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RESEARCH ARTICLE

Combined effects of two mild stresses (cold and hypergravity) on longevity, behavioral aging, and resistance to severe stresses in *Drosophila melanogaster*

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Abstract Mild stresses may have positive effects on aging, longevity and resistance to severe stresses at various ages in *Drosophila melanogaster*. However, no study has combined two mild stresses to know whether more positive effects would be observed than with each stress alone. Cold and hypergravity (HG) have positive effects on some traits, but negative ones can also be observed, particularly in females. This study combined in the same flies cold and HG exposure. When cold and HG had each positive or negative effects their combination had additive effects but, when only one of the pretreatments had some effect, the effect of their combination usually reflected this effect. Therefore, combining two mild stresses with positive effects on aging and longevity can be more efficient than each stress alone. However, if one of these mild stresses had negative effects and the other one positive effects, the net result of their combination could be the suppression of the positive effect of the second stress. On the whole, if the net result of the combination of two mild stresses would be negative, it would be preferable not to combine them.

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Introduction

It is now well established that a mild stress, i.e. a stimulus which disturbs the homeostasis of the organism but does not induce severe damages, can provoke an adaptive response which enhances the ability of the organism to resist other stresses: this phenomenon is called hormesis (reviews in Mattson and Calabrese 2010). For instance, heat and low doses of various chemicals can increase longevity in the nematode *Caenorhabditis elegans* (review in Le Bourg 2009a).

Mild stresses can also increase longevity and improve healthspan in flies. For instance, heat or cold shocks and hypergravity (HG) can slightly increase longevity in *Drosophila melanogaster* flies, delay behavioral aging and increase resistance to life-threatening stresses (review in Le Bourg 2009a). However, mild stresses can have deleterious effects in females, because HG can slightly decrease longevity (Le Bourg 2009b) and cold has been observed either to increase (Le Bourg 2007; Le Bourg et al. 2009) or decrease longevity (Le Bourg 2010) or to be neutral (Le Bourg 2007, 2011a).

The strength of the effects of the same mild stress on aging and longevity of flies can vary. For instance,

positive effects of heat shocks on longevity varied from a minor effect (2 days in Khazaeli et al. 1997 and Le Bourg et al. 2001) to a larger one (6 days in Hercus et al. 2003: other results in Le Bourg 2009a). Nevertheless, Le Bourg et al. (2009) failed to reproduce the positive effect of heat on longevity observed by Le Bourg et al. (2001). Regarding behavioral aging, both positive (Minois et al. 2001) and no effect (Le Bourg et al. 2001) of heat on spontaneous locomotor activity have been reported.

The effects of different mild stresses on resistance to a severe stress can also be variable. On the one hand, heat, HG, and cold increase resistance to lethal heat (reviews in Le Bourg 2009a, b) but decrease resistance to starvation (Minois and Le Bourg 1999; Minois 2001; Le Bourg 2007). Therefore, as often observed (e.g. Bubliy et al. 2012), cross-resistance to several severe stresses after a mild stress has been applied is not always observed (see also Hoffmann et al. 2005 for negative genetic correlations among stress resistance traits in a sex-dependent manner). On the other hand, different mild stresses can have different effects on resistance to a severe stress: for instance, HG and heat have no effect on resistance to fungal infection while cold increases it, in males only (Le Bourg et al. 2009).

While studies on mammals are rather scarce (reviews in Le Bourg 2009a and Le Bourg and Rattan 2008), most experts are of the opinion that hormesis is applicable as a pro-healthy aging intervention in mammals and human beings (see e.g. Le Bourg and Rattan 2010; Pardon 2010; Kahn and Olsen 2010). Therefore, hormesis has gained attention in recent years, particularly in biogerontology (see e.g. Mattson 2008; Rattan 2008), and time has come to deeply analyze the conditions to obtain the largest positive effects of a mild stress on the aging process. Particularly, one may wonder whether combining two mild stresses in the same animals would allow to synergize their positive effects or whether this combination could become a severe stress with negative effects. As indicated above, different mild stresses can have different effects on the same trait, either positive, negative or neutral, and it can be hypothesized that their combination would have different effects, depending on the effect of each stress alone. To sum up, any outcome could be observed when combining two mild stresses and, as emphasized by Sørensen et al. (2010), "research in this area is still rather limited", to say the very least.

A combination of heat and cold pretreatments on survival to cold has been tested in larvae of *D. melanogaster* (Rajamohan and Sinclair 2008). Heat pretreatment alone had no positive effect on survival but its combination with cold had a more positive effect than the cold pretreatment alone, particularly if the heat pretreatment preceded the cold one. Unfortunately, the authors did not test adult and aged flies. Le Bourg (2010) has combined in the same flies a cold pretreatment and a dietary manipulation, the suppression of live yeast from the food (i.e. mainly a reduction of the protein content), which increases longevity in some cases, delays the age-linked decline of climbing activity, but also decreases resistance to various stresses (Le Rohellec and Le Bourg 2009). If cold and suppression of live yeast had positive effects (e.g. a lower age-linked decline of climbing activity), their combination had additive effects, flies without live yeast and subjected to cold getting the highest scores. By contrast, when one of these treatments had deleterious but not tragic effects (e.g. suppressing live yeast decreases resistance to fungal infection), the positive effect of the other treatment could still be observed or not in flies subjected to both treatments. Finally, the highly deleterious effect of the absence of live yeast on resistance to cold (most of the flies do not recover from a 16 h 0°C shock) was not attenuated by a cold pretreatment which, when used alone, increases resistance to cold. The negative effect of a cold pretreatment on longevity of females was also reinforced if they had no access to live yeast.

This previous study seems to indicate that the combination of two treatments having each positive effects could have additive effects and that, when one treatment has a negative effect, trying to predict the effects of its combination with a second one is hazardous. It thus could be expected that combining two mild stresses with positive effects on aging and longevity such as HG and cold could be more efficient than each mild stress alone. However, in females, as cold has sometimes been observed to decrease longevity and HG to have no effect or even a slight negative effect, it is difficult to predict the effect of the two stresses in the same flies. Similarly, as HG has no effect on resistance to fungal infection, contrarily to cold which has positive effects, it is also risky to predict whether their combination would have positive effects or not.

The present article reports the results of the combination in the same flies of two mild stresses,

HG exposure and cold pretreatment, on longevity, resistance to severe stresses (lethal heat, cold, oxidative stress, starvation and fungal infection), and climbing activity at various ages. Flies were subjected either to no mild stress, to one of them, or to the two stresses.

Materials and methods

Flies

The experimental flies were adult males and females of the wild strain Meyzieu caught at the end of the 1970s in France, near the city of Lyon. The strain is maintained by mass mating (three bottles containing hundreds of flies being mixed) on the standard medium (agar, sugar, corn meal and killed yeast) containing a mold inhibitor (*para*-hydroxymethylbenzoic acid) and enriched with live yeast at the surface of the medium.

In order to obtain the parents of the experimental flies, flies were allowed to lay eggs for one night in a bottle containing the medium described above. About fifty pairs emerging from this bottle 9–10 days after egg-laying were transferred to bottles (ca. 25 pairs in a bottle) containing the medium previously described: these flies are the parents of the experimental flies. Experimental flies were obtained as follows: eggs laid by 5 day-old parents during a 15 h period on a Petri dish containing the usual medium colored with charcoal and a drop of live yeast 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.

Flies were transferred to new vials twice a week. Flies spent the first 2 weeks of their adult life in a room where is located a centrifuge (see below) and the remaining of their life in an incubator. In both the room and the incubator the rearing temperature was 25 ± 0.5°C; light was on from 07.00 to 19.00 h (fluorescent lamp). However, it happened that in one of the longevity experiments (see below) the temperature in the room of the centrifuge was ca. 26–27°C during the first week of adult life.

Pretreatment of flies by a mild stress

Cold pretreatment

Flies were exposed from 5 days of age to 0°C for 60 min a day during two periods of 5 days separated by 2 days with no cold shock. Flies were transferred without anesthesia in early morning to empty polystyrene vials (diameter: 17 mm, length: 63 mm) closed by a polypropylene plug. These vials were kept for 1 h in ice (0°C) and afterwards at room temperature for at least 20 min and, after that, flies were 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 when 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.

Hypergravity pretreatment

HG is obtained by putting flies in a continuously rotating centrifuge (for a picture, see Le Bourg 2008a) and the same procedure as in previous experiments is used. Flies were subjected to 5 g for 2 weeks from the second day of adult life and after that transferred to 1 g: these flies are called the HG-flies. Flies never subjected to HG were placed near the rotating centrifuge during the first two weeks of adult life: these flies are called the 1 g-flies. After the two weeks of centrifugation, all flies were transferred into the incubator described above. The centrifuge was stopped during the cold pretreatments and the recovery time from cold, i.e. for ca. 90 min. Thus, the days of cold pretreatments, all HG-flies were kept at 1 g for ca. 90 min, even if they were not subjected to the cold pretreatment.

Combination of pretreatments

Flies in each sex were subjected either to cold or HG pretreatments, to both pretreatments, or were not pretreated. Therefore, there was, respectively, 4 groups of flies: Cold flies, HG flies, HG–cold flies, and control flies not subjected to any pretreatment. HG exposure began the second day of life (1 day of age) and was stopped 2 weeks later (15 days of age), and

the cold pretreatments were done for 2 periods of 5 days separated by 2 days and beginning at 5 days of age, as indicated above. Thus, HG–cold flies were subjected to both pretreatments from 5 to 9 days of age and from 12 to 15 days of age, to HG only from 2 to 5 days of age and at 10 and 11 days of age, and to cold only at 16 days of age.

Longevity

Longevity of flies born during a 1-day interval in August 2010, December 2010 or October 2011 was recorded daily from emergence until the death of the last fly. For each experiment, longevity results were analyzed with factorial ANOVAs testing for the effect of sex, cold, HG and all interactions. In order to obtain a normal distribution of residuals, the data of the first and third experiments were squared before analysis.

Resistance to heat

The survival time in a water-bath set at 37°C was observed at various ages. Flies were transferred just before shock in groups of 3 flies into empty polystyrene vials (diameter: 17 mm, length: 63 mm), the plug containing absorbent cotton with distilled water to prevent desiccation. Flies were observed every 5 min and those totally immobile were considered to be dead (flies never recover after total immobility). A first experiment used a cohort of flies born during a 1-day interval in September 2010 at 3, 4, and 5 weeks of age. A second experiment used flies born during a one-day interval in January 2011 at 6–7 weeks of age, i.e. at 40, 42, 44, and 47 days of age, and males only at 48–49 days of age. The survival time results of each experiment were analyzed with a factorial ANOVA testing for the effect of sex, cold, HG, age and all interactions.

Resistance to cold

Resistance to cold was observed at various ages. Flies born during a one-day interval in February 2011 were kept at 0°C for 16 h overnight at 3, 4 or 5 weeks of age (19, 26 or 33 days). After this cold shock, flies were transferred back to their rearing vials at 25°C. The percentage of survivors 3 days after the cold shock was analyzed with a logistic model testing for the effect of sex, cold, HG, age and all interactions. A logistic model cannot be computed if there is an empty cell and it happened that no 3-week-old HG male among the 25 which were subjected to the cold pretreatment died. Therefore, in order to be able to use the logistic model, it was decided to arbitrarily consider that one fly of this group died.

A second experiment was done at 6 and 7 weeks of age. The results of 6-week-old flies were analyzed with a logistic model. As no 7-week-old male not cold-pretreated survived to the cold shock, the effect of the cold and HG pretreatments were analyzed with χ^2 tests. The results of 7-week-old females were analyzed with a logistic model.

Resistance to infection

The spores of the fungus *Beauveria bassiana* kept at −80°C in 20% glycerol were incubated at 25°C in 90 mm Petri dishes containing the appropriate medium (for one liter of distilled water, the autoclaved medium contained: peptone (Sigma P463): 1 g, glucose (Fluka 49159): 20 g, malt extract (Fluka 70167): 20 g, agar: 15 g). After sporulation, which occurs ca. 4 weeks after spreading spores on the medium, flies were infected. The day of infection, flies were transferred to new vials before to be very slightly anesthetized with ether and then shaken for ca. 1 min in a Petri dish containing a sporulating fungal culture. After having checked under stereomicroscope that all flies were well covered with spores, flies were transferred back to their vials. Longevity was recorded daily from the day following infection until the death of the last fly. Day 0 is the day of infection.

A first experiment used flies born during a one-day interval in September 2010. These flies were infected at 40 days of age and the longevity of non-infected flies was also observed from this age. The longevity results were square-rooted and analyzed with a factorial ANOVA testing for the effect of infection, sex, cold, HG and all interactions.

A second experiment used a cohort of flies born during a one-day interval in January 2011 and infected at 19, 26, and 33 days of age. Their longevity results were log-transformed and analyzed with a factorial ANOVA testing for the effect of sex, cold, HG, age and all interactions. The longevity of non infected flies was also observed from 19 days of age to confirm that infection decreased longevity. These longevity results were analyzed with a factorial ANOVA testing for the effect of sex, HG, cold, and all interactions.

Resistance to starvation

Resistance to starvation was observed in flies born during a one-day interval in March 2011. Flies of each vial were transferred without anesthesia at 18.30 h at 19, 26, or 33 days of age into a vial containing a medium made of agar, a mold inhibitor (*para*-hydroxymethyl-benzoic acid) and distilled water. Vials were renewed twice a week up to the death of the last fly. In this way, flies experienced starvation but not desiccation. The number of dead flies was recorded twice a day (06.30 and 18.30 h) until the last death. Logarithms of the survival times were analyzed with a factorial ANOVA testing for the effect of sex, cold, HG, age and all interactions.

Resistance to 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 (Le Bourg 2008b). 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 microliters of a M/2 saccharose solution (Prolabo 27478.296) were deposited on the strip with hydrogen peroxide (3.3% (w/v), i.e. 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.

Resistance to hydrogen peroxide was observed at 3, 4, 5 and 6 weeks of age in males but only at 3, 4, and 5 weeks of age in females, because no HG females subjected to the cold pretreatment were available after the experiments carried out at the previous ages. An additional experiment was performed with males only at 7 weeks of age in two replicates (flies born on two successive weeks; the replicate factor was considered as fixed in the statistical analysis). All flies were born during a one-day interval in March 2011.

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 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 a 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. The longevity of the 7-week-old males of the additional experiment was however observed twice a day (6.30 and 18.30 h), because it was expected that their survival times would be very low.

Survival time were analyzed in each sex with factorial ANOVAs testing for the effect of cold, HG, age and all interactions (no age effect for the additional experiment).

Climbing activity

In the climbing activity test, flies are individually placed in a vertical vial subjected to a mechanical stimulation provided by a single-vial shaker and the highest height reached within 20 s after cessation of the stimulation is recorded. Climbing scores decrease with age and this test has been routinely used in aging research for many years (e.g. Miquel et al. 1972; Feany and Bender 2000). The procedure has been described in detail (Le Bourg and Minois 1999) but a low stimulation intensity was used in the present experiment (ca. 145 rpm during 3 s). Climbing activity was observed in the morning at 19, 22, 26, 29, 33, 36, and 40 days of age, but females could not be observed at the two oldest ages. Ten flies with intact legs were observed in each group of sex, cold and HG pretreatments, but this number could be lower at the last age, due to a lack of flies (only 9 females in the control females at 33 days of age and, respectively, 6 and 8 flies in HG–Cold and Cold males at 40 days of age). All flies were born during a one-day interval in September 2010.

A log transformation was applied to the climbing scores before they were analyzed and, as females were not studied at 36 and 40 days of age, an ANOVA testing for the effect of cold, HG, age and all interactions was computed for each sex.

Results

Longevity

In the first experiment (flies born in August 2010), the sex effect was significant ($F(1, 760) = 58.41$, $p < 0.0001$) as well as all interactions involving the sex factor (data not shown). Therefore, the two sexes

were analyzed in separate ANOVAs. In males (Fig. 1a, b), both the cold pretreatment ($F(1, 391) = 30.68$, $p < 0.0001$) and HG increased longevity ($F(1, 391) = 5.39$, $p = 0.0207$). The interaction between these factors was not significant, which means that the effects of these two factors were additive. In females (Fig. 1a, c), both the cold pretreatment ($F(1, 369) = 25.78$, $p < 0.0001$) and HG ($F(1, 369) = 3.72$, $p = 0.0545$) decreased longevity, significantly or marginally. The significant interaction showed that the deleterious effect of cold was reinforced if females were also subjected to HG ($F(1, 369) = 6.73$, $p = 0.0099$). In this experiment, flies began to die at an early age (no mortality plateau: Fig. 1b, c), which is quite unusual, and the longevity means were rather low, particularly in control females (mean ± SEM: 38.39 ± 1.50 days). The high temperature during the first week of life (see "Materials and methods") could explain this low longevity and it is needed to replicate this experiment before reaching any conclusion regarding the effect of cold and HG pretreatments.

Longevities were higher in the second experiment (flies born in December 2010) than in the first one (compare Fig. 1a–c and d–f). In this experiment, the sex effect was also significant ($F(1, 982) = 133.45$, $p < 0.0001$) as well as the first-order interactions involving the sex factor (data not shown). Therefore, the two sexes were analyzed in separate ANOVAs. In males (Fig. 1d, f), the cold pretreatment ($F(1, 484) = 6.61$, $p = 0.0104$) increased longevity. The gravity effect and its interaction with the cold pretreatment were not significant. In females (Fig. 1d, g), both the cold pretreatment ($F(1, 498) = 31.91$, $p < 0.0001$) and HG ($F(1, 498) = 11.87$, $p = 0.0006$) decreased longevity. The interaction between these two factors was not significant.

Longevities in the third experiment (flies born in October 2011) were higher than in the previous ones

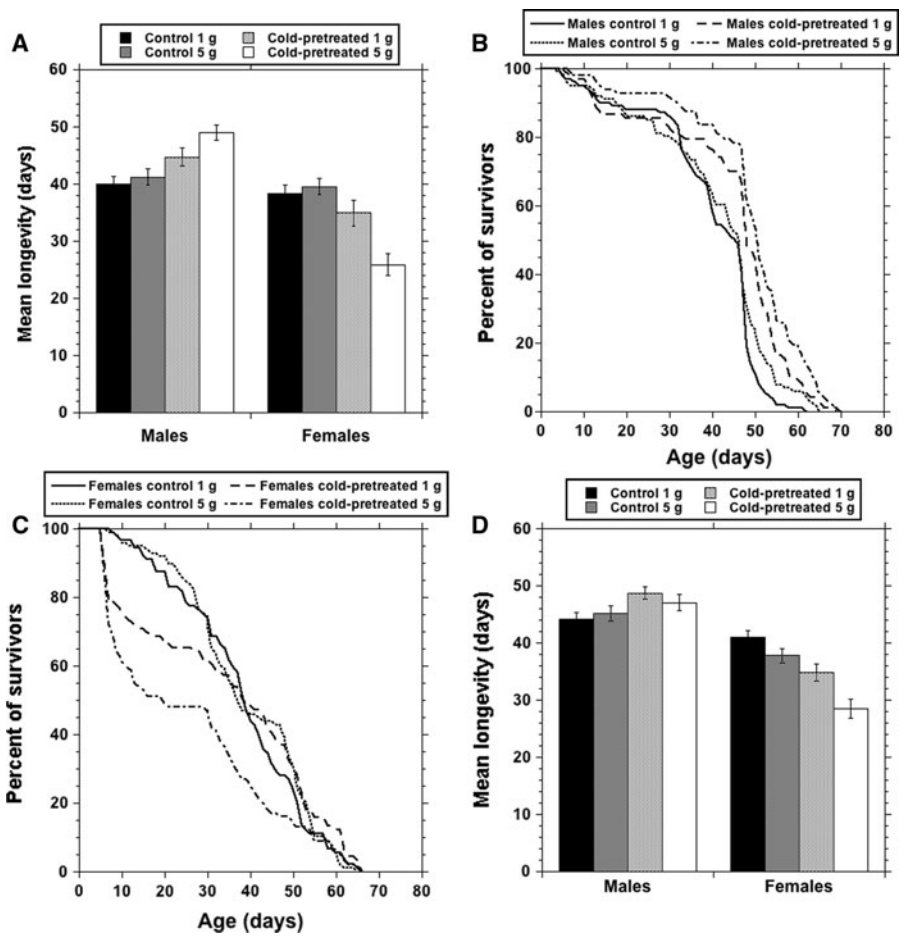
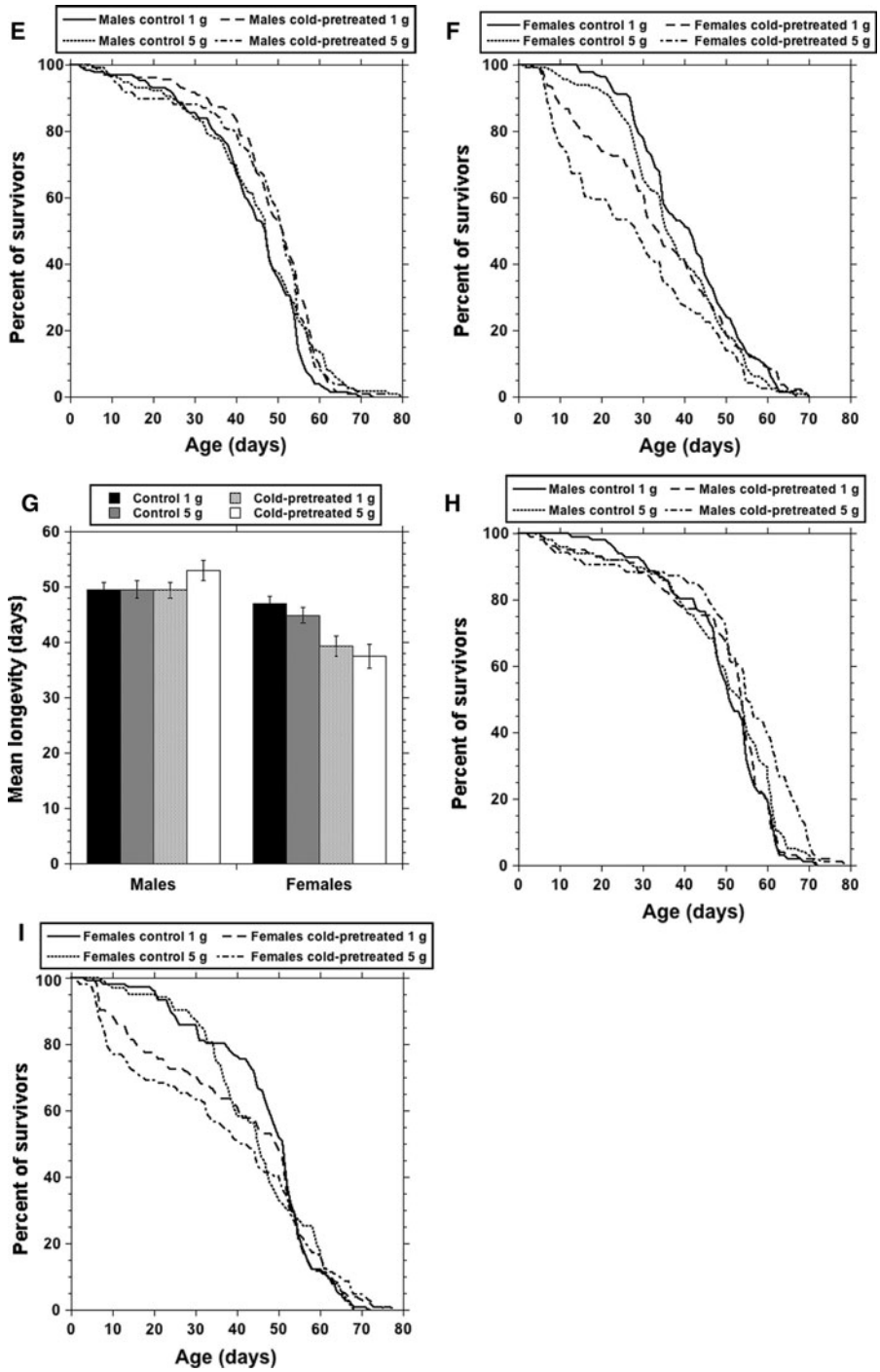


Fig. 1 First experiment: **a** mean longevity ± SEM as a function of sex and pretreatment. Flies were cold-pretreated daily (0°C for 60 min) or not during two periods of 5 days separated by 2 days, starting at 5 days of age, and/or subjected to 5 g for 2 weeks from the second day of life. Each *bar* is the mean of 86–101 flies; **b**, **c** survival curves of males and females. Second experiment: **d** mean longevity ± SEM as a function of sex and pretreatment. Each *bar* is the mean of 108–136 flies; **e**, **f** survival curves of males and females. Third experiment: **g** mean longevity ± SEM as a function of sex and pretreatment. Each *bar* is the mean of 86–106 flies; **h**, **i** survival curves of males and females

Fig. 1 continued



(compare Fig. 1a, d, g). The sex effect ($F(1, 790) = 46.00$, $p < 0.0001$) and the first-order interactions involving this factor were significant (data not shown). Therefore, the two sexes were analyzed in separate ANOVAs. In males (Fig. 1g, h), the cold pretreatment marginally increased longevity ($F(1, 378) = 3.72$, $p = 0.0544$) and HG increased longevity ($F(1, 378) = 4.21$, $p = 0.0408$), the interaction between these factors being not significant, even if males subjected to the two pretreatments lived the

longest: Fig. 1g shows that the positive effects of cold and HG on mean longevity were only observed in this group of flies. However, the survival curves (Fig. 1h) show that HG and cold-pretreated males had a higher survival at old age, as in the previous experiments (Fig. 1b, e), even if they had a lower one at young age (see "Discussion"). In females (Fig. 1g, i), the cold pretreatment decreased longevity ($F(1, 412) = 5.63$, $p = 0.0181$) but the gravity effect and its interaction with the cold pretreatment were not significant.

These three experiments show that, on the whole, the cold and HG pretreatments could increase longevity in males, these two effects being additive, but they had negative effects in females.

Resistance to heat

In the experiment subjecting 3–5 week-old flies to the heat stress, both the cold ($F(1, 403) = 18.28$, $p < 0.0001$) and HG pretreatments ($F(1, 403) = 4.54$, $p = 0.0336$) increased survival time at 37°C (Fig. 2), but the effect of the cold pretreatment was more important. Females survived longer than males ($F(1, 403) = 4.65$, $p = 0.0317$). The age effect and all interactions were not significant. Therefore, in both sexes, a cold or a HG pretreatment increased heat resistance at various ages and these effects were additive.

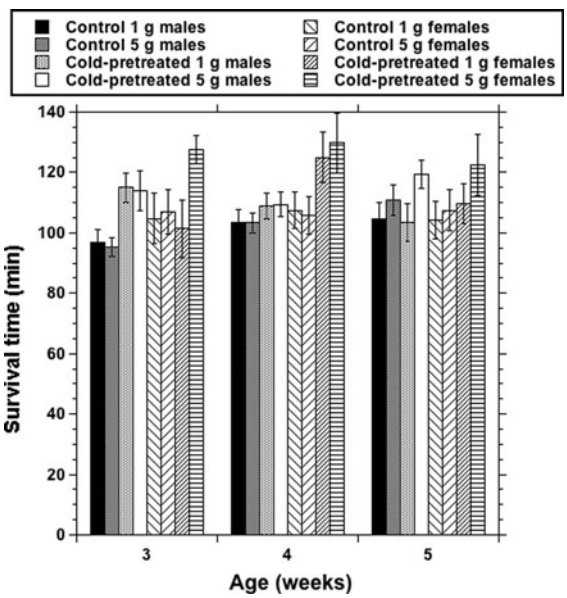


Fig. 2 Mean survival time at 37°C ± SEM as a function of sex, age and pretreatment. Flies were cold-pretreated daily (0°C for 60 min) or not during two periods of 5 days separated by 2 days, starting at 5 days of age, and/or subjected to 5 g for 2 weeks from the second day of life. Their survival time at 37°C was observed at 3, 4, or 5 weeks of age. Each *bar* is the mean of 18 flies, except for 3 and 4 week-old HG–Cold males ($n = 17$)

In the experiment subjecting 6–7 week-old flies to the heat stress, the HG pretreatment decreased survival time at 37°C ($F(1, 157) = 5.20$, $p = 0.0239$, respectively at 1 and 5 g, mean ± SEM: 94.04 ± 1.87 vs. 90.41 ± 2.07 min). The cold, sex, age effects and all interactions were not significant, even if the cold pretreatment ($F(1, 157) = 2.63$, $p = 0.1066$) slightly increased survival time (94.93 ± 1.95 vs. 89.51 ± 1.98 min), an effect being mainly due to males. Therefore, in both sexes, the positive effect of cold was not observed in old flies, even if there was still such a tendency. A positive effect of HG was however not observed at these old ages, as exposure to HG at young age decreased survival time at 37°C. An additional experiment with 48–49 day-old males ($n = 81$) confirmed that cold and HG had no positive effects at this old age (data not shown), even if there was still a slight tendency for a positive effect of cold.

To sum up, both HG and cold pretreatments increased survival time at 37°C and these effects were additive. However, these positive effects were lost at the oldest ages.

Resistance to cold

At 3–5 weeks of age (Fig. 3a), the cold ($F(1, 760) = 52.09$, $p < 0.0001$) and the HG pretreatments ($F(1, 760) = 3.84$, $p = 0.0503$) increased the percentage of survivors, but the HG effect bordered significance. Males better survived than females ($F(1, 760) = 46.73$, $p < 0.0001$) and the percentage of survivors decreased with age ($F(2, 760) = 13.73$, $p < 0.0001$). The interaction between age and the cold pretreatment showed that the effect of cold decreased with age ($F(2, 760) = 4.56$, $p = 0.0107$). All other interactions were not significant. Therefore, a cold pretreatment increased resistance to cold at various ages and the HG pretreatment had a similar effect, which however bordered significance. These two effects appear to be additive, since no interaction between the two pretreatments was observed.

At 6 weeks of age (Fig. 3b), the cold pretreatment also increased the percentage of survivors ($F(1, 190) = 4.34$, $p = 0.0385$ but this result was mainly due to males, as shown by the sex by cold pretreatment

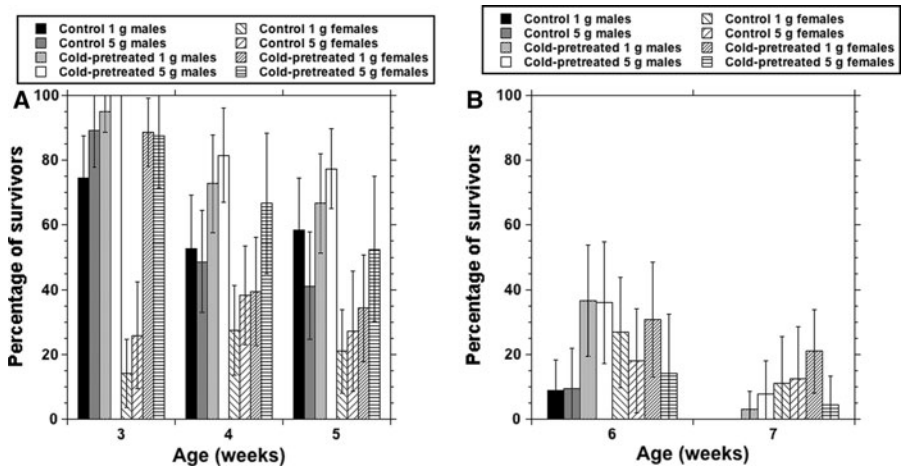


Fig. 3 Percentage of survivors (± confidence interval at $p = 0.05$) 3 days after a 16 h 0°C cold shock as a function of sex, age, and pretreatment. Flies were cold-pretreated daily (0°C for 60 min) or not during two periods of 5 days separated by 2 days, starting at 5 days of age, and/or subjected to 5 g for 2 weeks from the second day of life. Each *bar* is the mean of 16–44 flies. **a** Results of flies cold-shocked at 3, 4 or 5 weeks of age. **b** Results of flies cold-shocked at 6 or 7 weeks of age

interaction ($F(1, 190) = 4.88$, $p = 0.0284$). The HG effect and the other interactions were not significant. At 7 weeks of age (Fig. 3b), the pretreatments and their interaction had no effect on the percentage of survivors in females (Fs close or inferior to 1). No significant effects of cold and HG pretreatments were observed in 7-week-old males (χ^2 tests) and Fig. 3b shows that no interaction between these pretreatments was visually observed. These results at old ages confirm that a cold pretreatment can increase survival to a severe cold shock, but that the HG effect is of a lesser importance, if it indeed exists.

Thus, cold and HG increased resistance to a strong cold stress up to 5 weeks of age, and these effects were additive. However, these effects were erased at older ages.

Resistance to infection

In the experiment performed at 6 weeks of age, infection decreased longevity ($F(1, 350) = 57.13$, $p < 0.0001$), as expected. The other factors (sex, HG, cold) were not significant. All interactions were not significant but the second-order interaction between the two pretreatments and infection ($F(1, 350) = 7.30$, $p = 0.0072$): all infected flies survived for ca. 5 days and non-infected 1 g flies lived longer if they were subjected to cold (11.26 ± 1.02 vs. 9.05 ± 1.05 days), the contrary being observed in HG-flies (9.45 ± 0.90 vs. 12.36 ± 1.28 days). Therefore, no positive effect of the cold and HG pretreatments was observed on resistance to infection at 6 weeks of age. A second experiment was thus done to test whether positive effects would be observed if infection is done at younger ages.

In the experiment with flies infected at 3, 4 or 5 weeks of age (Fig. 4), being subjected to HG decreased survival time to infection ($F(1, 634) = 4.91$, $p = 0.0270$). Males survived longer than females ($F(1, 634) = 31.87$, $p < 0.0001$) and survival time decreased with age at infection ($F(2, 634) = 45.86$, $p < 0.0001$). The significant third-order interaction between sex, age, cold and HG pretreatments ($F(2, 634) = 3.65$, $p = 0.0265$) was mainly due to the erratic variations of 1 g females not subjected to the cold pretreatment. The cold pretreatment effect was not significant ($F < 1$), but the significant interaction with the HG pretreatment ($F(1, 634) = 6.08$, $p = 0.0139$) showed that cold increased survival time in 1 g flies but not in HG ones. This was confirmed by separate ANOVAs on 1 g flies and HG ones: cold slightly increased survival time in 1 g flies ($F(1, 356) = 4.03$, $p = 0.0455$) but not in HG ones ($F(1, 278) = 2.39$, $p = 0.1231$) for which there was a tendency for the opposite pattern to be observed. Anyway, even if the cold pretreatment increased survival time in 1 g flies, this is a weak effect of <1 day (11.44 vs. 10.62 days).

Infection decreased longevity because the mean longevity of non-infected flies was ca. 30 days after the infection at 19 days of age, i.e. twice higher than in

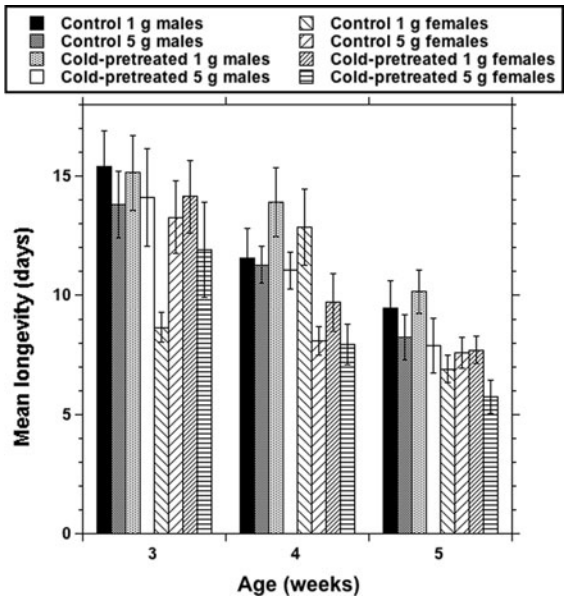


Fig. 4 Mean longevity ± SEM of flies infected by the fungus *B. bassiana* as a function of sex, age and pretreatment. Flies were cold-pretreated daily (0°C for 60 min) or not during two periods of 5 days separated by 2 days, starting at 5 days of age, and/or subjected to 5 g for 2 weeks from the second day of life, and they were infected at 3, 4 or 5 weeks of age. Each *bar* is the mean of 12–40 flies. Day 0 is the day of infection

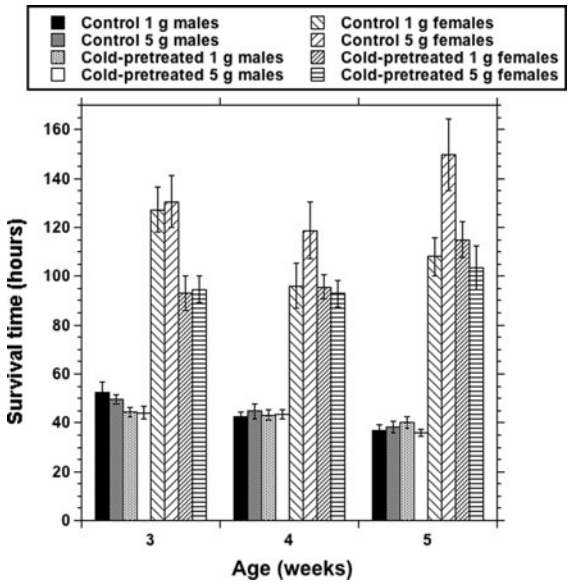


Fig. 5 Mean survival time ± SEM in starvation as a function of sex, age and pretreatment. Flies were cold-pretreated daily (0°C for 60 min) or not during two periods of 5 days separated by 2 days, starting at 5 days of age, and/or subjected to 5 g for 2 weeks from the second day of life. Their survival time in starvation was observed at 3, 4, or 5 weeks of age. Each *bar* is the mean of 18–30 flies

infected flies (see Fig. 4). An ANOVA of the longevity results of non-infected flies showed that the cold pretreatment increased longevity ($F(1, 196) = 11.81$, $p = 0.0007$, mean ± SEM of flies subjected or not subjected to cold, respectively: 32.30 ± 1.13 vs. 26.82 ± 0.98 days, day 0 is 19 days of age). The sex and gravity factors and all interactions were not significant (data not shown). However, as the longevity records began after 19 days of age, flies dying before that age are not taken into account in these results: previous longevity experiments have shown that females can severely suffer from the cold pretreatment while it is not the case in males.

In summary, a cold pretreatment slightly increased resistance to infection at 3–5 weeks of age, but HG decreased it. These effects were not observed at an older age.

Resistance to starvation

Females survived longer than males (Fig. 5, $F(1, 576) = 1033.60$, $p < 0.0001$). The cold pretreatment decreased survival time ($F(1, 576) = 10.66$, $p = 0.0012$), but this effect was less important in males than in females (sex by cold interaction: $F(1, 576) = 5.54$, $p = 0.0190$). HG had no effect but its interaction with the cold pretreatment ($F(1, 576) = 4.58$, $p = 0.0328$) showed that the deleterious effect of cold was more important in HG flies. Survival times were lower at 4 weeks of age ($F(2, 576) = 4.32$, $p = 0.0137$), an effect mainly due to females (sex by age interaction: $F(1, 576) = 12.38$, $p < 0.0001$) and the effect of cold was more important at 3 weeks of age than at older ages (age by cold interaction: $F(2, 576) = 4.45$, $p = 0.0121$). The other interactions were not significant. However, it is not easy to clearly see the effects of cold and HG in each sex and analyzing the results of each sex in separate ANOVAs can be helpful.

Survival times slightly decreased with age in males ($F(2, 294) = 16.90$, $p < 0.0001$). The HG and cold effects and all interactions were not significant in males. The age effect was also significant in females, the lowest survival times being observed at 4 weeks of age ($F(2, 282) = 4.20$, $p = 0.0159$). Cold decreased survival times of females ($F(1, 282) = 11.52$, $p = 0.0008$). The significant interaction between the cold

and HG pretreatments ($F(1, 282) = 5.03, p = 0.0257$) showed that HG females not subjected to cold survived the longest (mean: 131.67 ± 7.10 h), while HG–cold females (96.35 ± 3.65 h) and 1 g-cold ones (100.46 ± 3.88 h) had similar survival times which were lower than those of 1 g females not subjected to cold (110.89 ± 5.30 h). Therefore, being subjected to cold suppressed the positive effect of HG. The HG factor and the other interactions were not significant.

To sum up, no effects of cold and HG were observed in males. In females, cold had negative effects on survival time to starvation and HG could have positive effects in females not subjected to cold.

Resistance to hydrogen peroxide

Resistance to hydrogen peroxide decreased with age in females ($F(2, 274) = 7.09, p = 0.0010$) by less than half a day each week of age (mean ± SEM at 3, 4 and 5 weeks of age: 4.01 ± 0.11, 3.85 ± 0.12, and 3.43 ± 0.11 days). The significant HG by age interaction ($F(2, 274) = 7.90, p = 0.0005$) showed that 1 g females survived longer at 3 weeks of age than HG ones (means: 4.39 vs. 3.61 days), while this trend was erased at later ages (4 weeks of age, respectively for 1 g and HG flies: 3.65 vs. 4.07 days; 5 weeks of age: 3.40 vs. 3.47 days). The effects of the cold and HG pretreatments were not significant, as well as all other interactions.

In males, resistance to hydrogen peroxide also decreased with age ($F(3, 350) = 25.68, p < 0.0001$; mean ± SEM at 3, 4, 5 and 6 weeks of age: 3.22 ± 0.08, 2.66 ± 0.06, 2.36 ± 0.07 and 2.43 ± 0.09 days). The cold pretreatment slightly increased survival time ($F(1, 350) = 8.40, p = 0.0040$; mean ± SEM of cold-pretreated and not pretreated males: 2.79 ± 0.06, and 2.63 ± 0.06 days) and its significant interaction with age ($F(3, 350) = 3.60, p = 0.0138$) showed that the effect of cold was only observed at the oldest ages (means at 3, 4, 5 and 6 weeks of age for cold-pretreated and not-cold-pretreated males, respectively: 3.22 vs. 3.21 days, 2.66 vs. 2.65 days, 2.46 vs. 2.27 days, 2.82 vs. 2.17 days, for each group n was in a 28–56 range). The effect of the HG pretreatment and the other interactions were not significant.

All factors and interactions were not significant in the additional experiment using only 7-week-old males, if we except a just significant interaction between the replicate and the HG factors ($F(1, 140) = 4.04, p = 0.0464$). Flies subjected to the cold pretreatment survived for 56.61 ± 1.73 h and those not cold-pretreated for 54.48 ± 2.19 h. Therefore, this experiment does not confirm the better resistance of cold-pretreated males observed at 6 weeks of age, even if there is a similar trend, not significant however.

Thus, even if significant at 3–6 weeks of age, tiny positive effects of cold were observed, in males only.

Climbing activity

In females (Fig. 6a), the cold pretreatment increased the climbing scores ($F(1, 180) = 10.42, p = 0.0015$) but HG marginally decreased them ($F(1, 180) = 3.64, p = 0.0581$). The significant interaction between the two pretreatments ($F(1, 180) = 4.77, p = 0.0302$) showed that 1 g flies subjected to cold got the highest scores and that the three other groups had similar scores (post hoc analysis). The scores decreased with age ($F(4, 180) = 10.45, p = 0.0026$). The age by HG interaction ($F(4, 180) = 3.76, p = 0.0058$) and that between age and the cold pretreatment ($F(4, 180) = 3.25, p = 0.0132$) were mainly due to the high score of control 1 g flies at 22 days of age and to the decreased variability at older ages (floor effect). The second-order interaction between HG, cold, and age was not significant.

In males (Fig. 6b), the cold pretreatment increased the climbing scores ($F(1, 246) = 7.39, p < 0.0001$) and these scores decreased with age ($F(6, 246) = 13.47, p < 0.0001$). The gravity factor and all interactions were not significant.

Therefore, cold increased climbing scores in both sexes and, in females, HG could be slightly detrimental and suppress the positive effect of cold. In contrast, HG had not any effect in males.

Discussion

These experiments have combined in the same flies two mild stresses known to have some positive effects on longevity and aging (summary of the results in Table 1). Combining these two stresses required to slightly modify the procedures to apply them, which prohibits to strictly compare the present results to the previous studies of HG and cold pretreatments. In the present experiments the centrifuge was stopped, as in previous experiments studying HG, for a few minutes

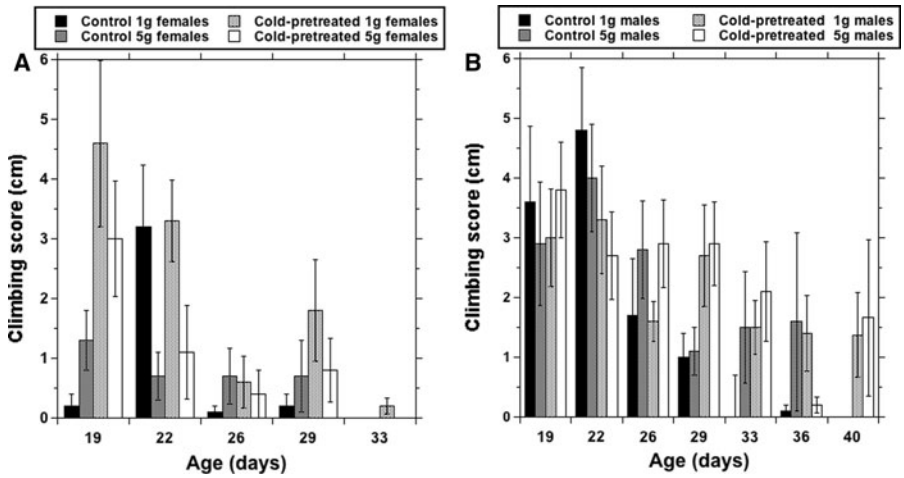


Fig. 6 Mean climbing score ± SEM as a function of sex (**a** females; **b** males), age and pretreatment. Flies were cold-pretreated daily (0°C for 60 min) or not during two periods of 5 days separated by 2 days, starting at 5 days of age, and/or subjected to 5 g for 2 weeks from the second day of life. The climbing score is the height (cm) reached by a fly in 20 s after the cessation of a 3 s mechanical stimulation delivered by a single-vial shaker. Each point is the mean of 10 flies, but there was only 9 females in the control females at 33 days of age and, respectively, 6 and 8 flies in HG–Cold and Cold males at 40 days of age

daily to check longevity and twice a week to renew the vials, but also for ca. 90 min each day the cold pretreatment was applied. Could the lower survival at young age of males observed in one of the longevity experiments (Fig. 1h) be linked to some difficulty to readapt to HG after this rather long stay at 1 g? In addition, flies subjected to the cold pretreatment only were not always stored in an incubator, as in previous studies of the effects of cold pretreatments, but they were located in the room containing the rotating centrifuge for the first 2 weeks of life. These warnings being said, what are the effects of the cold and HG pretreatments in the present experiments?

On the one hand, a cold pretreatment at young age increased longevity and very slightly improved resistance to oxidative stress in males, but it decreased longevity of females and had no effect on their resistance to oxidative stress. In both sexes, cold improved resistance to heat, cold or fungal infection and delayed the age-related climbing ability decline. The cold pretreatment decreased resistance to starvation in females and had no effect in males. These results are similar to previous ones, if we except that, in females, cold could increase longevity and had no positive effect on resistance to infection (Le Bourg 2007, 2008b, 2010, 2011a, Le Bourg 2010). In addition, in the present experiment, cold had no positive effect on resistance to heat at 6 weeks of age in both sexes, contrarily to previous results (Le Bourg 2007, 2011a).

On the other hand, a HG treatment at young age increased longevity of males and resistance to heat in both sexes, had no effect on resistance to oxidative stress in both sexes, and decreased longevity of females, all these results being similar to previous ones (Le Bourg 2008ab). However, HG decreased resistance to infection in both sexes and had no positive effect on climbing ability: previous studies reported no effect on resistance to infection at 3 weeks of age and a positive effect on climbing ability, at least in males (Le Bourg 2008a, 2010). It was also observed that HG females not subjected to cold had a higher resistance to starvation than 1 g ones, no matter they were subjected or not subjected to cold, while a previous study using both younger and older flies reported a negative effect of HG in females (Minois and Le Bourg 1999). Finally, HG increased resistance to cold while a previous study using a cruder procedure reported no positive effect (Minois and Le Bourg 1999).

Table 1 shows that, on the whole, positive effects were more often observed in males than in females. Positive effects were however observed in females, which confirms the conclusion of Sarup and Loeschcke (2011) that females are not less "likely to show

Table 1 Summary of the effects of HG and cold pretreatments and of their combination on longevity, behavioral aging and resistance to various stresses

	Males			Females		
	Cold	HG	Cold + HG	Cold	HG	Cold + HG
Longevity						
First experiment	+	+	+ +	−	−	− −
Second experiment	+	0	+	−	−	− −
Third experiment	(+)	+	+ +	−	0	− −
Heat						
3–5 weeks of age	+	+	+ +	+	+	+ +
6–7 weeks of age	0	−	−	0	−	−
Cold						
3–5 weeks of age	+	(+)	+ +	+	(+)	+ +
6 weeks of age	+	0	+	0	0	0
7 weeks of age	0	0	0	0	0	0
Infection						
3–5 weeks of age	+	−	0	+	−	0
6 weeks of age	0	0	0	0	0	0
Starvation						
3–5 weeks of age	0	0	0	−	+	−
Hydrogen peroxide						
3–5 weeks of age				0	0	0
3–6 weeks of age	+	0	+			
7 weeks of age	0	0	0			
Climbing ability						
3–5 weeks of age				+	(−)	0
3–6 weeks of age	+	0	+			

The cold and HG columns report the effect of each pretreatment, as shown in the statistical analyses (see text). A plus, minus or 0 sign indicates whether the effects were beneficial, deleterious or neutral (e.g. a beneficial effect on resistance to heat is a longer survival time). However, marginally significant results ($0.05 < p \leq 0.06$) are not reported as 0, but as (+) or (−), depending on the observed trend. The Cold + HG column reports the effects of both pretreatments: a duplication of the plus or minus signs is used when the combination of the two pretreatments appears to be more important than the effect of each pretreatment alone, i.e. when these flies had the lowest of highest score, as shown in figures

a hormetic response to a given treatment". It has been argued that the lower positive effects in females are linked to reproduction (e.g. Vaiserman et al. 2003; Sørensen et al. 2010), but HG did not decrease fecundity, even if it modified its expression during life (Lints and Le Bourg 1989). Therefore, this study adds to previous ones showing that positive effects of mild stress can be lower in females than in males, but does not provide any explanation.

On the whole, it can be said that the present results are in accordance with previous ones. In some cases, both cold and HG have positive effects on the trait under study while, for other traits, their effects differed: what is the net result of their combination on each of these traits?

Table 1 shows that when cold and HG had each positive effects, as it is the case for longevity of males (first and third experiments) and resistance to heat or cold in both sexes, their combined effects were additive: the combination was more efficient than each stress alone. Similarly, when both pretreatments had negative effects, their combination was more negative than each stress alone, as it was the case for the longevity of females. When the two pretreatments had no effect, their combination had no effect (resistance to infection at 6 weeks of age in both

sexes or to hydrogen peroxide in females). When one of the pretreatments was neutral and the other one had positive or negative effects, the combination had respectively positive or negative effects in most of the cases. This was observed in males for the resistance to oxidative stress, climbing ability and longevity in the second experiment, and in both sexes for the resistance to heat at old age. Finally, when cold and HG had opposite effects, as it is the case for resistance to infection in 3–5 week-old flies or for resistance to starvation and climbing ability in females, their combination had either no effect (infection, climbing ability) or was negative (starvation). In any case, the positive effect of one of the pretreatments was erased.

To sum up, when cold and HG had each positive or negative effects their combination had additive effects but, when only one of the pretreatments had some effect, the effect of their combination usually reflected this effect. Therefore, combining two mild stresses with positive effects on aging and longevity is more efficient than each stress alone. However, if one of these mild stresses has negative effects and the other one positive effects, the net result of their combination can be the suppression of the positive effect of the second stress. A rather similar picture emerged when a cold stress was combined with the suppression of live yeast (Le Bourg 2010), a treatment having both positive (climbing ability, longevity of females) and negative effects (longevity of males, resistance to cold or to fungal infection). Other studies showed that when a mild stress was applied to flies subjected to another treatment having negative effects on longevity (e.g. increased temperature or being mated), the positive effect of this mild stress was diminished. HG increased longevity of virgin males at 25°C but transferring them to 30°C after the end of HG exposure suppressed the positive effect of HG. Similarly, no positive effect of HG was observed on the longevity of mated males (Le Bourg et al. 2004).

Deciding to combine two mild stresses in the hope to get better effects than those of each pretreatment thus requires that each pretreatment has no negative effect on some traits, which implies to make a comprehensive study of the effects of each stress, and not to only measure longevity or resistance to a single strong stress, such as resistance to heat. Furthermore, if it happens that one of the pretreatments has both negative and positive effects, one may wonder whether it is preferable not to combine it with another pretreatment known to have mainly positive effects, as the net result of the combination could be negative.

For instance, combining dietary restriction and exercise in the same old rats restores the efficiency of cardiac ischemic preconditioning more than each treatment alone (Abete et al. 2005) and thus appears to be beneficial. In such conditions, it could be proposed that elderly overweight people increase their activity level and restrict their diet to decrease the risk for cardiovascular diseases (discussion in Abete and Rengo 2008). However, it would be going too far in supporting the idea that normal-weight elderly people should restrict their diet to improve their cardiac risk profile, because diet restriction in normal-weight people can be more hazardous than beneficial (review in Fontana and Klein 2007).

The present study has shown that two mild stresses with positive effects do not necessarily equate a strong stress with negative effects when combined, since this combination can be more efficient than each single stress. However, this positive effect of the combined stresses is not so important that it deserves the extra work needed to implement the second stress. As an outcome, it is probably better to select a single mild stress with large positive effects on aging and resistance to stress, as is a cold stress, than to combine two mild stresses such as HG or heat stress which have lower effects than cold in the hope that, when combined, these effects would be more important.

For the time being, cold appears to be an efficient stress to increase longevity and resistance to strong stresses, and to delay the age-related climbing ability decline, even if some positive effects can be observed only in males. Furthermore, cold stress can have positive effects even if applied at rather old ages and not only in young flies (Le Bourg 2011a). Finally, applying a cold stress in flies is not a technical challenge as the only needed equipment is an ice flaker machine, an equipment routinely used in biology labs. By contrast, using HG is more challenging since it is necessary to design and build a centrifuge able to contain dozens of vials containing the flies, which is not possible for many labs.

Using cold stress in future studies aiming to discover the mechanisms of hormesis thus seems appropriate. Previous studies using HG as a mild stress have shown that the 70 kDa heat-shock protein (HSP70) was a key to explain the increased resistance

to heat and that the activity of two antioxidant enzymes, superoxide dismutase and catalase, was not modified at any age if flies were subjected to HG at a young age (review in Le Bourg 2008a). It could be of interest to know whether antioxidant enzymes are at play in flies previously subjected to a cold mild stress because, contrarily to HG, cold very slightly increases survival time to hydrogen peroxide (Le Bourg 2008b and the present study). In the same way, it would be of interest to confirm that HSP70 (or other HSPs) can explain the higher resistance to heat of cold-pretreated flies. For the time being, the causes of the positive effects of a cold mild stress remain obscure even if some studies have focused on the mechanisms of resistance to cold (e.g. Colinet et al. 2010; Hoffmann et al. 2003; Overgaard et al. 2005; Sinclair et al. 2007). Studying the effect of a transcription factor such as dFOXO could also be of interest because its nematode homologue DAF-16 can explain some hormetic effects in this species (Cypser and Johnson 2003; Galbadage and Hartman 2008) and dFOXO mutants are unable to mount a hormetic response when subjected to a low dose irradiation at the larval stage (Moskalev et al. 2011). Similarly, studying the effect of the DIF transcription factor, which induces antimicrobial peptide genes (Lemaître and Hoffmann 2007), could be useful since a cold stress increases survival time after infection with the fungus *B. bassiana*. Could it be that this transcription factor is a key to explain the better resistance of cold-pretreated flies (see however Le Bourg 2011b)?

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