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RESEARCH ARTICLE

Combining three mild stresses in *Drosophila melanogaster* flies does not have a more positive effect on resistance to a severe cold stress than combining two mild stresses

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Abstract Among other positive effects, mild stresses can increase resistance to severe stresses. Previous studies combining two mild stresses showed that when each mild stress had positive effects their combination had more positive effects than each mild stress. The present study tested whether combining three mild stresses could still provide positive additive effects, or whether this combination has negative effects because it is no longer a mild stress but rather a strong stress with negative effects. Flies were subjected to either fasting, hypergravity for one or two weeks, or cold shocks, to combinations of two or of the three mild stresses, and survival to a severe cold stress was observed at 13 or 20 days of age. Positive effects of each mild stress and of combining two stresses could be observed, but combining the three stresses provided a similar survival or a lower survival than the combination of two stresses. Thus, combining three stresses was not more efficient than combining two stresses.

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Introduction

A mild stress is a stimulus disturbing the homeostasis of the organism but without severe damages. Mild stresses can induce an adaptive response enhancing the ability to resist other stresses (e.g. Calabrese et al. 2015). These positive effects of mild stress are called hormetic effects and it is now a well established result that mild stresses can have various positive effects in animal models and human beings (reviews in Le Bourg and Rattan 2008, Mattson and Calabrese 2010, Rattan and Le Bourg 2014, but see also, for recent studies in *Drosophila melanogaster*: McClure et al. 2014, Le Bourg 2015, Le Bourg and Massou 2015, Gomez et al. 2016, Henten et al. 2016). In addition, these positive effects appear to last for life, at least in flies, as a mild cold stress at young age increases survival time at 37 °C at very old ages (Le Bourg 2016).

Increasing the intensity of a mild stress with hormetic effects can make it a strong stress with deleterious effects. For instance, living in hypergravity (HG) for two weeks, i.e. at gravity levels higher than the Earth's gravity (1 g), increases longevity of *D. melanogaster* male flies, but living in HG for life decreases it (compare Lints et al. 1993 and Le Bourg et al. 2000). Similarly, Sarup and Loeschcke (2011) showed that exposing flies to 34 °C at 3, 6 and 9 days of age could increase lifespan, provided the heat stresses were not too long (i.e. 3 vs. 1 or 2 h). Because increasing the strength of a mild stress can make the threshold for a strong stress being reached, increasing this strength in the hope to increase positive effects of the mild stress is maybe not a good idea.

As another strategy to increase the positive effects of mild stress, one could combine two mild stresses with hormetic effects in the same flies, in the hope that the threshold for a strong stress is not reached but, rather, that this combination has additive positive effects. It was the rationale of a previous study combining HG and exposure to cold at young age before observing longevity and resistance to severe stresses (Le Bourg 2012). When both mild stresses had positive or negative effects their combination had additive effects. Thus, when each mild stress had positive effects, combining them had more positive effects than each stress alone. However, if one of the mild stresses had negative effects and the other one had positive effects, their combination could result in the suppression of the positive effect of the second stress. In a second study, fasting, which can increase resistance to a severe cold stress (Le Bourg 2013), was combined with either HG, cold or heat mild stresses, and resistance to cold was observed (Le Bourg 2015). When each mild stress had positive effects (fasting, cold, and hypergravity in males only), their combination had additive effects, and thus positive effects. By contrast, if one of the mild stresses had no positive effect or even a negative effect (heat), combining it with fasting did not increase the positive effect of fasting or even decreased it.

These two studies (Le Bourg 2012, 2015) combined two mild stresses and showed that, provided each mild stress has positive effects, their combination had more positive effects than each stress alone. However, one could wonder what happens when three mild stresses are combined: would we observe that their positive effects are still additive or that a threshold for a strong stress with negative effects has been reached?

The purpose of the present study is thus to combine in the same flies three mild stresses, namely HG, cold, and fasting, and to observe the resistance to a severe cold stress. Each stress was applied alone, combined with each of the other mild stresses or with the two other ones, thus for a total of 8 groups of flies in each sex (no mild stress, HG, cold, fasting, HG + cold, HG + fasting, cold + fasting, HG + cold + fasting). In addition, in order to make to vary the strength of the mild stresses and the age at the severe cold stress, flies lived in HG at 3 or 5 g, either for one or two weeks, and were respectively subjected to 5 or 10 daily 1 h 0 °C cold stresses, the severe cold stress being applied respectively at 2 or 3 weeks of age. In such conditions, one can expect that flies subjected to the severe cold stress at 3 weeks of age have been confronted with more intense mild stresses, and also that their resistance to the severe cold stress is lower than at 2 weeks of age.

Materials and methods

Flies

The wild strain Meyzieu caught at the end of the 1970s in France, near the city of Lyon, was maintained by mass-mating in bottles. Flies were fed on a medium (agar, sugar, corn meal and killed yeast) containing a mould inhibitor (para-hydroxymethyl-benzoic acid) and enriched with live yeast at the surface of the medium. In order to obtain the parents of the experimental flies, flies laid eggs for one night in a bottle. About 50 pairs emerging from this bottle 9–10 days after egg-laying were transferred to bottles (ca 25 pairs in a bottle): these flies are the parents of the experimental flies. Experimental flies were obtained as follows: eggs laid by ca 5 day–old parents during a ca 15 h period on a Petri dish containing the medium coloured with charcoal and a drop of live yeast were transferred by batches of 25 into 80 ml glass vials. At emergence, virgin flies with a duration of preimaginal development of 9–10 days were transferred under ether anaesthesia in groups of 15 flies of the same sex to 20 ml polystyrene vials containing ca 5 ml of the medium described above. Flies were transferred to new vials twice a week. In the following, the date of emergence is indicated by the number of the week in the calendar year (e.g. the first week of 2016 is 1/2016). Except for the first two weeks of adult life, during which flies were kept in the room with the centrifuge (see below), flies spent their life in an incubator. In both the room of the centrifuge and the incubator the rearing temperature was 25 ± 0.5 °C and light was on from 07.00 to 19.00 h. Viability and sex-ratios of the groups of flies used in this study are reported in the Supplemental material section available online (Table S1).

Cold pretreatment

Flies transferred from their vials into empty Falcon 2045 vials (19 ml, 16 × 150 mm) were stored in ice flakes at 0 °C and, after one hour, transferred back to their rearing vials at 25 °C. Flies were exposed from 5 days of age to 0 °C for 60 min a day during one or two periods of 5 days separated by 2 days with no cold shock. The centrifuge described below was stopped during the cold shock and for ca 20 min after the end of this shock, in order for flies to recover from the cold shock before resuming centrifugation. 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 achieved by putting flies in a continuously rotating centrifuge (102 rpm, see Picture S1 in the Supplemental material section), the centrifuge being stopped the days of cold pretreatments (see above) for ca 80 min. Flies were subjected to 3 or 5 g for either 8 or 15 days from the second day of adult life. Flies not subjected to HG were placed near the rotating centrifuge during these 8 or 15 days and thus subjected to the same environment (see Picture S1), except the increased gravity level. After the period of centrifugation, all flies were transferred into the incubator described above.

Fasting pretreatment

At 12 or 19 days of age, flies were transferred from their vials into empty Falcon 2045 vials for 24 h, the polypropylene plug containing absorbent cotton with distilled water to prevent desiccation. After that, flies were transferred back to their rearing vials for 6 h before being subjected to the severe cold stress described below.

Combination of pretreatments

Each pretreatment was applied alone, combined with each of the other pretreatments, or combined with the two other pretreatments, thus for a total of 8 groups of flies in each sex (no pretreatment, HG, cold, fasting, HG + cold, HG + fasting, cold + fasting, HG + cold + fasting). In order to make to vary the strength of the mild stresses and the age at the severe cold stress, flies lived in HG, at 3 or 5 g, either for one or two weeks and were subjected to 5 or 10 daily 1 h 0 °C cold stresses, the severe cold stress being applied respectively at 2 or 3 weeks of age. Tables 1 and 2 summarise the procedure.

Resistance to cold procedure

Flies were transferred from their vials into empty Falcon 2045 vials, stored in ice flakes at 0 °C for 21 h and, after that, transferred back to their rearing vials at 25 °C. The percentage of survivors was observed 3 days after the end of the cold shock.

Statistical analysis

The effects of pretreatments, age, and their interaction on the percentage of survivors were analysed with logistic models. Because the aim of these experiments was knowing whether a combination of two or three pretreatments is more efficient than a single one, pretreatments were considered as a single factor in the logistic model (i.e. no pretreatment, HG, cold, fasting, HG + cold, HG + fasting, cold + fasting, HG + cold + fasting) and not as separate factors (i.e. cold, HG, and fasting factors). The effect of pretreatments and age at the severe cold stress were analysed for each HG condition (3 or 5 g) and in each sex because sex and/or its interactions with other factors had large effects (data not shown). As the 21 h cold shock was applied after cold and/or HG pretreatments lasting for one or two weeks, at 13 or 20 days of age, a significant effect of this age factor could be due either to the length of the HG and/or cold pretreatments (one or two weeks) or to the age at cold shock.

Results

Flies kept at 5 g

These experiments used flies of the 9/2016, 20/2016, 35/2016 and 40/2016 groups with ca 32 vials of 15 flies for each sex in each of these groups. The two first

Table 1 Summary of the experimental procedure for flies subjected to the severe cold stress at 2 weeks of age

Days	1 g flies				Hypergravity flies (HG : 3 g or 5 g)			
	Cold + fasting	Cold	Fasting	1 g	Cold + fasting	Cold	Fasting	HG
0	Emergence							
1	Flies in the room of the centrifuge				1 g ➡ HG			
2-4	Flies in the room of the centrifuge				HG			
5-8	1 h/day at 0 °C				HG ➡ 1 g 1 h/day at 0 °C 20 min at 25 °C 1 g ➡ HG		HG ➡ 1 g for ca 80 min 1 g ➡ HG	
9	1 h at 0 °C				HG ➡ 1 g 1 h at 0 °C		HG ➡ 1 g	
9-17	Flies in the incubator							
12	24 h fasting	No fasting	24 h fasting	No fasting	24 h fasting	No fasting	24 h fasting	No fasting
13	21 h at 0 °C							
17	Percentage of survivors							

The arrows indicate when flies were transferred from 1 g to HG or from HG to 1 g. The centrifuge was stopped for ca 80 min when flies were subjected to 1 h at 0 °C

groups were subjected to the HG and cold stresses for two weeks and tested for cold resistance at 20 days of age, and the two last groups were subjected to the HG and cold stresses for one week and tested for cold resistance at 13 days of age. Only a few males died because of HG and cold pretreatments, but more than 100 males of the 9/2016 and 20/2016 groups died after the fasting pretreatment, while no such effect was observed in the 35/2016 and 40/2016 groups. Thus, older males were less able to sustain the fasting pretreatment than younger ones. By contrast, up to 150 females died in all groups because of the cold pretreatment and nearly no female died because of fasting.

In males, older flies survived less to the cold shock than younger ones [Fig. 1, $F(1, 1322) = 25.65$, $p < 0.0001$, percentage of survivors after a cold shock at 20 or 13 days of age (±confidence interval at $p = 0.05$), respectively: 12.75 ± 2.68 vs. 24.80 ± 3.11]. The interaction with the pretreatments factor was not significant ($F < 1$). The pretreatments significantly modulated survival but this effect was not very important, as shown by the rather low F-value (Fig. 1; Table 3, $F(7, 1322) = 4.36$, $p < 0.0001$, percentage of survivors: no pretreatment, 12.57 ± 4.70; HG, 14.69 ± 4.78; cold, 15.47 ± 5.27; fasting, 29.88 ± 7.01; HG + cold, 25.49 ± 6.91; HG + fasting, 16.11 ± 5.37; cold + fasting, 31.30 ± 8.48; HG + cold + fasting, 16.78 ± 6.13). Therefore, fasting, HG + cold, and cold + fasting increased survival but the other pretreatments, and particularly the combination of the three pretreatments, had no effect. In addition, HG + cold or cold + fasting did not give rise to a higher survival than fasting only.

In females, as in males, older flies survived less than younger ones (Fig. 2, $F(1, 1576) = 93.67$, $p < 0.0001$, 26.18 ± 3.04 vs. 51.14 ± 3.49%). The significant age by pretreatments interaction ($F(7, 1576) = 2.47$, $p = 0.0161$) showed that the age effect could be more important in some groups, but survival was always lower at 20 days of age in all groups (Fig. 2). Pretreatments strongly modulated survival

Table 2 Summary of the experimental procedure for flies subjected to the severe cold stress at 3 weeks of age

Day	1 g flies				Hypergravity flies (HG : 3 g or 5 g)			
	Cold + fasting	**Cold**	**Fasting**	**1 g**	**Cold + fasting**	**Cold**	**Fasting**	**HG**
0	Emergence							
1	Flies in the room of the centrifuge				1 g ➡ HG			
2-4	Flies in the room of the centrifuge				HG			
5-9	1 h/day at 0 °C				HG ➡ 1 g 1 h/day at 0 °C 20 min at 25 °C 1 g ➡ HG		HG ➡ 1 g for ca 80 min 1 g ➡ HG	
10-11	Flies in the room of the centrifuge				HG			
12-15	1 h/day at 0 °C				HG ➡ 1 g 1 h/day at 0 °C 20 min at 25 °C 1 g ➡ HG		HG ➡ 1 g for ca 80 min 1 g ➡ HG	
16	1 h at 0 °C				HG ➡ 1 g 1 h at 0°C		HG ➡ 1 g	
16-23	Flies in the incubator							
19	24 h fasting	No fasting	24 h fasting	No fasting	24 h fasting	No fasting	24 h fasting	No fasting
20	21 h at 0 °C							
23	Percentage of survivors							

The arrows indicate when flies were transferred from 1 g to HG or from HG to 1 g. The centrifuge was stopped for ca 80 min when flies were subjected to 1 h at 0 °C

(Fig. 2; Table 3, $F(7, 1576) = 25.22$, $p < 0.0001$, percentage of survivors: no pretreatment, 13.25 ± 4.34; HG, 14.80 ± 4.66; cold, 54.23 ± 6.89; fasting, 38.57 ± 6.39; HG + cold, 47.02 ± 7.96; HG + fasting, 37.89 ± 6.31; cold + fasting, 65.92 ± 6.94; HG + cold + fasting, 51.95 ± 7.89). Thus, all pretreatments excepting HG increased survival. However, the combination of the three pretreatments was less efficient than that of cold + fasting and not different from the effect of cold only. In addition, only cold + fasting provided a slightly higher survival than that observed with cold only, while combining cold or fasting with HG had no more positive effect than each pretreatment alone, probably because HG had not any positive effect (Fig. 2).

Flies kept at 3 g

These experiments used flies of the 10/2016, 47/2016, 46/2016 and 49/2016 groups with ca 32 vials of 15 flies for each sex in each of these groups. The two first groups were subjected to the HG and cold stresses for two weeks and tested for cold resistance at 20 days of age, and the two last ones were subjected to the HG and cold stresses for one week and tested for cold resistance at 13 days of age. Like in the previous experiment, only a few males died because of HG and cold pretreatments, but ca 50, 85, 80 and 10 males of the 10/2016, 47/2016, 46/2016, and 49/2016 groups died after the fasting pretreatment. Thus, particularly at an older age, males were less able to sustain fasting

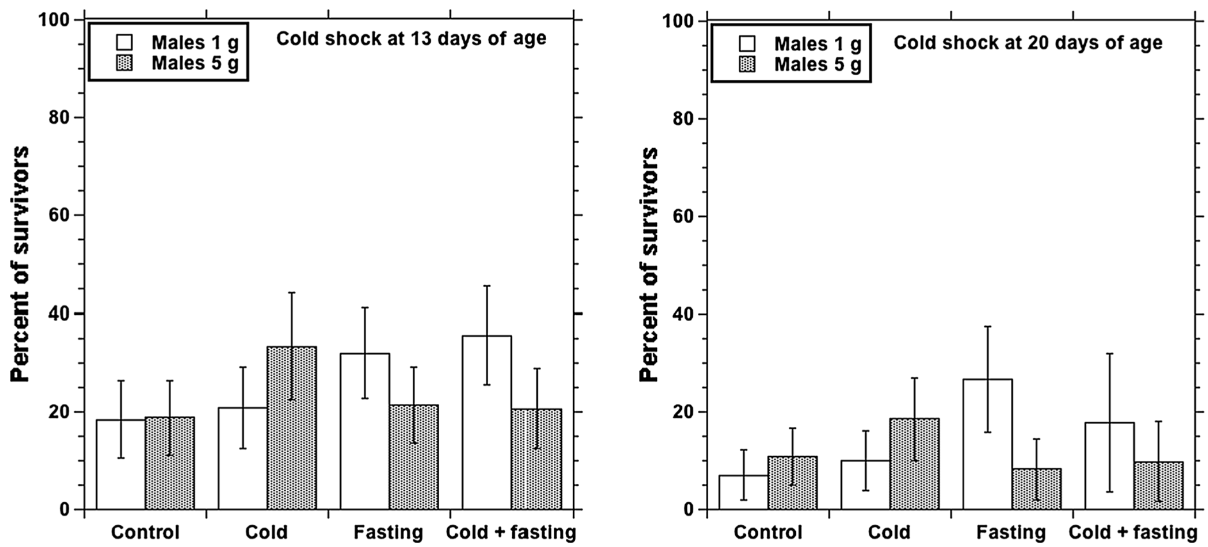

Fig. 1 Percentage of male survivors (±confidence interval at p = 0.05) 3 days after a 21 h cold stress (0 °C) as a function of gravity (1, 5 g), fasting, or cold pretreatments, and age at cold shock (13 or 20 days). Each *bar* is the mean of 72–107 flies for males subjected to the cold shock at 13 days of age, and 28–110 ones for males cold-shocked at 20 days of age

than females. Like in the previous experiment, too, many females died because of the cold pretreatment and nearly no female died because of fasting.

In males, older flies survived less to the cold shock than younger ones (Fig. 3, $F(1, 1454 = 19.11$, $p < 0.0001$, 36.53 ± 3.54 vs 50.20 ± 3.55). The interaction between the two factors was not significant (F close to 1). The pretreatments modulated survival (Fig. 3 and Table 3, $F(7, 1454) = 10.29$, $p < 0.0001$, percentage of survivors: no pretreatment, 29.66 ± 5.83; HG, 41.13 ± 6.35; cold, 63.82 ± 6.68; fasting, 43.00 ± 6.74; HG + cold, 45.83 ± 7.53; HG + fasting, 36.69 ± 7.27; cold + fasting, 60.74 ± 8.24; HG + cold + fasting, 31.20 ± 8.12). Therefore, each pretreatment alone, or in combination with another one, increased survival, the effect being of a low importance in the HG + fasting group. By contrast, combining the three pretreatments did not increase survival.

Older females survived less than younger ones, but the effect boarded significance (Fig. 4, $F(1, 1602) = 3.75$, $p = 0.0531$, 45.92 ± 3.38 vs 50.13 ± 3.50%). The significant age by pretreatments interaction ($F(7, 1602) = 2.31$, $p = 0.0243$) showed that, in contrast with the other groups, survival was slightly better at 20 days of age in the HG + cold and HG + cold + fasting groups than at 13 days of age (Fig. 4). Pretreatments strongly modulated survival (Fig. 4 and Table 3, $F(7, 1602) = 34.84$, $p < 0.0001$, percentage of survivors: no pretreatment, 27.90 ± 5.76; HG, 19.05 ± 5.07; cold, 70.18 ± 6.86; fasting, 42.51 ± 6.17; HG + cold, 67.88 ± 7.12; HG + fasting, 36.53 ± 6.38; cold + fasting, 84.04 ± 5.23; HG + cold + fasting, 56.10 ± 7.60). Thus, HG slightly decreased survival and all other pretreatments increased it, but HG + fasting had the lowest positive effect. While cold + fasting provided the highest survival (84%), combining the three pretreatments was not more efficient than combining two pretreatments, except when compared to the HG + fasting group (56 vs 36%). However, this last group had a rather low survival, probably because HG slightly decreased survival (19 vs 28% in the no pretreatment group).

Discussion

These experiments tested whether combining two or three mild stresses, namely daily 1 h 0 °C shocks, HG, and 24 h fasting, could increase survival to a severe cold shock (21 h at 0 °C) applied at 2 or 3 weeks of age. In all experiments, flies had a lower survival at 3 weeks of age, but this age effect only marginally modified the effect of the mild stresses. Varying the strength of mild stresses was obtained by subjecting flies either to 5 or 10 daily

Table 3 Summary of the results

no pretreatment M: n = 236 F: n = 233	3 g	Cold	Fasting
3 g	M: 39 (231) F: –32 (231)	M: 55 (168) **F: 143 (165)**	M: 24 (169) F: 31 (219)
Cold		**M: 115 (199)** **F: 152 (171)**	**M: 105 (135)** **F: 201 (188)**
Fasting			M: 45 (207) F: 52 (247)
3 g + cold + fasting M: 5 **F: 101**			
no pretreatment M: n = 191 F: n = 234	5 g	Cold	Fasting
5 g	M: 17 (211) F: 12 (223)	M: 103 (153) **F: 155 (151)**	M: 28 (180) **F: 186 (227)**
Cold		M: 23 (181) **F: 309 (201)**	M: 149 (115) **F: 398 (179)**
Fasting			**M: 138 (164)** **F: 191 (223)**
5 g + cold + fasting: M: 33 **F: 292**			

Flies subjected to a 3 g level are in the upper table and those subjected to a 5 g level in the lower table. The number of flies in the no pretreatment group is reported in the first left cell of the first row (M: males, F: females). Pretreatments are shown in the top row and first left column: when a single pretreatment is applied the intersection is identical (e.g. 3 and 3 g) and when two pretreatments are combined it is different (e.g. 3 g and Cold). The combination of the three pretreatments is shown on the last line of the table. Each cell reports the survival increase compared to the no pretreatment group, the two ages being pooled, and the number of flies is reported in parentheses. For instance, the survival of the no pretreatment male group of experiments using a 3 g HG level is 29.66% (n = 236) and that of the 3 g group is 41.13% (n = 231): the value of the 3 g cell (cross of 3 g and 3 g) is (41.13/29.66−1) × 100 = 38.67%, rounded to 39%. Significant increases compared to the no pretreatment group (post hoc tests after analysis with a logistic model) are shown in bold. Absolute percentages of survival are reported in the text

1 h 0 °C shocks, and to 3 or 5 g for one or two weeks before being subjected to the severe cold stress.

Up to ca 25% of males could die after fasting, mainly if fasting occurred at an older age (19 vs 12 days of age), as previously observed in 4 week-old flies of the same strain, one third of flies being unable to survive to a 24 h fasting (Le Bourg 2013), or in 3 week-old *cn bw* males (Le Bourg and Massou 2015). Thus, fasting can be either a mild or a strong stress, depending on sex, age and starvation duration (Le Bourg 2013). Similarly, a number of females could not survive to the daily cold stresses, as also observed in a previous study (see lifespan curves in Le Bourg 2012), showing that a mild stress in one sex can be a strong stress in the other sex. This shows, as stressed by Sarup and Loeschcke (2011) that “if we are to use hormesis

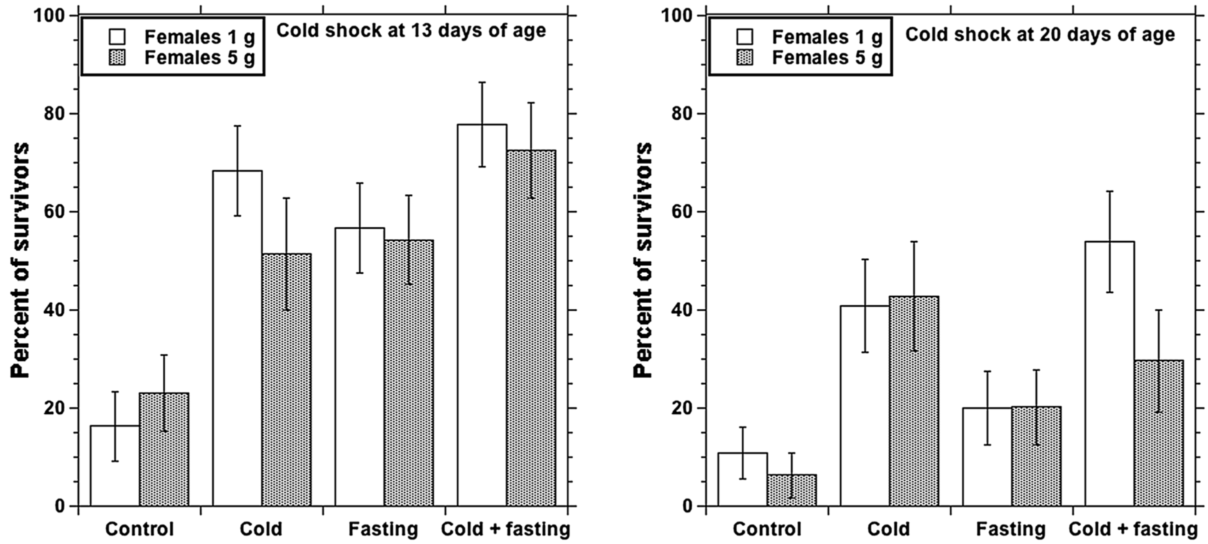

Fig. 2 Percentage of female survivors (±confidence interval at p = 0.05) 3 days after a 21 h cold stress (0 °C) as a function of gravity (1, 5 g), fasting, or cold pretreatments, and age at cold shock (13 or 20 days). Each *bar* is the mean of 74–118 flies for females subjected to the cold shock at 13 days of age, and 74–130 ones for females cold-shocked at 20 days of age

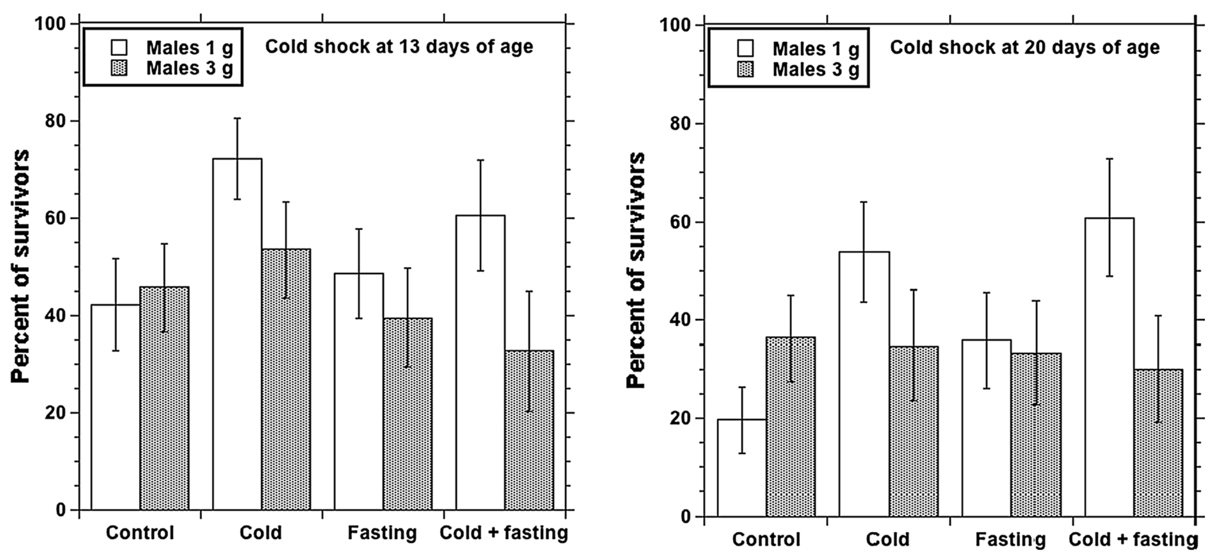

Fig. 3 Percentage of male survivors (±confidence interval at p = 0.05) 3 days after a 21 h cold stress (0 °C) as a function of gravity (1, 3 g), fasting, or cold pretreatments, and age at cold shock (13 or 20 days). Each *bar* is the mean of 55–118 flies for males subjected to the cold shock at 13 days of age, and 64–132 ones for males cold-shocked at 20 days of age

therapeutically it is important that the treatment is beneficial on the individual level and not just on average over the population". In addition, other strains could better resist to fasting and cold, and this could be tested in future studies.

Excepting females subjected to 3 g (see Table 3), survival was higher in all pretreatment groups than in the group not subjected to any pretreatment. However, this better survival could be important or very weak (see Table 3 for post hoc tests).

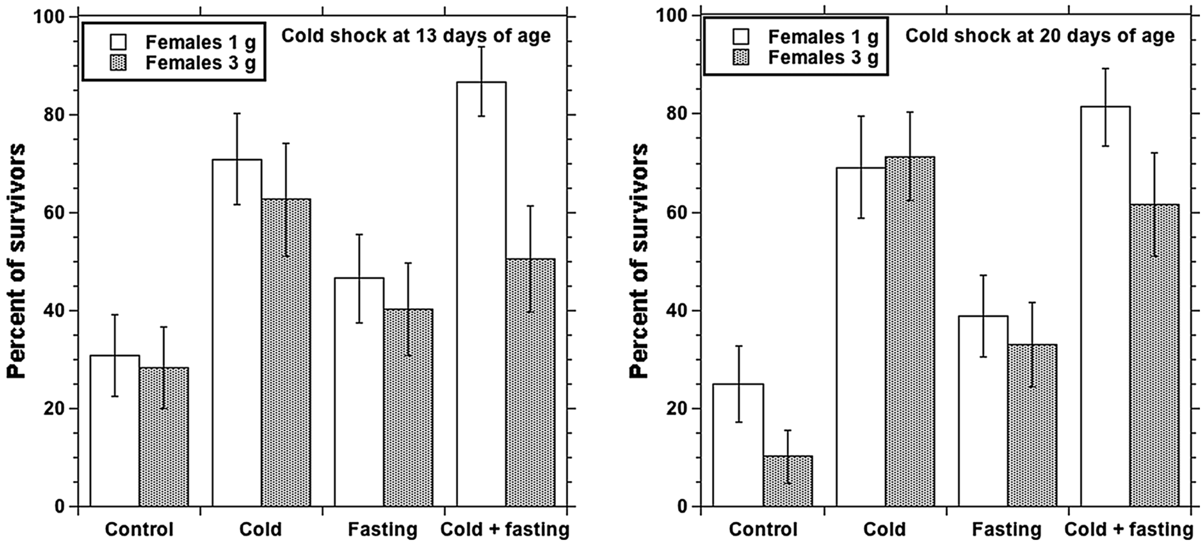

Fig. 4 Percentage of female survivors (±confidence interval at p = 0.05) 3 days after a 21 h cold stress (0 °C) as a function of gravity (1, 3 g), fasting, or cold pretreatments, and age at cold shock (13 or 20 days). Each *bar* is the mean of 67–117 flies for females subjected to the cold shock at 13 days of age, and 78–131 ones for females cold-shocked at 20 days of age

A poor survival of males was observed in experiments involving the 5 g pretreatment (Fig. 1). Because this poor survival also affected the no pretreatment condition and 1 g groups, this result is not linked to the HG level, but probably to random variations between experiments. Only low positive effects of fasting alone and of cold combined with HG or fasting were observed in experiments involving the 5 g pretreatment in males (Fig. 1). By contrast, a higher survival was observed in experiments involving the 3 g pretreatment (Fig. 3), and cold alone or combined with fasting strongly increased survival.

In all experiments with males, combining two pretreatments increased or did not increase survival more than a single pretreatment. In the experiments involving the 5 g pretreatment, cold (15%) or 5 g alone (15%) did not increase survival, when compared to the no pretreatment group (13%), but their combination was efficient (25%). By contrast, the fasting group had a 30% survival, but the cold + fasting one only reached 31%. In the experiment involving the 3 g pretreatment, the cold group had a 64% survival and the cold + fasting group only 61% (no pretreatment group: ca 30%), even if fasting alone slightly increased survival (43%).

Combining the three pretreatments decreased survival of males. In the experiment involving the 5 g pretreatment, the cold + HG group had a 25% survival and the fasting group 30%: combining the three pretreatments lowered survival to 17%, close to the value of the no pretreatment group (13%). In the experiment with the 3 g pretreatment, the cold + fasting group had a 61% survival and the 3 g group 41%: combining the three pretreatments decreased survival to 31%, close to the value of the no pretreatment group (30%).

In females, like in males, combining two pretreatments increased or did not increase survival more than a single pretreatment. In the experiments with the 3 g pretreatment, cold (70%) or fasting (43%) increased survival when compared to the no pretreatment group (28%), and their combination was even more efficient (84%). Similarly, in the experiments involving the 5 g pretreatment, cold (54%) or fasting (39%) increased survival (no pretreatment group: 13%), and their combination was also more efficient (66%). However, when HG (15%) was combined with either cold (54%) or fasting (39%), survival was slightly lower (HG + cold: 47%; HG + fasting: 38%) than with cold or fasting alone: this is in contrast to males for which cold and 5 g had no effect, but their combination increased survival (see above). Like in males, however, combining the three pretreatments did not provide extra survival: in the experiment involving the 3 g pretreatment, the cold + fasting group had a 84% survival but combining the three pretreatments lowered it to 56%.

Similarly, in the experiment with the 5 g pretreatment, the cold + fasting group had a 66% survival but only 52% of females survived in the three pretreatments group. However, in this experiment, combining the three pretreatments in females tested at 13 days of age (Fig. 2, left) was slightly more efficient than combining cold and HG or fasting and HG, but post hoc tests showed that these effects were not significant ($p \geq 0.25$).

Therefore, the main conclusion of these experiments is that while a mild stress can increase survival to a severe stress and combining two stresses can be more efficient than a single one, combining three mild stresses is less efficient than combining two stresses (females) and can even suppress any positive effect when compared to the no pretreatment group (males). Thus, it seems that the no observed adverse effect level (NOAEL, Calabrese et al. 2015) of mild stress has been reached when three pretreatments are combined.

The present experiments combining two mild stresses have shown rather similar effects to those of previous studies (e.g. combining cold and HG, see Le Bourg 2012, or combining fasting with cold, HG, or heat, see Le Bourg 2015), but also contrasting results, as combining HG and fasting had positive additive effects in males of the latter study but not in the present one. Le Bourg (2015) concluded that it is of interest to combine two mild stresses only when both have positive effects, but the present study shows that even if each mild stress has no positive effect (i.e. cold or 5 g in males: 15 vs 13% in the no pretreatment group, see Fig. 1), it can happen that their combination is nevertheless efficient (25%). Such a result has been observed in humans, for whom a combination of exercise and heat reduced pain of patients with a painful knee, while each treatment alone had no significant effect (Kim et al. 2013). However, there is a caveat in the fact that some pretreatments killed flies before they were subjected to the severe cold stress, i.e. fasting in males and cold in females, as observed in previous studies (Le Bourg 2012, 2015).

Mild stresses are thus a double-edged sword that can either provide benefit or induce a disaster: in any case, it seems that while combining two mild stresses can be more efficient than using a single stress, combining three stresses does not provide any more benefit and can even be deleterious. This is probably linked to the shape of the hormetic curve (see Calabrese et al. 2015 or Le Bourg 2015): the window for beneficial effects can be narrow and thus deleterious effects can be easily observed. To use a metaphor, the present study shows that, regarding the use of mild stresses, the Tarpeian Rock is close to the Capitol.

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