Increased Longevity, Reduced Fecundity, and Delayed Development in Fruitfly (*Zaprionus paravittiger*) Fed on Butylated Hydroxy Anisole (44133)

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Abstract. **Oxygen free radicals are generated as a by-product during normal metabolism, and they cause damage to proteins, lipids, and DNA in organisms. The defense system of the body counteracts these highly reactive chemical moieties and neutralizes them. However, a small fraction of free radicals escapes, which causes lipid peroxidation and hence aging of the organism. It has been hypothesized that, if the free radicals are arrested/reduced, then aging can be delayed or life span could be enhanced. To test the above hypothesis, we fed butylated hydroxy anisole (BHA) to a drosophilid insect, *Zaprionus paravittiger,* and observed its effect on life span, fecundity, and developmental period. The insects were reared and maintained on standard corn meal agar (CMA) medium at 26° ± 2°C. Various concentrations (1, 5, 10, 25, 50, and 100 m*M*) of BHA were mixed with CMA medium, and the cultures were reared and maintained on these mixtures to study the life span of insects. Survivor curves showed that lower concentrations (5, 10, 25 m*M*) of BHA increased the life span, while higher concentrations (50, 100 m*M*), which were rather toxic, decreased life span. The most suitable concentration was 10 m*M,* which increased median (LT_{50}) (27% and 15% in male and female) and maximum (LT_{100}) (18% and 27% in male and female) life spans of insects maximally. Females exhibited longer life spans compared with males. The cultures were fed on the optimal concentration (10 m*M*) of BHA to study its effect on developmental period and egg-laying rate. The total developmental period was delayed by 7.2%, and egg-laying rate was reduced by 19.7% on BHA feeding. The extension in developmental period and reduction in egg-laying capacity could be the contributory factors to the favorable effect of BHA on life span.**

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Oxygen free radicals (OFRs) play a major role in aging and in the pathogenesis of a variety of diseases (1, 2). OFR reactions can produce age-related changes due to their chemical nature and ubiquitous presence in living organisms (3). According to free radical theory, the life span of an organism can be increased by slowing down the rate of initiation of free radical reactions. However, free radical reactions in the body are countered in part by enzymatic (superoxide dismutase, glutathione peroxidase, catalase, etc.) and nonenzymatic (tocopherols, ascorbic acids, glutathione, etc.) means.

Dietary antioxidants can be beneficial in protection against most human diseases (associated with free radicals) including cancer and cardiovascular diseases (4). Overexpression of both superoxide dismutase and catalase in transgenic Drosophila has increased the life span by 30% (5). Kitani and co-workers (6) observed that l-deprenyl increased the average life span by 34% in rats. However, butylated hydroxy toluene (BHT) showed contradictory results in mice (7). The life-prolonging effect of antioxidants

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may be due either to protection against free radicals or to their ability to lower the metabolic rate. According to Sohal (8), oxidative stress increases during aging because of two plausible reasons: one is the decline in antioxidant defenses, and the second is the increase in generation of hydrogen peroxide (H_2O_2) and superoxide anion radical ($O_2^{\cdot -}$). A dynamic equilibrium between antioxidant defense and rate of free radical generation exists which acts as a set point for regulation of gene expression (9).

Previously, we and others have reported that propyl gallate increased the average life span of insects (10, 11). The life-extending effects of several antioxidants are directly related to a reduction in oxygen consumption by Drosophila (12). Caloric restriction has been known to increase longevity, which could be due to retardation of growth, reduction of body fat, and decrease in oxidative damage. The selection of late-age reproducing Drosophila strains showed significant increase in life span after 21 generations (13). The measure of egg-laying rate and developmental period might be useful in establishing the relationship of these two factors with life span.

Butylated hydroxy anisole (BHA) is a phenolic antioxidant which is used as food additive to protect against auto-oxidation of lipids (14). It inhibits $O_2^{\cdot -}$ radical (15) and blocks tumor necrosis factor (TNF)–induced cytotoxicity (16). BHA shows both carcinogenic and anticarcinogenic activity. It has increased life span and antioxygenic enzyme activities in a bruchid (17), and glutathione reductase activity in brain and liver of pre-reproductive-age-group male mice (18).

Although antioxidants have been used to prolong the longevity of various organisms, their mechanism of action is not clear. The information regarding the effect of antioxidants on developmental period, fecundity, and longevity is scanty. The present investigation delineates the possible effect of BHA on longevity, developmental period, and egg-laying capacity in the banana fruit fly, *Zaprionus paravittiger.*

Materials and Methods

Insects. Banana fruit fly (*Z. paravittiger*) adults were collected from Guru Nanak Dev University Amritsar Campus lawns in the month of September. They were identified by their size (approximately 4 mm) and presence of four vertical lines on the thorax. Flies of both sexes were reared at 26° ± 2°C on standard corn meal agar (CMA) medium. Pairs of male and female flies were selected from the F_1 generation, and they were mated. The process was repeated for the F_2, F_3, and F_4 generations to obtain relatively homogeneous cultures. The use of flies for research purposes and all the procedures done were approved by the Animal Care and Use Committee of Guru Nanak Dev University, Amritsar, India.

Life Span Studies. Life span studies were performed on freshly emerged insects. Each set of experiments comprised 25 vials (4″ × 1″), each containing diet medium (¼th filled) and five male and five female (freshly emerged) flies. BHA was added to final concentrations of 0, 1, 5, 10, 25, 50, and 100 m*M*, and the cultures were maintained at 26° ± 2°C. The dead insects of both sexes were collected, counted, and discarded after a 24-hr interval until the last individual died. Survivors were transferred to fresh medium every week to avoid mixing of egg laying.

Median (LT_{50}—mean number of days when 50% of individuals of population were dead or 50% survival time) and maximum (LT_{100}—mean number of days when last individual of the population was no longer alive or 0% survival time) life spans, age mortality correlationship (r), age-independent susceptibility to death (a_0), and aging rate (a_1) were computed as reported earlier (19).

Developmental Period. Effect of BHA (10 m*M*) on various developmental phases of flies was studied. Charcoal was mixed in the respective medium to darken its surface to make the eggs and larvae clearly visible. Media were poured in culture bottles and plugged. Approximately 100 male and 150 female flies from respective cultures were put into the medium for egg laying. After 1 hr, flies were removed. Hatching takes place after approximately 40 hr. The observations were recorded for different developmental stages.

Fecundity. Freshly emerged flies were taken from control (without BHA) and BHA-fed cultures. Ten male and ten female insects were taken in each vial, and one set of experiments comprised 25 vials. The flies were transferred to fresh medium everyday. The medium containing eggs was poured into a petri dish containing water. Silvery white eggs, which were floating on the upper surface of the water, were counted.

Statistical Analysis. The experiments were repeated thrice to confirm the reproducibility. The data for life span, developmental period, and fecundity were analyzed by using nonparametric Mann-Whitney U test followed by one-way analysis of variance (ANOVA). Aging rate and age-independent susceptibility to death were calculated from Gompertz plots. The relationship between age and log mortality rate was analyzed using simple linear regression.

Results

The effect of feeding various concentrations (0, 5, 10, 25, 50, and 100 m*M*) of BHA on life span of flies was determined. Three sets of experiments were conducted serially, each time with a separate control group. Each group contained 250 flies raised under identical conditions. BHA increased survival and decreased mortality at 5, 10, and 25 m*M* concentrations, whereas higher concentrations, namely 50 and 100 m*M*, were rather deleterious and reduced the life span of the insects. At higher concentrations, the effect of BHA can be due to its chemical toxicity and can restrict food intake. The most suitable concentration was 10 m*M*, which shifted the survivorship curve maximally (Fig. 1). The median (LT_{50}) and maximum (LT_{100}) life spans experienced maximum respective increase of 26.95% and

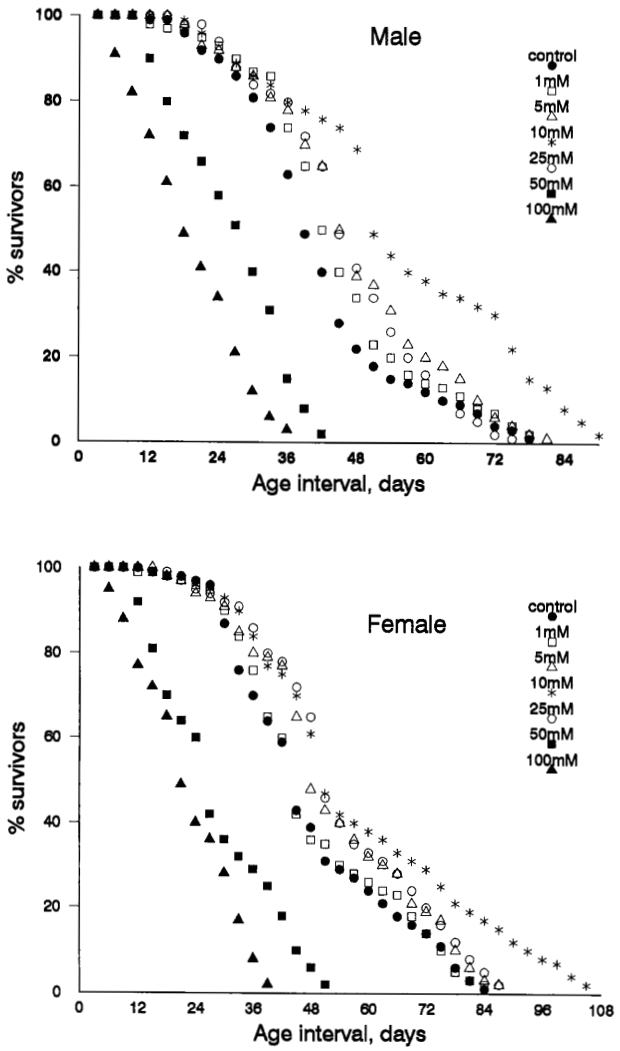

Figure 1. Survival curves of male (top) and female (bottom) *Z. paravittiger* fed on various concentrations (0, 1, 5, 10, 25, 50, 100 m*M*) of BHA. Data were expressed as percent survivors with respect to age (days) of flies. Number of flies of each sex used for each concentration was 375. The values are the mean of three set of experiments. % survival, the proportion of flies alive at the end of each sampling interval. BHA-fed flies indicated an increase in life span at 10 m*M* ($P < 0.01$).

17.62% in males and 15.13% and 27.01% in females (Table I).

Gompertz plots of mortality in general showed an exponential increase in mortality rate with age in both the sexes. The correlation coefficient (*r*—age versus mortality rate) was 0.733 and 0.801 for the flies fed on control diet. It varied from 0.88 to 0.98 for males and 0.79 to 0.95 for females on BHA feeding (Fig. 2, a and b). The aging rate (a_1) was 0.036–0.082 (males) and 0.032–0.059 (females). Age-independent susceptibility to death (a_0) was −3.97 and −3.71 for male and female flies, respectively. It decreased to −5.37 (males) and −5.77 (females). Both aging rate and age-independent susceptibility to death were maximum with 100 m*M* BHA feeding. The most suitable concentration (10 m*M*) reduced age-independent susceptibility to death in both the sexes, although the effect was sex specific. In general, BHA increased the longevity of insects by decreasing age-independent susceptibility to death although the aging rate was increased.

The effect of BHA was also studied in relation to egg-laying rate and development time. Both these parameters were affected by BHA feeding. The flies started laying eggs between 24 and 36 hr after emergence. Maximum egg laying was observed on the 15th day of adult life in control as well as BHA-fed cultures. However, antioxidant feeding prolonged the egg-laying capacity up to 36 days, compared with 33 days in control flies. A significant reduction in fecundity (egg laying) was noted during the peak period of reproduction (Table II). BHA-fed cultures showed a 19.7% decrease in the total number of eggs laid during the entire reproductive age of female flies (not shown).

The life cycle of *Z. paravittiger* is categorized into egg, three larval (L_1, L_2, L_3) stages, two pupal (P_1, P_2) stages, and adult flies. The total developmental time was 285.7 ± 2.5 hours at 26 ± 2°C. BHA delayed the total developmental time by 7.21% (Table III). Significant changes were observed during L_3, P_1, P_2 and freshly emerged phases. Maximum delay (16.99%) was observed in the P_2 stage of development.

Discussion

BHA-induced alterations in mortality rate and in medium and maximum longevity revealed that BHA prolongs the life span in *Z. paravittiger*. BHA shows antioxidative as well as carcinogenic properties depending upon its dietary levels (14, 20). It may also act as a pro-oxidant at high concentrations, as has been reported for ascorbic acid (21, 22) and sodium hypophosphite (23).

The rightward shift of survivorship curve with lower concentrations (5, 10, 25 m*M*) of BHA depicted that life span ascended to an equal degree in all members (24). The leftward shift with higher antioxidant concentrations (50, 100 m*M*) due to increased age-independent susceptibility to death (a_0) resulted in diagonal survivorship curves. Lamb (25) and Lints *et al.* (26) suggested that a diagonal survivorship curve is not due to senescence but to other causes. The reduction in life span with high doses of BHA reflects its toxicity and/or pro-oxidative nature. BHA-induced changes in mortality rate and in median and maximum life spans showed that BHA extended longevity in *Z. paravittiger*. Similar results have already been noted in our laboratory with other antioxidants, such as sodium hypophosphite (19), ascorbic acid (22), and propyl gallate (27).

The following possibilities may explain the mechanism of action of BHA in prolonging the life span of insects. One possible mechanism is that BHA induces or activates existing defense mechanisms that protect the organism from free radical damage. Propyl gallate increased catalase and glutathione reductase activities and enhanced life span of *Z. paravittiger* (28). BHA has increased glutathione reductase activity in the pre-reproductive age group of male mice (18). However, free radicals may also impair the activities of the

Table I. Median (LT_{50}) and Maximum Life Span of Male and Female *Z. paravittiger* Fed on Different Concentrations of BHA

	Male		Female	
	Median	Maximum	Median	Maximum
Con	36.59 ± 1.22	75.67 ± 0.58	40.06 ± 0.92	82.67 ± 2.31
1 m*M*	38.06 ± 0.42 (+4.02)	76.67 ± 1.53 (+1.32)	40.72 ± 0.26 (+1.65)	83.33 ± 2.08 (+0.80)
5 m*M*	43.33 ± 1.52[a] (+18.42)	80.67 ± 0.58[b] (+6.61)	43.21 ± 1.33[c] (+7.86)	88.33 ± 2.08 (+6.85)
10 m*M*	46.45 ± 1.24[a] (+26.95)	89.00 ± 1.00[b] (+17.62)	46.12 ± 1.18[a] (+15.13)	105.00 ± 1.00[a] (+27.01)
25 m*M*	44.10 ± 0.66[a] (+20.52)	77.33 ± 0.58[c] (+2.19)	45.29 ± 1.81[c] (+13.06)	86.33 ± 0.58 (+4.43)
50 m*M*	24.98 ± 0.46[a] (−31.73)	40.67 ± 0.58[b] (−46.25)	21.66 ± 0.89[b] (−45.93}	49.00 ± 2.00[b] (−40.73)
100 m*M*	13.25 ± 1.39[b] (−63.78)	35.00 ± 1.00[b] (−53.74)	17.14 ± 0.99[b] (−57.21)	37.00 ± 1.73[b] (−55.24)

Note. Values are mean ± SD. Data were analyzed by using a nonparametric Mann-Whitney *U* test followed by one way ANOVA. The values in parentheses represent percent change with respect to controls in each column. +, increase; −, decrease compared with control.
[a] $P < 0.01$, [b] $P < 0.001$, [c] $P < 0.05$, significantly different from control.

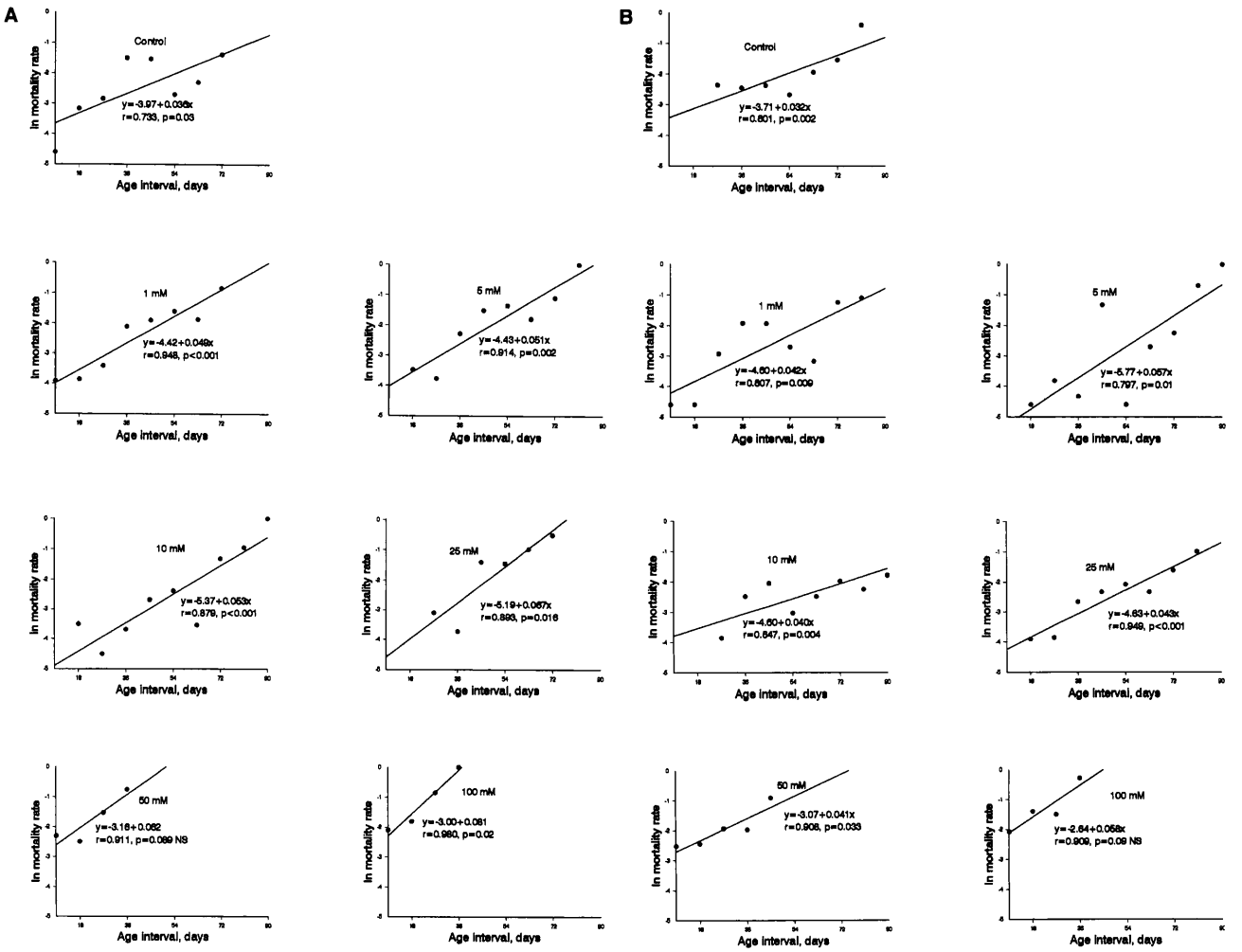

Figure 2. Gompertz plots of *Z. paravittiger* fed on various concentrations of BHA. (A) Male. (B) Female. Data were plotted between ln mortality rate and age interval (days). Simple linear regression was used for the statistical analysis.

Table II. Number of Eggs/10 Female Flies of *Z. paravittiger* Fed on BHA (10 m*M*)

Age (days)	Control	BHA	% change[a]
1	37.0 ± 6.2	40.0 ± 2.0	+8.11
8	91.3 ± 7.1	80.0 ± 5.0	−12.38
15	164.3 ± 14.4	127.0 ± 7.0[b]	−22.70
22	54.0 ± 4.6	61.3 ± 1.2	+13.51
29	14.7 ± 5.0	25.3 ± 6.8	+72.11
36	—	2.0 ± 1.0	—

Note. Values are mean ± SD. Data were analyzed by Mann-Whitney *U* test followed by one-way ANOVA.
[a] With respect to control.
[b] $P < 0.05$, significantly different from control.

Table III. Developmental Time (hr) of *Z. paravittiger* Fed on BHA (10 m*M*)

Developmental stage	Control	BHA	% change[a]
Egg	0	0	
Larva			
L_1	37.3 ± 2.1	37.6 ± 1.5	−0.80
L_2	63.0 ± 2.6	63.3 ± 2.5	+0.48
	(25.7 ± 4.7)	(25.7 ± 4.0)	
L_3	86.3 ± 1.5	92.0 ± 3.0[b]	+6.60
	(23.3 ± 4.0)	(28.7 ± 0.6)	
Pupa			
P_1	134.7 ± 4.2	144.0 ± 3.6[b]	+6.90
	(48.0 ± 2.9)	(52.0 ± 4.0)	
P_2	150.7 ± 3.1	176.3 ± 3.5[c]	+16.99
	(16.0 ± 5.3)	(32.3 ± 1.6)	
Adult	285.7 ± 2.5	306.3 ± 6.7[d]	+7.21
	(135.0 ± 3.0)	(130.0 ± 6.2)	

Note. Values in parentheses show duration of developmental phases (hr). Data were analyzed by using Mann-Whitney *U* test followed by one-way ANOVA. +, increase; −, decrease compared with respective control.
[a] With respect to control.
[b] $P < 0.05$, [c] $P < 0.001$, [d] $P < 0.01$, significantly different from control.

antioxygenic enzymes. In the presence of antioxidants, the enzyme activities could be higher without any induction by the antioxidant. This leads to the second possible mechanism, that BHA acts through its antioxidant effect, causing trapping of free radicals (16). BHA is commonly used as a food preservative due to its antioxidative properties, which inhibit the autooxidation of lipids (14). It quenches superoxide anion radical ($O_2^{\cdot-}$) (15), inhibits lipid peroxidation and peroxide induced DNA damage (29). Increase in longevity at 10 m*M* indicates that BHA has antioxidative effects at this low concentration. It did not produce any adverse effect to a large degree. However, the developmental time was delayed and the fecundity was reduced (Table II).

The third possible mechanism could be dietary restriction. Caloric restriction lowers steady-state levels of oxidative stress, extends the maximum life span in mammals (30), and delays age-associated degenerative diseases by decreasing rate of initiation of free radicals (31). In the present study, BHA may restrict food intake thereby increasing average and maximum life span. The antioxidant feeding decreases food intake (32), which leads to increased life span. It may be reasonable to interpret that some antioxidants can also show their effect by a mechanism similar to caloric restriction. However, in the present study BHA did not change the body weight of the flies (data not shown). If the caloric restriction mechanism was operational here, then one might expect a decrease in the total body weight of insect. Hence, the above possibility seems unlikely in the present study.

Reduction in egg-laying capacity observed in the present study could be the resultant of conservation of energy and low metabolic rate contributing towards the longer life span. The total developmental time was significantly delayed by BHA. However, Wadhwa (19) did not find any change in duration of developmental time on feeding sodium hypophosphite to *Z. paravittiger.* This reflects the differential effect of different antioxidants. BHA does not, however, hamper development at 10 m*M* concentration. The delay in development might be due to slowing down of a genetic program that governs the process of development. Lints and Lints (33), and Lints and Soliman (34) showed similar reports on *Drosophila melanogaster* and *Tribolium castaneum,* in which longevity and growth rates were linked. Similarly, Harrington and Harley (35) found that vitamin E slowed down the development and increased the life span of a nematode, *Caenorhabditis elegans.* Sohal and

Allen (36) stated that aging is a continuation of development and a genetically programmed phenomenon. Therefore, any change in developmental time could lead to alteration in life span, as observed presently. Although the longevity and senescence are under genetic control (37), it is suggested that by changing the developmental period of various organisms, including insects, the longevity can be prolonged (38). The present results corroborate with the above statement. Recently, we reported that kinetin, a plant growth hormone, delays aging, prolongs life span, and slows down development in *Z. paravittiger* (39), which corroborates the present observations. Although the actual mechanism of action of BHA is not clear, it is reasonable to suggest that BHA increases longevity, slows down development, and reduces egg-laying rate at its optimal range of concentrations.

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