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Three Mild Stresses Known to Increase Longevity in *Drosophila melanogaster* Flies do not Increase Resistance to Oxidative Stress

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Abstract: Exposure to a mild stress at a young age (hypergravity, cold or heat) increases longevity and resistance to heat shocks in *Drosophila melanogaster* flies and it may also delay behavioral aging and increase resistance to other stresses. The effect of these mild stresses on resistance to oxidative stress has not been studied. Flies were thus fed on a saccharose solution containing hydrogen peroxide after being subjected or not to one of these three mild stresses at a young age. Hydrogen peroxide decreased survival time to the same extent in control flies and in those subjected to a mild stress, which shows that these mild stresses do not increase resistance to oxidative stress. Therefore, the longevity increase induced by these mild stresses is probably not explained by a better protection against oxidative stress.

Key words: Hormesis, hydrogen peroxide, life span

INTRODUCTION

The idea that a mild stress can positively affect longevity and aging is now widely accepted (e.g.[1-5]). A mild stress does not kill the animal but disturbs homeostasis and the organism implements an adaptive response to cope with this mild stress[6].This adaptive response, called hormesis, eventually improves the functional ability of the organism to resist other stresses.

Exposure to mild stresses, such as hypergravity (HG: gravity levels higher than 1 g, the Earth gravity level, e.g.[7]), heat (37°C) or cold (0°C) shocks (e.g.[8-11]), or X-irradiation[12,13] can slightly increase longevity in *Drosophila melanogaster*, or in the nematode *Caneorhabditis elegans* (heat shock and hyperbaric oxygen:[14]). However, some mild stresses do not increase longevity as, for instance, UV or gamma-irradiations in *C. elegans*[14] or increase longevity of *D. melanogaster* males but not of females (HG:[7,15]; X-irradiation:[13]). While a mild stress can increase longevity, it does not always delay behavioral aging because the ability to climb up the side of a vertical vial decreases to a lower extent with age when flies are subjected at young age to cold or HG[11,16], but heat has no effect on the aging of this behavior[9].

Exposure to a mild stress at young age increases resistance to some strong stresses. For instance, survival time at 37°C is increased after pretreatment with heat, cold or HG (e.g.[9,11,17]). Furthermore, exposure to HG at young age increases survival time of males exposed at 4 weeks of age to a single or several non-lethal heat shocks, but no positive effect is observed in males heat-shocked at later ages and in females heat-shocked at 4, 5 or 6 weeks of age[18,19]. Cold shocks at young age also increase survival time of flies exposed at 4 weeks of age to several non-lethal heat shocks[11]. In other words, a treatment applied at *young* age can partially protect flies at *middle* age from non-lethal heat shocks. However, HG has no effect on resistance to dessiccation, starvation, or cold shock[16]. Cold shocks at young age have a negative effect on resistance to starvation, but a positive effect on survival to long cold shocks up to 3 weeks after a pretreatment with cold[11].

Oxidative stress has been used for a long time in gerontology research to test the free radical theory of aging[20]. For instance, authors expose flies to hydrogen peroxide and record survival time (e.g.[21]). A previous article has shown that a low dose of hydrogen peroxide can slightly increase the survival time of flies living on a poorly nutritious medium, i.e., a M/2 saccharose solution on which the longevity is shortened by half[22] and confirmed that high doses of hydrogen peroxide decrease survival time. Another article has shown that HG exposure did not vary the activity of the antioxidant enzymes superoxide dismutase and catalase[23], but no study has been done on the effects of mild stresses known to increase longevity on resistance to oxidative stress. The purpose of the present article is thus to test whether exposing flies at young age to one of three different mild stresses known to increase longevity, i.e., heat, cold or HG, can increase survival time of flies

living on a M/2 saccharose solution containing hydrogen peroxide.

MATERIAL AND METHODS

Flies: The experimental flies were adult males and females of the wild strain Meyzieu caught at the end of the seventies in France, near Lyon. This strain is maintained by mass mating (several bottles containing hundreds of flies mixed every three weeks) on the standard medium (agar, sugar, corn meal and killed yeast) containing a mold inhibitor (para-hydroxymethyl-benzoic acid) and enriched with live yeast at the surface of the medium. Experimental flies were obtained as follows: eggs laid by 5 day-old flies during a 15 h period by 50 pairs were transferred by batches of 25 into 80 mL glass vials containing the medium described above. At emergence, virgin flies with a duration of preimaginal development of 9-10 days were transferred under ether anesthesia in groups of 15 flies of the same sex to 20 mL polystyrene vials containing the standard medium with a drop of live yeast. In all experiments, flies of each vial were transferred without anesthesia to a new one containing fresh medium twice a week up to the day of transfer into the vials containing saccharose only or saccharose with hydrogen peroxide (see below). Flies spent their life in an incubator (but see below the HG experiment): the rearing temperature was 25±0.5°C; light was on from 08.00-20.00 h (fluorescent lamp).

Heat pretreatment: One half of the flies was not subjected to heat shocks. The other flies were transferred just before heat shock from their rearing vials to empty polystyrene vials (diameter: 17 mm, length: 63 mm) closed by a polypropylene plug containing absorbent cotton soaked with distilled water. Flies were subjected daily during 5 days to a 5 min 37°C heat shock in a water-bath, the first shock being applied at 5 days of age, as in a previous article[9]. After each heat shock, flies were transferred back to their rearing vials. At 12 days of age, i.e., 3 days after the last heat shock, flies were transferred to vials containing saccharose only or saccharose with hydrogen peroxide (see below).

Hypergravity pretreatment: Flies were transferred one day after emergence either to 1, 3.02 or 5.02 g (1, 3 and 5 g in the following). Vials were kept vertically within the same centrifuge rotating at 102±0.2 rpm at different distances from the axis, as in previous articles (e.g.[15]). The centrifuge, containing the 3 and 5 g groups and the 1 g groups were in a room 25±0.5°C, lit from 08.00-20.00 (fluorescent lamp). At two weeks of age, centrifugation was stopped and flies were transferred to the incubator previously described. One day (16 days of age), 3 days (19 days of age), or two weeks (27-28 days of age) later, depending on experiment, they were transferred to vials containing saccharose only or saccharose with hydrogen peroxide.

Cold pretreatment: One half of the flies was not subjected to cold shocks. The other flies were exposed from 5 days of age to 0°C for 60 min a day during two periods of 5 days separated by two days, as in a previous article[11]. Flies were transferred without anaesthesia in early morning to empty polystyrene vials (diameter: 17 mm, length: 63 mm) closed by a polypropylene plug. These vials were kept for one hour in ice (0°C) and afterwards at room temperature for at least 20 min before being transferred back to their rearing vials. The vials used for the cold shock did not contain food to avoid any delay of the temperature fall and prevent flies from being stuck to food while asleep. Therefore, control flies were kept in their rearing vials, because, as these flies are not knocked down by cold, transferring them to empty vials would be a period of starvation to which flies transferred to 0°C are not subjected since they are asleep. At 19 days of age, i.e., 3 days after the last cold shock, or 33 days of age, flies were transferred to vials containing saccharose only or saccharose with hydrogen peroxide (see below).

Longevity with hydrogen peroxide: Flies of each vial were transferred to polystyrene vials (diameter: 17 mm, length: 63 mm) closed by a polypropylene plug, as in a previous article[22]. This plug was cut with a razor blade in order to insert into it a strip of chromatography paper (Whatman, 3MM Chr, ca 10 by 30 mm). One hundred μL of a M/2 saccharose solution (Prolabo 27478.296) were deposited on the strip. Depending on the experiment, the saccharose solution contained various concentrations of hydrogen peroxide (0, 0.8, 3.3% (w/v), i.e., 0, 245, 979 mM) diluted from 30% (w/w) hydrogen peroxide (Prolabo 23622.298). New solutions of saccharose were prepared each week and solutions were stored at 4°C. In order to prevent desiccation, the vials containing the flies were stored in closed wet boxes.

Every day and up to the death of the last fly, the number of dead flies was recorded, the plug and the strip were replaced by new ones and 100 μL of the appropriate saccharose solution were deposited on the new strip. Old strips were discarded and plugs were rinsed with hot tap water and dried in order to be used again the next day. As the plug was tightly inserted into

the vial, the old strips were still wet when they were discarded, i.e., flies were not subjected to desiccation.

During the course of the experiment, humidity could condense on the inner wall of the vials. If drops of water were seen by the experimenter, flies were transferred to another vial without anaesthesia and dead flies were removed. Flies were transferred to new vials ca weekly and it was then attempted to remove corpses. However, some dead flies were not removed if there was a risk of escaping of the other flies.

Statistics: In all experiments, day 0 is the day of transfer into the vials containing saccharose and mean survival time is life expectancy after this day. Life expectancy data were transformed if needed to obtain a normal distribution of residuals and were analyzed with factorial ANOVA.

RESULTS AND DISCUSSION

Heat pretreatment: A first experiment tested the effect of 3.3% hydrogen peroxide (January 2007, 5 vials for each sex and pretreatment level). A two-way ANOVA (sex and pretreatment factors) showed that females survived for a longer time than males ($F(1, 284) = 37.58$, $p<0.0001$: mean survival time of males and females, respectively: 3.05 and 3.60 days). The heat pretreatment effect and its interaction with sex were not significant ($F<1$).

A second experiment tested the effect of 0.8% hydrogen peroxide, two replicates being done during a 2 months period (February-March 2007, 5 vials for each sex and pretreatment level in each replicate). The replicate factor was considered as fixed and not as random, because the differences between replicates are not purely random due for instance to seasonal effects. The pretreatment factor was significant ($F(1, 555) = 7.83$, $p = 0.0053$), showing that heat-shocked flies survived for a slightly lower time (mean: 9.77 days) than control ones (10.44 days). Males survived for a longer time than females (sex effect: $F(1, 555) = 4.46$, $p = 0.0351$; mean survival time of males and females, respectively: 10.42 and 9.80 days) and the nearly significant sex by treatment interaction ($F(1, 555) = 3.62$, $p = 0.0576$) suggested that the pretreatment effect was mainly due to females. The replicate factor was significant ($F(1, 555) = 33.72$, $p<0.0001$, means of the two replicates: 9.41 and 10.83 days). The sex by pretreatment interaction was also significant ($F(1, 555) = 5.77$, $p = 0.0166$), showing that the pretreatment effect was observed in one of the replicates only.

Therefore, these experiments have shown that a heat pretreatment had either no effect on resistance to oxidative stress or slightly decreased it.

Hypergravity pretreatment: A first experiment tested the effect of 3.3% hydrogen peroxide at 19 days of age (January 2007, 5 vials for each sex and gravity level). A two-way ANOVA (sex and gravity factors) on the logarithm of survival time (in order to normalize residuals) showed that females survived for a longer time than males ($F(1, 402) = 235.77$, $p<0.0001$: mean survival time of males and females, respectively: 2.45 and 3.51 days). The gravity effect was significant ($F(2, 402) = 4.02$, $p = 0.0187$), showing that 3 g flies survived for a lower time (mean: 2.85 days) than both 1 g (3.02 days) and 5 g ones (3.10 days). Bonferroni post-hoc tests showed that the differences between the 3 g group and the two other gravity groups were significant ($p<0.05$), while that between 1 and 5 g groups was not. The sex by gravity interaction was not significant (F close to 1).

A second experiment tested the effect of 0.8% hydrogen peroxide at 2 and 4 weeks of age (16 and 27-28 days of age), three replicates being done at each age during a 5 months period (September 2004-January 2005, 3 vials for each sex and gravity level in each replicate). For each age, an ANOVA tested the effect of sex, gravity and replicate. The replicate factor was considered as fixed and not as random.

In 2-week old flies, the gravity factor was significant ($F(2, 742) = 3.33$, $p = 0.0363$), showing that 3 g flies survived for a slightly lower time (mean: 8.12 days) than both 1 g (8.55 days) and 5 g ones (8.70 days). Bonferroni post-hoc tests showed that the difference between the 3 g group and the 5 g one was significant ($p = 0.0359$), while those between the 1 g group and the two other gravity groups were not. The replicate factor was significant ($F(2, 742) = 52.21$, $p<0.0001$, means of successive replicates: 8.45, 9.73, 7.27 days). The sex by replicate interaction was also significant ($F(2, 742) = 9.35$, $p<0.0001$), showing that the replicate effect was mainly due to females. The sex effect and the other interactions were not significant (data not shown).

In 4-week old flies, the sex factor was significant ($F(1, 693) = 81.82$, $p<0.0001$), showing that females survived for a slightly lower time (mean: 6.12 days) than males (7.52 days). The replicate factor was significant ($F(2, 693) = 87.41$, $p<0.0001$, means of successive replicates: 8.13, 5.97, 6.09 days). The second-order interaction between sex, gravity and replicate interaction was also significant ($F(4, 693) = 5.44$, $p = 0.0003$). The gravity effect and the other interactions were not significant (data not shown).

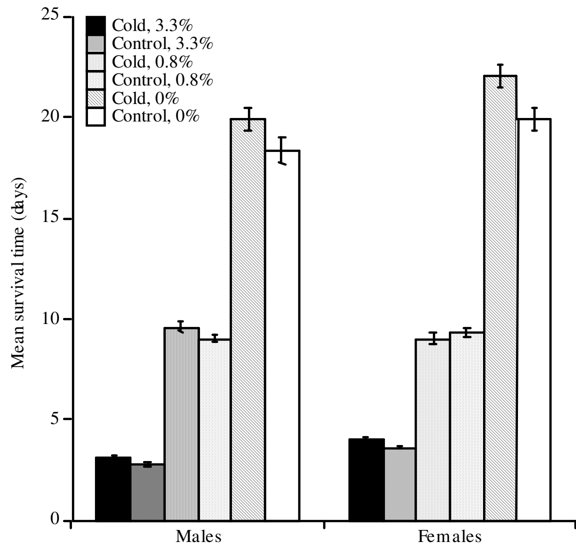

Fig. 1: Mean survival time in days (±SEM) after transfer at 19 days of age to vials containing a M/2 saccharose solution as the only food source. Hydrogen peroxide was added or not added to this solution (hydrogen peroxide concentrations: 0, 0.8 or 3.3% w/v). One half of the flies was not subjected to cold shocks and the other flies were exposed from 5 days of age to 0°C for 60 min a day during two periods of 5 days separated by two days

To sum up all these experiments done at 2 or 4 weeks of age with 0.8 or 3.3% hydrogen peroxide concentration, it can be said that being subjected at young age to HG had no clear effect on resistance to oxidative stress.

Cold pretreatment: Figure 1 summarizes the results of experiments done at 19 days of age. A first experiment tested the effect of 3.3% hydrogen peroxide at 19 days of age, two replicates being done during a 3 months period (February-April 2007, 3-5 vials for each sex and pretreatment level in each replicate). The replicate factor was considered as fixed and not as random. A three-way ANOVA (sex, pretreatment and replicate factors) on the logarithm of survival time (in order to normalize residuals) showed that females survived for a longer time than males ($F(1, 476) = 111.61$, $p<0.0001$: mean survival time of males and females, respectively: 2.97 and 3.81 days). The cold pretreatment effect was significant ($F(1, 476) = 18.24$, $p<0.0001$), showing that the cold pretreatment very slightly increased survival time (mean of respectively cold-shocked and control flies, 3.54 and 3.22 days). The replicate factor was significant ($F(1, 476) = 123.57$, $p<0.0001$, means of the two replicates: 3.01 and 3.91days), as well as its interaction with sex ($F(1, 476) = 5.76$, $p = 0.0168$) and the second-order interaction between replicate, sex and pretreatment ($F(1, 476) = 8.22$, $p = 0.0043$). The other interactions were not significant.

A second experiment tested the effect of 0.8% hydrogen peroxide at 19 days of age, two replicates being done during a 2 months period (February-March 2007, 5 vials for each sex and pretreatment level in each replicate). The replicate factor was considered as fixed and not as random. The replicate factor was significant ($F(1, 525) = 8.00$, $p = 0.0049$; means of the two replicates: 8.92 and 9.61 days). The sex and pretreatment factors and all interactions were not significant ($F<1$). However, the nearly significant interaction between sex and pretreatment ($F(1, 525) = 3.72$, $p = 0.0541$) suggested that cold-shocked males survived slightly longer than control ones (means, respectively: 9.62 and 9.02 days) while the opposite pattern was observed in females (9.02 vs 9.32 days).

Finally, a control experiment tested whether the pretreatment had some effect on longevity of flies feeding on saccharose from 19 days of age, two replicates being done during a 3 months period (April-June 2007, 4 or 5 vials for each sex and pretreatment level). The replicate factor was considered as fixed and not as random. A three-way ANOVA (sex, pretreatment and replicate factors) showed that females survived for a longer time than males ($F(1, 425) = 13.08$, $p = 0.0003$: mean survival time of males and females, respectively: 19.11 and 20.96 days). The cold pretreatment effect was significant ($F(1, 425) = 10.10$, $p = 0.0016$), showing that the cold pretreatment slightly increased survival time (mean of respectively cold-shocked and control flies, 21.04 and 19.16 days). The replicate factor was not significant ($F<1$), but its interaction with sex ($F(1, 425) = 12.33$, $p = 0.0005$) and with the pretreatment ($F(1, 425) = 3.96$, $p = 0.0473$) were significant. The sex effect was nearly erased in one of the replicates while the pretreatment effect was more important in this replicate. The other interactions were not significant.

These experiments with 0.8 and 3.3% hydrogen peroxide have shown that a cold pretreatment had either no effect on resistance to oxidative stress or slightly increased it. The control experiments showed that the cold pretreatment increased longevity when flies feed only on saccharose. Therefore, a cold pretreatment increases longevity when flies feed on the standard medium (agar, sugar, corn meal, dry and live yeast)[11], but also on a poorly nutritious medium known to decrease longevity[22]. Since a small positive effect of

cold pretreatment on resistance to oxidative stress was observed, other experiments at an older age were carried out in order to confirm and extend these results.

The effect of 3.3% hydrogen peroxide was tested at 33 days of age (May 2007, 5 vials for each sex and pretreatment level). A two-way ANOVA (sex and pretreatment factors) showed that females survived for a longer time than males ($F(1, 275) = 56.69$, $p<0.0001$: means of males and females, respectively: 2.64 and 3.39 days). The cold pretreatment effect was nearly significant ($F(1, 275) = 3.56$, $p = 0.060$), suggesting that cold pretreatment very slightly decreased survival time (mean of respectively cold-shocked and control flies, 2.94 and 3.09 days). The interaction between sex and pretreatment was not significant.

The effect of 0.8% hydrogen peroxide was also tested at 33 days of age (May 2007, 4 vials for each sex and pretreatment level). The sex and pretreatment factors and their interaction were not significant (F close to 1, mean survival time of all flies: 7.29 days, n = 207).

A control experiment tested whether the pretreatment had some effect on longevity of flies feeding on saccharose from 33 days of age (May 2007, 4 vials for each sex and pretreatment level). A two-way ANOVA (sex and pretreatment factors) showed that females survived for a longer time than males ($F(1, 185) = 10.48$, $p = 0.0014$: means of males and females, respectively: 16.42 and 19.23 days). The cold pretreatment effect was not significant ($F<1$) and the just significant sex by pretreatment interaction showed that the cold pretreatment slightly increased longevity of males and decreased that of females ($F(1, 185) = 3.90$, $p = 0.0497$, means of cold pretreated and control males: 17.58 and 15.11 days; means of cold pretreated and control females: 18.69 and 19.71 days).

To sum up experiments using the cold pretreatment, this pretreatment had no clear effect on resistance to oxidative stress at 3 or 5 weeks of age. When a significant and positive effect of cold pretreatment on survival time was however observed (3 week-old flies subjected to 3.3% hydrogen peroxide), it was not important.

Previous studies have shown that three mild stresses, namely heat, HG and cold, can increase longevity of flies and resistance to a lethal heat shock. Hypergravity and cold, but not heat, also delay behavioral aging and the two first mild stresses increase resistance to successive non-lethal heat stresses. Finally, cold, but not HG, increases resistance to a severe cold shock but decreases resistance to starvation while HG has no effect on this trait. Therefore, on the whole, it may be said that mild stresses have positive effects on resistance to various strong stresses.

In such conditions, knowing whether they also increase resistance to oxidative stress is of interest, because the free radical theory of aging[20] postulates that oxidative stress is a crucial factor to explain the aging process and longevity variability. An increased resistance to oxidative stress of flies subjected to mild stresses could also explain their increased longevity if the free radical theory of aging explains longevity variability (e.g.[24]). Testing this hypothesis is of importance because, for the time being, we know that a mild stress can increase longevity but we do not know why. Previous studies have shown that the increased longevity of flies subjected to HG cannot be explained by the synthesis of the 70 kDa heat shock protein. This protein is not synthetized at 25°C, the rearing temperature of flies, but its synthesis after a heat shock is more important in flies exposed to HG at a young age than in flies always kept at 1 g[25]. As far as HG is concerned, it is doubtful that antioxidant enzymes are a critical factor explaining the longevity increase induced by this mild stress, because being subjected to HG at a young age has no effect on superoxide dismutase and catalase activities at young, middle and old age[23]. Such a study has however not been carried out in flies subjected to the other mild stresses, i.e., heat and cold.

Nevertheless, it could be that resistance to oxidative stress is increased after a mild stress, even if the activity of antioxidant enzymes does not vary. In the present study, flies exposed to one of the three mild stresses known to increase longevity were subjected to one of two hydrogen peroxide concentrations at various ages. These concentrations strongly decreased longevity (e.g. Fig. 1), as expected[22], but HG or heat pretreatments did not attenuate this effect and thus did not increase resistance to oxidative stress.

However, figure 1 shows that a cold pretreatment very slightly decreased the negative effect of the 3.3% hydrogen peroxide concentration — but not that of the 0.8% one — if flies were subjected to hydrogen peroxide at 19 days of age. Cold pretreatment also increased survival time of flies feeding on saccharose from 19 days of age, which shows that cold can increase longevity and has only minor effects on resistance to hydrogen peroxide. A similar effect was observed in males feeding on saccharose from 33 days of age, but not in females. However, the slight positive effect of a cold pretreatment on resistance to oxidative stress was not confirmed when flies were subjected to hydrogen peroxide at 33 days of age.

Therefore, mild stresses can increase resistance to several strong stresses but not to oxidative stress. It thus

seems that the increased longevity induced by a mild stress cannot be explained by a higher resistance to oxidative stress, which confirms that resistance to oxidative stress and longevity are not always tightly linked[21,26].

Since mild stresses increase resistance to several stresses, but not to oxidative stress, it could be either that hormesis has simply no effect on resistance to oxidative stress or, more importantly, that oxidative stress is not a critical factor for life processes. In other words, the importance of the free radical theory of aging to explain aging and longevity has perhaps been overestimated (e.g.[27]). However, the present results are not sufficient to infer such a strong conclusion, because this experiment only tested resistance to hydrogen peroxide and using another oxidant, for instance paraquat, could bring to the fore a positive effect of mild stresses on resistance to oxidative stress, since flies of long-lived lines have an increased resistance to paraquat[24]. Secondly, coping with the low level of oxidative stress normally encountered *in vivo*[28] has probably nothing to do with surviving to poisoning by a high oxidant concentration. Yet, for the time being, it can be concluded that mild stresses known to slightly increase longevity do not increase resistance to oxidative stress.

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