of space.

Selection of Relatively Short- and Long-Lived Cohort Flies. Loss of flight ability, toward the end of life, is a universal feature of senescence in flies, whereby senescent flies can be distinguished from their chronologically similar but "physiologically younger" cohorts, which still retain the ability to fly (10, 13). Flies unable to fly, and referred to as "crawlers," were separated from their flying cohorts, the "fliers," at 10 days of age and housed in separate cages with similar population density.

Exposure to Hyperoxia. Insect cages were placed within sealed Plexiglas containers, connected via a gas manometer, to a gas cylinder containing 100% oxygen. Oxygen was first passed through water and then the chamber under a low positive pressure at a steady rate of flow.

Determination of Protein Carbonyl Content. Protein carbonyl content was measured according to Levine *et al.* (14) using the 2,4-dinitrophenylhydrazine (DNPH) procedure. In each experiment a 10% (wt/vol) homogenate of ≈30 flies was made in 5 mM phosphate buffer (pH 7.5) containing the protease inhibitors leupeptin (0.5 μg/ml), aprotenin (0.5 μg/ml), and pepstatin (0.7 μg/ml), and 0.1% Triton X, using a ground-glass homogenizer. (Addition of streptomycin sulfate had no detectable effect on carbonyl content.) The homogenate was filtered through eight layers of muslin gauze to remove exoskeleton. From the resulting filtrate, 300-μl aliquots containing 1.6–2.0 mg of protein were treated with 300 μl of 10 mM DNPH dissolved in 2 M HCl or with 2 M HCl in the controls. Samples were then incubated for 1 hr at room temperature, stirred every 10 min, precipitated with 10% trichloroacetic acid (final concentration), and centrifuged for

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Abbreviations: DNPH, 2,4-dinitrophenylhydrazine; HA, high levels of activity; LA, low levels of activity; MRDT, mortality rate-doubling time; SOD, superoxide dismutase.

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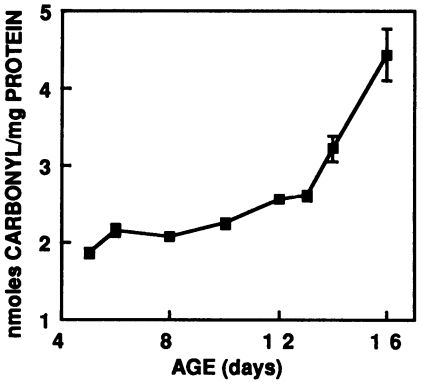

FIG. 1. Comparison of the protein carbonyl content in whole-body homogenates of houseflies of different ages. Carbonyl content was measured by the DNPH method of Levine *et al.* (14). Flies were kept under HA conditions with 200 flies per cage. Values are the average ± SEM of three to five determinations.

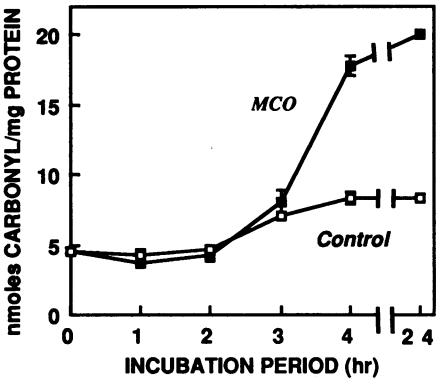

FIG. 2. Accumulation of protein carbonyls in homogenates of the housefly of mixed ages and sexes exposed to a metal-catalyzed oxidation (MCO) system. The reaction mixture consisted of protein at 1 mg/ml/2 mM ADP/16 μM $FeCl_3$/0.5 mM ascorbate/phosphate buffer. Values represent the average ± SEM of three to five determinations.

3 min. The pellet was washed thrice with 1 ml of ethanol/ethyl acetate, 1:1 (vol/vol) and redissolved in 1 ml of 6 M guanidine in 10 mM phosphate buffer/trifluoroacetic acid, pH 2.3. Any trace insoluble material was removed by centrifugation. The difference in absorbance between the DNPH-treated and the HCl-treated samples was determined at 366 nm, and the results were expressed as nmol of carbonyl groups per mg of protein, using the extinction coefficient of 22.0 $mM^{-1} \cdot cm^{-1}$ for aliphatic hydrazones.

Biochemical Assays. Superoxide dismutase (SOD) activity was measured in the whole-body homogenates of the flies by the direct method of Misra and Fridovich (15), as described (16). Catalase activity was assayed according to Luck (17), as described in detail (16). Glutathione content was determined by the method described by Tietze (18), as outlined by us (16).

Metabolic Rate. The rate of oxygen consumption of the flies was measured with a differential Gilson respirometer using standard procedures. For each measurement, single flies were weighed and placed in the Warburg flask, allowed to equilibrate for 15 min with open stopcock, followed by 15 min of shut stopcock. Oxygen intake was measured in the subsequent 2-hr period.

Determination of Mortality Rate-Doubling Time (MRDT). The MRDT was determined by using Finch's equation (19): MRDT = $\ln 2/G$, where G is the slope of the logarithm of the mortality-rate plot (Gompertz plot).

RESULTS

Effect of Age on Protein Carbonyl Content. Previous studies in this laboratory have shown that cross-sectional sampling of aging populations should be restricted to the period before the onset of dying phase because the survivors progressively represent subsets of the population undergoing slower rates of aging (20). In the housefly, the dying phase, indicated by the sharp downward slope of the survivorship curve, begins ≈16 days of age. The carbonyl content, determined in the whole-body homogenates of male flies, ranging up to 16 days of age, increased with age ≈250%, exhibiting an exponential pattern (Fig. 1).

To ascertain whether the carbonyl content of the housefly homogenates may, indeed, be a product of metal-catalyzed oxidation, homogenates were exposed to an Fe-ADP/ascorbate metal-catalyzed oxidation system. Protein carbonyl content showed a semiexponential time-dependent increase (Fig. 2). It is thus possible that similar processes may be involved *in vitro* and *in vivo* in the carbonyl accumulation, but further studies are needed to elucidate the mechanisms.

Relationship Between Life Expectancy of Flies and Carbonyl Content: Effect of Physical Activity. The effect of physical activity on life-span of houseflies is shown in Fig. 3*A*. Both the average (48.1 ± 2.1 days) and the maximum life-span of the flies, kept under conditions of relatively LA in urine-specimen jars, was >2-fold longer (20.6 ± 0.4 days) than those kept under conditions of relatively HA in cages.

Age-specific death rates of flies exhibited a logarithmic or Gompertzian pattern (Fig. 3*B*). MRDT, calculated from the slope of the Gompertz plots, is widely believed to be the single most important measure of the aging rate of a population (19), whereas the intercepts of the Gompertz plots

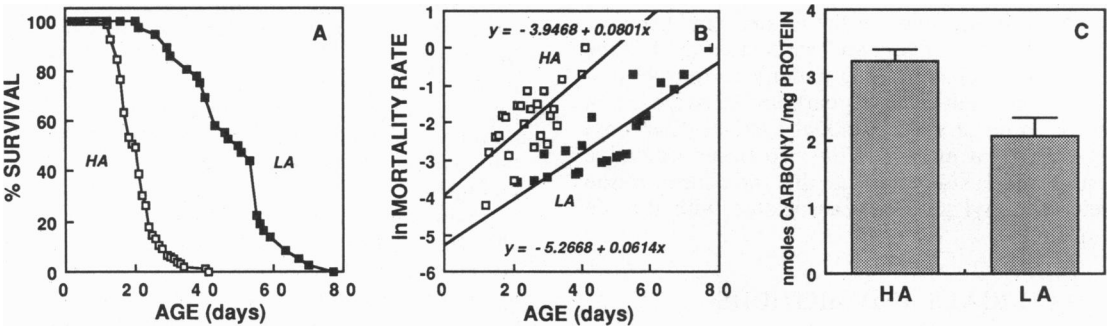

FIG. 3. (*A*) Survivorship curves of houseflies kept under conditions of HA and LA. HA flies were placed in 1-ft^3 cages where flies were able to fly, whereas LA flies were individually confined in 150-ml urine-specimen jars, fitted with a cardboard maze, where flies were able to walk but could not fly due to space restriction. Data are based on the mortality of 200 flies in the HA group and 47 flies in the LA group. (*B*) Mortality rates, graphed on a semilogarithmic scale (Gompertz plots), of houseflies kept under conditions of relatively HA and LA (data from groups in *A*). (*C*) Comparison of protein carbonyl content in the homogenates of 14-day-old houseflies raised under conditions of relatively HA and LA. Values are the average ± SEM of four determinations (P = 0.01).

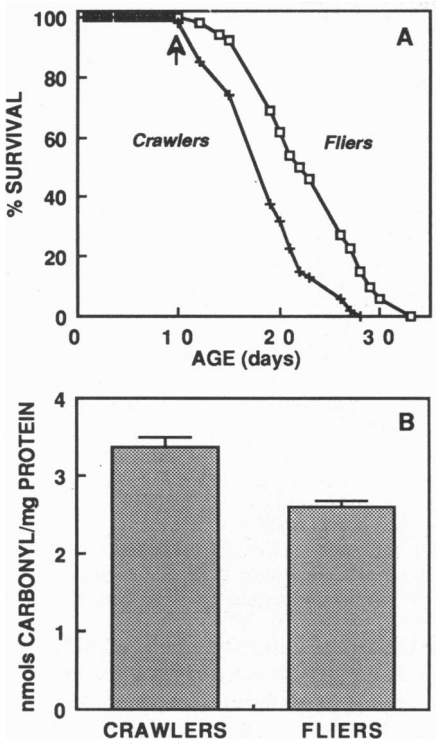

FIG. 4. (*A*) Survivorship curves of crawlers and fliers, isolated from cohort populations of houseflies at 10 days of age. Crawlers are flies that had lost the ability to fly, whereas fliers still retained it. Data are based on mortality of 50 flies in each group. (*B*) Comparison of protein carbonyl content in the homogenates of 12-day-old crawlers and fliers. Each value is an average ± SEM of four determinations ($P = 0.002$).

reflect the incidence of age-independent death rate. MRDT of HA and LA flies was 8.6 days and 11.3 days, respectively.

Carbonyl content of the flies, measured at 14 days of age, was 55% greater ($P = 0.01$) in the HA than in the LA flies (Fig. 3*C*). Using the average life-span as the end-point (1.0), at 14 days of age the HA flies had reached 0.68 of their physiological age or life expectancy, whereas LA flies had reached only 0.29 of their average life expectancy.

Comparison of Short- and Long-Lived Flies. Loss of flight ability is a phenotypic characteristic associated with senility in the flies, permitting the separation of senescent from presenescent flies of the same chronological age (21). Survivorship curves of flies that had lost their flight ability (or crawlers) and their cohorts still possessing flight ability (fliers), separated from an aging population at 10 days of age and housed separately, are presented in Fig. 4*A*. The subsequent average survival of the crawlers was 9.0 ± 0.6 days, and the average survival of the fliers was 13.3 ± 0.7 days. Thus, from the same cohort population two subgroups with 48% difference in their subsequent average life expectancy were separated. It should be pointed out that the survivorship of crawlers and fliers was studied under relatively low levels of population density, which itself affects the life-span.

The carbonyl content, measured at 12 days of age, was 29% higher ($P = 0.002$) in the crawlers than in the fliers (Fig. 4*B*). The physiological age at this age was 0.63 in crawlers and 0.51 in fliers.

Effect of Hyperoxia on Aging and Carbonyl Content. Houseflies, exposed to 100% oxygen, started exhibiting stress symptoms, such as slowing down of the flying and walking activity, after ≈24 hr, followed by heavy mortality starting at ≈4 days of age (Fig. 5*A*). The average survival under hyperoxia was 5.1 ± 0.1 days (group A). To study the effects of sublethal exposure to hyperoxia, flies were removed from the hyperoxic environment to air at 3.5 days of age (group B)—i.e., just before heavy mortality would have set in. Paradoxically, the subsequent average life-span of group B flies (25.9 ± 0.6 days) was 47% longer ($P = 0.0001$) than that of the control flies (17.6 ± 0.4) kept throughout life in the air (group C) (Fig. 5*A*).

The Gompertz plots of age-dependent mortality rates, presented in Fig. 5*B*, indicate that life-lengthening effect of sublethal hyperoxia is not due to an increase in MRDT (or slowing down of the aging rate) but is due to a decrease in the incidence of age-independent mortality, as indicated by the intercept values. In fact, MRDT of the group B flies (7.8 days) was shorter than the group C control flies (8.6 days).

Carbonyl content of group B flies was monitored during the period of hyperoxic exposure and in the subsequent recovery period. The carbonyl content accumulated at a faster rate ($P = 0.001$) under hyperoxic conditions than in air (group C), as indicated by the progressive increase in the difference in the carbonyl content between the two groups (Fig. 5*C*). The carbonyl content of flies in the postexposure phase, measured twice in 3 days, remained higher in the group B than in the control group C flies; however, the differences between the two groups were somewhat narrowed with time.

To investigate the possible causes of chronologic life-lengthening effect of sublethal hyperoxia, the possibility of

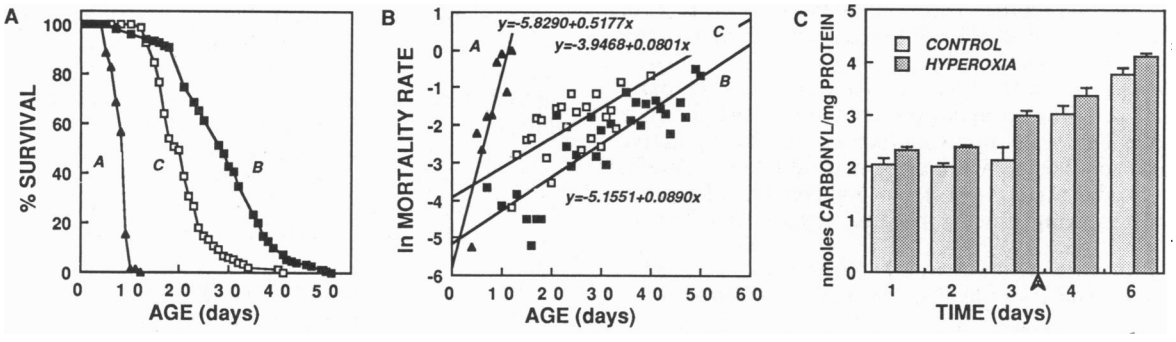

FIG. 5. (*A*) Effect of hyperoxia (100% oxygen) on survival of the houseflies. In group A (▲), flies were kept under 100% oxygen throughout life. In group B (■), 4-day-old flies were placed under 100% oxygen atmosphere for 3.5 days, after which they were transferred to air. Group C (□) flies were kept in air throughout life. In each group, 200 male flies were housed in a 1-ft³ cage. (*B*) Mortality rates, graphed on a semilogarithmic scale, of houseflies in *A*. Slopes of the age-related mortality-rate plots correspond to the length of hyperoxic exposure. (*C*) Effect of hyperoxia (100% oxygen) on carbonyl content of houseflies. Five-day-old flies, kept under HA conditions with 200 flies per cage, were placed in 100% oxygen for 3 days and subsequently transferred to air, as indicated by the arrowhead. The control flies were kept in air throughout. Carbonyl content was measured each day during hyperoxic exposure and twice in the 3-day postexposure period. Hyperoxia causes an increase in carbonyl content, which persists in the postexposure period ($P = 0.001$, ANOVA test). Each value is an average ± SEM of four determinations.

adaptive (over)compensatory responses was examined. No differences were observed between the group B flies, kept in hyperoxia for 3.5 days, and the control group C flies in the activities of SOD and catalase; however, glutathione concentration was marginally lower in the former (Table 1). In the postexposure period, the metabolic rate (rate of oxygen consumption) of the hyperoxia-exposed group B flies was significantly lower ($P = 0.003$) than that of the controls (Fig. 6), suggesting that the lengthening of average life-span in the group B flies in response to sublethal exposure to hyperoxia may be due to a decrease in the level of physical activity.

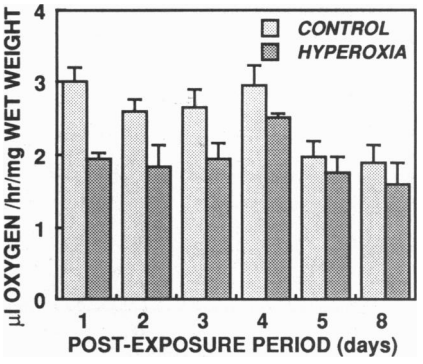

FIG. 6. Effect of hyperoxia (100% oxygen) on the rate of oxygen consumption of flies. Five-day-old flies were placed under 100% oxygen for 3 days and subsequently transferred to air; the control flies were kept in air throughout the experiment. Rate of oxygen consumption was measured by a differential Gilson respirometer in the postexposure period. Data are averages ± SEMs of 5–16 determinations for each bar. Statistical comparison, by ANOVA, indicated that hyperoxic exposure caused a significant postexposure depression of the rate of oxygen consumption ($P = 0.003$).

DISCUSSION

Stadtman, Oliver, and his colleagues (3, 5–9) have shown that protein carbonyl derivatives are formed *in vitro* as a result of metal-catalyzed oxidations and accumulate during the aging process in disparate model systems. Results of the present study further extend the relevance of this concept to the aging process by demonstrating that carbonyl content is associated with the life expectancy or the physiological age of the flies and is not associated with the chronological age, thereby, strengthening the link between protein oxidative damage and the aging process. In addition, results of this study clarify the paradoxical effects of sublethal oxidative stress by hyperoxia on subsequent aging by showing that such a treatment increases the carbonyl content and has a deleterious effect on the flies by reducing their metabolic potential.

Results of this and previous studies (2, 11, 12) indicate that reduction of physical activity of the flies, achieved by limitation of flying space or by confinement in the dark, extends not only the average but also the maximum life-span, as well as the MRDT. The latter two values are widely believed to reflect the underlying aging rates (19). It has also been previously shown that rates of mitochondrial O_2^- (22) and H_2O_2 (23) generation in the flies increase with age and are higher in HA than LA flies and in crawlers than in fliers of the same chronological age (24), suggesting the existence of a relationship between life expectancy and mitochondrial oxidant generation. The concentrations of the molecular products of free radical reactions, such as lipofuscin (25), Schiff base-containing fluorescent material (26), and *n*-pentane exhalation (27), were also inversely associated with life expectancy of the flies. Pentane exhalation is of particular interest because it indicates the *in vivo* level of oxidative stress (28). The present finding that accumulation of protein carbonyls is associated with life expectancy provides further support to the hypothesis that there is a correlation between the rates of oxidant generation, level of oxidative molecular damage, and the rate of physiological aging.

Previous studies in the literature have reported the paradoxical lack of a life-shortening effect by sublethal hyperoxia on subsequent survival, implying that any resulting putative damage is reversible and without permanent effects (29, 30). If true, it would argue against the involvement of oxygen radicals in the aging process because it has been demonstrated that the rate of mitochondrial O_2^- and H_2O_2 generation is directly related to ambient oxygen concentration (31, 32). In contrast, aging, by definition, is progressive and irreversible in nature. Present results indicate that although the average chronological length of life of flies was slightly extended after hyperoxia, the MRDT was shortened, and the metabolic rate and, possibly, the metabolic potential of the flies were depressed. Thus, sublethal exposure to hyperoxia had distinct deleterious effects on the flies. The increased survival, after sublethal hyperoxia, is most likely attributable to the decreased metabolic rate, which itself has an ameliorative effect on survival, as shown by the life-lengthening effects of reduced physical activity on the flies (see Fig. 3*A*). A similar increase in average life-span was previously observed by us in the houseflies in response to sublethal intake of diethyldithiocarbamate, an inhibitor of Cu,Zn SOD (33).

Table 1. Comparison of the activities of SOD and catalase and concentration of glutathione in houseflies kept in 100% oxygen for 3 days and control flies

Parameter	Control	Hyperoxia-exposed
SOD, units/mg of protein	25.8 ± 1.5	22.0 ± 3.0
Catalase, units/mg of protein	21.2 ± 1.2	22.6 ± 2.1
Glutathione, μg/g (wet body weight)	82.0 ± 3.4	72.0 ± 4.1

Control flies were maintained in air during the entire experiment. Values are averages ± SEMs of three to four determinations.

The profile of carbonyl-content augmentation in the flies in response to hyperoxia partially parallels that previously seen in the rat liver (7, 34). Carbonyl content of flies is increased in response to hyperoxia, the highest increase occurring between day 2 and day 3 of exposure. In the postexposure period, the difference in the carbonyl content between the treated and the control group somewhat narrowed but still persisted (see Fig. 5*C*). Such narrowing of the difference could be from the stimulation of alkaline protease activity by hyperoxia, as reported by Starke-Reed and Oliver (7). An additional factor could be that the decreased level of physical activity (metabolic rate) of the treated flies contributed to the lowered rate of carbonyl accretion. Although it is difficult to separate the relative contributions of these two factors on the basis of the present study, the deleterious effects of sublethal exposure to hyperoxia seem irreversible, as indicated by both the overall level of carbonyl content and the depression of the metabolic rate.

Altogether, results of this study confirm the predictions of the oxidative-stress hypothesis of aging. Studies in the housefly support the general concept that the rates of mitochondrial O_2^- and H_2O_2 increase with age, causing oxidative molecular damage at a rate corresponding to the physiological rate of aging. Severe experimental oxidative stress results in the shortening of the physiological length of life.

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