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Dietary Nordihydroguaiaretic Acid Increases the Life Span of the Mosquito (42389)

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Abstract. Our previous findings indicated that a major characteristic of aging organisms is a decrease in reducing capacity. Our objectives were to correct this impairment by administration of nordihydroguaiaretic acid (NDGA), a potent reducing agent, and to determine the effect on adult longevity of the mosquito. NDGA supplements were included in the axenic larval medium or adult diet of mosquitoes of different ages. The mean adult life spans of both sexes increased 42–64% over controls ($P < 0.025$), and the most effective doses were 0.001% for females and 0.005% for males. This NDGA effect was dependent on the age when feeding was initiated, since only biosynthetically active larvae and young adults were responsive. Also the effect was not due to dietary restriction. These results confirm the life span-enhancement effect of NDGA using defined conditions and establish the importance of redox status in the aging process.

Our previous results indicated that a decrease in reducing capacity is a key factor in the aging process. This was based on findings of lower levels of $NADP^+$-linked enzyme activities (1, 2), NADPH/$NADP^+$ ratios (3), protein biosynthesis, and DNA replication *in vivo* (4–6). Later we found that this loss in reducing capacity could be explained by a deficiency in glutathione (GSH), the major intracellular reducing agent, as aging-specific decreases in GSH levels were found in a variety of tissues in several aging organisms (7–10). Most importantly, the correction of this deficiency in the mosquito resulted in a concomitant increase in life span (11). Thus, the maintenance of a high reducing capacity could delay the aging process and result in increased longevity. A practical method to maintain this capacity would be the dietary administration of antioxidants.

There is evidence that nordihydroguaiaretic acid (NDGA), a potent antioxidant, could be effective in delaying the aging process (12, 13). NDGA administration inhibited the accumulation of the aging pigment, lipofuscin, and increased the clonal "life span" of *Neurospora crassa* (14), and increased adult longevity in *Drosophila melanogaster* (15). Although a dietary supplement of NDGA apparently caused an increase in rat survival (16), the vague methodology and insufficient number of animals gave inconclusive results. All these findings were suggestive, but studies with standardized aging models were needed to establish definitively the role of NDGA and aging.

Our objective was to determine systematically the effects of NDGA feeding on longevity using a well-characterized aging model. To this end the yellow fever mosquito was selected because of several unique advantages. It is a multicellular, eukaryotic organism that resembles mammals in its intermediary metabolism and nutritional requirements for growth (17–19). The mosquito has been validated as an aging model by extensive biochemical and biological characterization, and techniques for laboratory culture and aging have been standardized and used routinely. Finally, the mosquito grows and ages rapidly, an important consideration in life span measurements.

Materials and Methods. The yellow fever mosquito, *Aedes aegypti* (Louisville), has been colonized in this laboratory for over 30 years and 300 generations. At an optimal temperature of 29 ± 1°C, the larval period lasts approximately 7 days, and the pupal period, 2 days.

The chronological and biological ages of adult mosquitoes are as follows: 0–5 days, late metamorphosis; 6–22 days, mature; 23–35 days, old; 35+ days, very old. These age periods were based on survival curves and DNA and protein profiles (20) of cohort populations that were cultured and aged under our conditions. The median survival time of these mosquitoes was 29 ± 2 days for females and

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14 ± 1 day for males, values which have been reproducible for over 25 years.

Standard culture. The standardized culture procedure which was used to produce mosquitoes of all ages of the life span (18) is outlined briefly as follows. Eggs stored at 22°C for less than 1 month were hatched by agitating them for several minutes in distilled water with a vortex mixer. All larvae emerged within 15 min and were transferred and reared in pans containing a desiccated liver suspension to maintain a bacterial infusion that was the principal source of nutrients for the larvae.

Pupae were transferred to distilled water for adult emergence. Adult mosquitoes were fed from cotton pads moistened with 10% (w/v) sucrose solution which simulates the normal diet of plant and fruit nectars for adult mosquitoes. Blood meals, which are required only for ovarian development in females (21), were withheld to avoid this complication. Indeed, results comparing blood-fed vs sucrose-fed adults indicated a much shorter life span for the blood-fed mosquitoes (22). An environmental temperature of 29°C and a 12-hr light cycle was maintained throughout the life span.

Axenic culture. Mosquito larvae were grown individually on a chemically defined medium as described by Lang *et al.* (17). The use of axenic culture was of key importance to determine the specific and direct effect of NDGA without complications due to microbial flora. Of special note is that axenic and standard cultured mosquitoes have the same rates of growth, pupation, and adult emergence.

Feeding and survival studies. NDGA in various concentrations was given as a supplement to the axenic medium of newly hatched larvae or to the 10% sucrose solution of adults. Feeding of adults was initiated at different ages between 1 and 29 days and was continued through the entire life span.

All adults were transferred within 24 hr of emergence to nonsterile, survival cages. These cages consisted of glass lantern chimneys of about a 1-liter volume fitted with nylon net tops and screened petri dish bottoms. Approximately 25–35 mosquitoes were placed in each cage and kept at 29°C and 70% humidity for the entire life span. They were fed from cotton pads soaked with a 10% sucrose solution containing NDGA in concentrations ranging from 0 to 0.01% (w/v). These pads were placed on the net tops of the cages and were replaced every other day. The cages were monitored daily, and the dead mosquitoes were counted and removed.

Statistical methods. Student's *t* test was used for statistical comparisons of mean survival times (23).

Results. The feeding of low levels of NDGA to newly emerged adult mosquitoes increased adult longevity of both sexes (Table I, Fig. 1). The most effective concentration for males was 0.005% which increased the mean survival time 64%, and for females, 0.001%, which increased the median survival time 43%. The reason for this dosage difference was unclear, but not based on size, for the females were about two times heavier and yet required less NDGA.

The possibility was unlikely that the increases in longevity were due to restricted dietary intake, a phenomenon observed in ro-

TABLE I. EFFECT OF NDGA FEEDING ON THE LIFE SPAN OF ADULT MOSQUITOES

NDGA concentration (%)	Males			Females		
	No. of mosquitoes	Mean survival time (days)[a]	% of Control	No. of mosquitoes	Mean survival time (days)[a]	% of Control
0 (control)	319	14.3 ± 1.81	(100)	84	33.0 ± 2.40	(100)
0.001	232	16.0 ± 2.20	112	82	47.1 ± 1.97*	143
0.005	166	23.5 ± 1.82*	164	41	35.2 ± 2.40	107
0.01	—	—[b]	—	38	37.5 ± 2.31	114

[a] Results are shown as means ± SEM.
[b] Not tested.
* Difference from control is statistically significant, $P < 0.0005$.

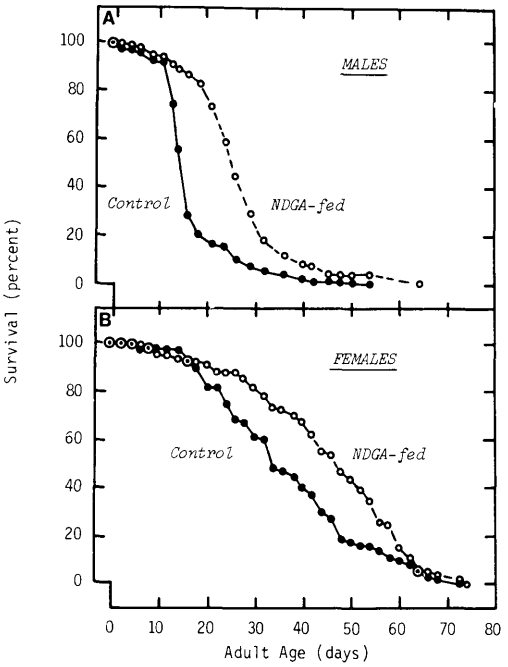

FIG. 1. Increased survival of NDGA-fed adult mosquitoes. NDGA-feeding was initiated in 1-day-old adult mosquitoes and continued throughout the life span. The NDGA concentration used was 0.005% (w/v) for males (A) and 0.001% for females (B). Percentage survival was plotted for control (●) and NDGA-fed (○) adults. Each group consisted of 80–120 male or female mosquitoes. Experimental details are given in text.

dent models (13), because the mosquitoes, which were fed *ad libitum,* had sufficient calories as the body weights were the same in all groups. In contrast, a control group of starved mosquitoes lost weight and died within 3 days.

The NDGA enhancement of life span was dependent on the age when feeding was initiated (Table II). Life span increased 24–65% when 0.001% NDGA was given to newly hatched larvae or to young adults up to 10 days of age. There was no effect in older adults.

Finally the NDGA effect was not due to inhibition of growth as it was equally effective when fed to adults only. Also, 0.001% NDGA feeding of larvae had no effect on larval development time, percentage pupation, and percentage adult emergence compared to controls.

Discussion. These results demonstrated in a well-defined aging model that NDGA feeding will increase longevity. This was due only to a decrease in the rate of adult aging, as the growth and development rates were unchanged. Moreover, this finding extended and verified earlier reports in other model systems (14–16). There is teleological support for the importance of NDGA for longevity as it is the major chemical component of the creosote bush, one of the longest lived organisms known (24, 25).

TABLE II. NDGA-SENSITIVE PERIODS OF THE MOSQUITO LIFE SPAN

Life span stage	Age feeding initiated (days)	Mean survival time of adults: Control (days)	Mean survival time of adults: NDGA (days)	% Change[a]
Larval	Newly hatched	29.9 ± 1.41[b]	45.1 ± 2.01	51**
Young adult	1	34.0 ± 1.28	56.8 ± 2.31	67**
	5	35.2 ± 1.76	44.0 ± 1.91	26*
	10	35.0 ± 2.11	47.0 ± 2.97	34*
Mature adult	15	27.0 ± 1.52	25.0 ± 2.43	−7
	21	29.1 ± 0.76	27.7 ± 1.30	−5
Old adult	29	36.5 ± 2.20	36.6 ± 1.22	0

Note. Experiments were carried out with 25–35 axenic larvae or adult female mosquitoes per control and NDGA-fed group. The NDGA concentration was 0.001% (w/v) in the axenic medium of adult feeding solution.

[a] NDGA-control/control × 100.

[b] Results are shown as means ± SEM.

* Difference from control is statistically significant $P < 0.001$.

** Difference from control is statistically significant $P < 0.0001$.

The findings support our hypothesis that a decreased reducing capacity, probably due to a GSH deficiency, is a biochemical cause of aging. It has been suggested that the redox state of tissues is maintained within a narrow range that variations could give rise to a variety of pathologic changes at the cellular, subcellular, and molecular levels (26). Indeed the loss of reducing capacity may explain many aging changes, including free radical effects (27–30) such as lipid peroxidation and the accumulation of lipofuscin and fluorescent age pigments, decreased NADPH/$NADP^+$ ratios (3), oxidative damage to membranes, and alterations in long-lived macromolecules such as collagen and elastin (27). For these reasons this hypothesis is consistent with the free radical theory of aging and, together with our finding of a GSH deficiency of aging, suggests that a loss of protection against toxicants and endogenously formed free radicals is due to a deficiency in GSH, the major intracellular antioxidant (8).

In contrast to our present findings most of the reducing agents tested by us or by others do not increase longevity (12–14, 27, 31). Although increases were observed in some studies, most were complicated by the use of poorly defined experimental organisms or lack of proper controls. In several instances, suboptimal conditions for maximal life span resulted in shorter than expected, control life spans. Thus, the beneficial effect of the antioxidant probably was due to the correction of an abnormal condition in the controls and not to a decrease in the rate of aging.

The most important role of NDGA in aging may be other than a general antioxidant activity. Indeed NDGA may have a specific sparing effect on GSH status, as our previous results showed that a GSH precursor with little antioxidant activity, thiazolidine carboxylic acids, caused a concomitant increase in GSH levels and longevity in the mosquito (11). Thus, only antioxidants that increase or spare GSH may have the potential to increase longevity. However, the relationship of NDGA and GSH has not been determined.

The significance of the NDGA-sensitive time period of the life span is unknown but may provide a clue to its mechanism of action. NDGA was effective only when feeding was initiated in mosquito larvae or young adults, which are periods of high biosynthetic activity as compared to older adults (6, 20). During these periods NDGA may be needed to protect against harmful byproducts of metabolism which may accelerate the aging process. However, NDGA probably does not act by inhibition of biosynthesis, for it had no effect on growth and development of the mosquito. Further studies are required to elucidate the mechanism of NDGA action.

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