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# Biogerontology

## Fasting can protect young and middle-aged *Drosophila melanogaster* flies against a severe cold stress --Manuscript Draft--

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<b>Abstract:</b>	<p>Flies were starved with water before being subjected to various severe stresses (heat, cold, fungal infection, hydrogen peroxide) immediately after starvation or after a delay. Starvation of young and middle-aged flies increased resistance to a long cold stress (0°C for up to 48 h), mainly if there was a 2-6 h delay between starvation and the cold stress, but positive effects in old flies were hardly observed. No positive effect was observed on resistance to the other stresses and starvation rather decreased resistance to them. It thus seems that fasting increases frailty but also puts at play mechanisms increasing resistance to cold. Starvation also increased learning scores but this could be linked to decreased positive phototaxis tendencies, and not to a better learning ability. Starvation appears to be a mild stress with limited hormetic effects, but studying the mechanisms of these effects is of interest because fasting is maybe of therapeutic value in human beings.</p>
<b>Response to Reviewers:</b>	See the file "answers to referees".

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**Fasting can protect young and middle-aged *Drosophila*  
*melanogaster* flies against a severe cold stress**

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## Abstract

Flies were starved with water before being subjected to various severe stresses (heat, cold, fungal infection, hydrogen peroxide) immediately after starvation or after a delay. Starvation of young and middle-aged flies increased resistance to a long cold stress (0°C for up to 48 h), mainly if there was a 2-6 h delay between starvation and the cold stress, but positive effects in old flies were hardly observed. No positive effect was observed on resistance to the other stresses and starvation rather decreased resistance to them. It thus seems that fasting increases frailty but also puts at play mechanisms increasing resistance to cold. Starvation also increased learning scores but this could be linked to decreased positive phototaxis tendencies, and not to a better learning ability. Starvation appears to be a mild stress with limited hormetic effects, but studying the mechanisms of these effects is of interest because fasting is maybe of therapeutic value in human beings.

## Key-words

Fasting — heat stress — cold stress — oxidative stress — fungal infection — learning — phototaxis — *Drosophila melanogaster*

## Introduction

A mild stress, i.e. a stimulus disturbing the homeostasis of the organism without inducing severe damages, can provoke an adaptive response enhancing the ability to resist other stresses: this phenomenon is called hormesis (reviews in Mattson and Calabrese 2010). Mild stresses, such as heat, cold and hypergravity (HG) can increase longevity or resistance to severe stresses and improve healthspan in *Drosophila melanogaster* (for a review in various species, see Le Bourg 2009; for heat in *D. melanogaster* see also Lagisz et al. 2013), **but sex and genetic background can modulate the effects of mild stress on longevity (e.g. Sarup and Loeschcke 2011)**. However, deleterious effects of mild stresses can be observed in female flies, because HG can slightly decrease longevity (Le Bourg et al. 2000) and cold has been observed either to increase (e.g. Le Bourg 2007) or decrease longevity (Le Bourg 2010a) or to be neutral (Le Bourg 2007, 2011). Positive effects of mild stress can be observed at old age, even if the mild stress is applied at various ages (Le Bourg 2011), and the positive effects of two mild stresses, HG and cold, can be additive (Le Bourg 2012). One of the features of hormetic treatments is that a too mild stress cannot give rise to positive effects while severe stresses have deleterious effects, intermediate stresses providing positive effects (Calabrese et al. 2012). In flies, positive effects are observed after a rather short exposure to a mild stress while longer exposures can be detrimental. For instance, keeping male

53 flies in HG for one week does not increase longevity (Le Bourg et al. 2000) and life-long exposures  
54 combined with a high HG level decrease it (Le Bourg and Lints 1989; Lints et al. 1993). In contrast,  
55 2-4 weeks exposures can increase longevity in males (e.g. Le Bourg et al. 2000).

56 Another treatment, dietary restriction (DR), is considered by many authors as a nearly  
57 universal means to increase longevity and improve healthspan (reviews in Everitt et al. 2010), even  
58 if it does not seem to increase lifespan in various species and mouse genotypes (reviews in Le  
59 Bourg 2010b; Nakagawa et al. 2012; Swindell 2012). However, there are major differences between  
60 mild stress and DR. Firstly, mild stresses can increase longevity and severe ones (in duration or  
61 intensity) decrease it but, in rodents, DR is more efficient as its duration and the percentage of food  
62 reduction increase (Bertrand et al. 1999), provided a malnutrition threshold is not reached (see Fig.  
63 4 in Speakman and Mitchell 2011). Secondly, DR increases mean longevity (up to +50%) and  
64 maximal longevity while mild stress only increases mean longevity (+20% at a maximum, see Fig.  
65 1 in Minois 2000). Therefore, DR cannot be considered as a mild stress with hormetic effects,  
66 because the features and effects of DR and mild stress are different (discussion in Le Bourg 2009).

67 Most studies of DR in flies have applied a food reduction along adult life but one could  
68 wonder whether a short starvation (or fasting), i.e. the complete absence of food for a limited  
69 period, could be considered by the organism as a signal for impaired environmental conditions. In  
70 such a case, an appropriate strategy would be to prepare for even worse living conditions by  
71 increasing resistance to severe stresses such as heat or cold shocks. In other words, a short  
72 starvation could be a stimulus disturbing homeostasis without inducing severe damages, and  
73 provoking an adaptive response enhancing the ability to resist other stresses: this is the very  
74 definition of a mild stress with hormetic effects. By contrast, a long starvation could put the  
75 organism at risk, as expected when a too severe stress is applied.

76 One could oppose to this rationale that DR in flies can impair resistance to severe stresses.  
77 For instance, removing live yeast from the nutritious medium decreases resistance to cold, fungal  
78 infection and starvation (Le Rohellec and Le Bourg 2009) and Burger et al. (2007) showed that DR  
79 decreased resistance to starvation and oxidative stress and, to a lesser extent, to cold. These studies  
80 subjected flies to DR for life and not to starvation for a short period but Vigne et al. (2009) showed  
81 that feeding young flies on a life-shortening poor medium for 2 days before an anoxia followed by  
82 reoxygenation (this is similar to a cardiac ischemia-reperfusion insult in mammals) strongly  
83 increased survival to this stress.

84 Starving wild-type flies for 24 h induced the expression of the anti-gram-negative-bacterial  
85 gene *Diptericin* and of the antifungal gene *Drosomycin* (Brown et al. 2009). These authors showed  
86 that, via nitric oxide release (which is active against gram-negative bacteria: Foley and O'Farrell

87 2003), starvation protected *relish* flies against gram-negative bacteria despite the fact that the Imd  
88 pathway protecting against these bacteria is deficient in this mutant. Starvation also stimulated the  
89 Toll pathway protecting against gram-positive bacteria and fungi, which culminates in the  
90 translocation to the nucleus of the NF- $\kappa$ B-like factor DIF and the synthesis of drosomycin. Thus,  
91 starvation did not protect *Dif*<sup>Δ</sup> mutants against gram-positive bacteria, because these flies cannot  
92 mount an immune response and because nitric oxide does not protect against gram-positive  
93 bacteria (Brown et al. 2009). It could therefore be hypothesized that, if starvation would protect  
94 wild-type flies against fungal infection, it would not be the case for *Dif*<sup>Δ</sup> flies. A 6 h starvation in  
95 larvae also induced the expression of antimicrobial peptide genes, particularly *Drosomycin*, but this  
96 was not observed in *dFOXO* mutants (Becker et al. 2010). Feeding 2-day-old adult flies for 4 days  
97 with sucrose only, which is not starvation however, induced the translocation in the nucleus of the  
98 transcription factor dFOXO but, here again, *dFOXO* mutants were unable to display this response  
99 (Puig and Tjian 2005, for a review on the links between dFOXO and stress resistance, see Puig and  
100 Mattila 2011).

101 All these results allow suspecting that starvation could increase resistance of flies to fungal  
102 infection and other severe stresses. Positive effects of short starvation do also exist in rodents  
103 because fasting mice for 3 days (with water ad libitum) or spending 6 days on a protein-free diet  
104 strongly improved survival after renal ischemia-reperfusion injury (Mitchell et al. 2010; Peng et al.  
105 2012). Similarly, fasting rats for 3 days protected against deleterious consequences of cardiac  
106 ischemia-reperfusion (Šnorek et al. 2012) and fasting them for 2 days decreased mortality after  
107 brain ischemia (Marie et al. 1990). Subjecting mice to every other day feeding for 8 days also  
108 increased survival after cecal ligation and puncture, an experimental model of sepsis (Hasegawa et  
109 al. 2012). Finally, the possible use of starvation in cancer patients (review in Lee and Longo 2011)  
110 and its general clinical relevance (Robertson and Mitchell 2013) have been envisaged.

111 Therefore, in the present study, wild-type flies of various ages were subjected to a short  
112 complete starvation (with water ad libitum) to test whether this could increase resistance to severe  
113 stresses (cold, heat, oxidative stress and fungal infection). The effect of starvation on learning to  
114 suppress photopositive tendencies and on phototaxis was also observed in young flies because a  
115 previous study has shown that a mild stress, a cold pretreatment, had some effects on these traits  
116 (Le Bourg 2007).

117

## 118 **Material and methods**

### 119 **Flies**

120 The wild strain Meyzieu caught at the end of the 1970s in France, near the city of Lyon, was  
121 maintained by mass-mating in bottles. Flies were fed on a medium (agar, sugar, corn meal and  
122 killed yeast) containing a mold inhibitor (para-hydroxymethyl-benzoic acid) and enriched with  
123 live yeast at the surface of the medium.

124 In order to obtain the parents of the experimental flies, flies laid eggs for one night in a  
125 bottle. About 50 pairs emerging from this bottle 9–10 days after egg-laying were transferred to  
126 bottles (ca 25 pairs in a bottle): these flies are the parents of the experimental flies.  
127 Experimental flies were obtained as follows: eggs laid by ca 5 day-old parents during a ca 15 h  
128 period on a Petri dish containing the medium colored with charcoal and a drop of live yeast were  
129 transferred by batches of 25 into 80 ml glass vials. At emergence, virgin flies with a duration of  
130 preimaginal development of 9–10 days were transferred under ether anesthesia in groups of 15  
131 flies of the same sex to 20 ml polystyrene vials **containing ca 5 ml of the medium described**  
132 **above**. In the following, the date of emergence is indicated by the number of the week in the  
133 calendar year (e.g. the first week of 2012 is 1/2012).

134 Flies spent their life in an incubator and were transferred to new vials twice a week; the  
135 rearing temperature was  $25 \pm 0.5^\circ \text{C}$ ; light was on from 07.00 to 19.00 h (fluorescent lamp).

136

### 137 **Starvation procedure**

138 Flies were transferred from their vials to empty 19 ml Falcon 2045 vials ( $16 \times 150 \text{ mm}$ ) for  
139 several hours, the duration being depending on experiments and the plug containing absorbent  
140 cotton with distilled water to prevent desiccation. After that, flies were transferred back to their  
141 vials if there was a delay before being subjected to the experiments described below, or  
142 immediately subjected to these experiments if there was no delay after starvation. At young age,  
143 less than ca 1% of flies were observed to die during starvation.

144

### 145 **Resistance to cold**

146 Flies were kept in empty polystyrene vials (Falcon 2045) stored in ice at  $0^\circ \text{C}$  and, after  
147 that, transferred back to their rearing vials at  $25^\circ \text{C}$ . The percentage of survivors three days  
148 after the cold shock was recorded. This percentage was analyzed with a logistic model testing for  
149 the effect of sex and starvation and of their interaction. However, a  $\chi^2$  test was used when only

150 one sex was analyzed.

151 *Resistance to a cold stress at one week of age*

152 The effect of the length of a cold stress (16, 20, 24, 32, 48 or 72 h at 0° C) was studied in  
153 a series of successive experiments at 6 days of age in flies not subjected to starvation and in flies  
154 starved for 24 h, with no delay after starvation and after various delays (16 h cold stress: 8 h  
155 delay, group 13/2012; 20 h cold stress: 2, 4, 8 h delays, group 14/2012; 24 h cold stress: 2, 4,  
156 6 h delays, groups 17/2012 and 07/2013; 24 h cold stress: 6, 24, 48 h delays, group 20/2012;  
157 32 h cold stress: 2, 4, 6 h delays, groups 17/2013; 48 h cold stress: 2, 4, 6 h delays, groups  
158 22/2013; 72 h cold stress: 2, 4, 6 h delays, groups 24/2013).

159 *Resistance to a cold stress at 4 weeks of age*

160 Survival to a 24 h cold stress was observed in 27 day-old flies not subjected to starvation  
161 and in flies starved for 24 h (group 19/2012), either at the end of starvation (no delay) or after a  
162 2, 4, or 6 h delay. As many males did not survive **the starvation treatment** and no one survived  
163 to the cold stress, a 20 h starvation period and a 20 h cold stress were used in a new experiment  
164 (group 23/2012). As only a few males survived to this cold stress and many ones died during the  
165 starvation period, a 16 h starvation period and a 16 h cold stress were used in a new experiment  
166 (group 24/2012).

167 *Resistance to a cold stress at 6 weeks of age*

168 Flies were subjected at 41 days of age to a 20 h starvation and to a 20 h cold stress (group  
169 18/2012), either at the end of starvation (no delay) or after a 2, 4, or 6 h delay. As nearly no fly  
170 survived to this cold stress, a 20 h starvation period and a 16 h cold stress were used in a new  
171 experiment (group 22/2012). As nearly no male survived to this cold stress, a new experiment  
172 used a 16 h starvation period and a 16 h cold stress (group 34/2012). Thereafter, other experiments  
173 used a 16 h starvation period and either an 8 h cold stress (group 36/2012), a 6 h cold stress (group  
174 40/2012), or a 4 h cold stress (group 38/2012).

175

176 **Resistance to heat**

177 *Resistance to heat at one week of age*

178 Resistance to heat was observed at 6 days of age in flies not subjected to starvation and in  
179 flies starved for 24 h, either at the end of starvation (no delay) or after a 2, 4, or 6 h delay. Flies



180 were transferred just before the heat shock into empty polystyrene vials (Falcon 2045), the plug  
181 containing absorbent cotton with distilled water to prevent desiccation, and kept in a water-bath  
182 set at 37° C for 90 or 120 minutes (respectively, groups 15/2012 and 16/2012). Thereafter,  
183 they were transferred back to their vials and the percentage of survivors one day after the heat  
184 shock was recorded. For each heat shock duration, this percentage was analyzed with a logistic  
185 model testing for the effect of sex, starvation group, and their interaction. However, in order to  
186 take into account the death of flies observed to be moribund one day after the heat shock,  
187 survival was also recorded up to 3 days after the heat shock but this did not modify the results  
188 of statistical analyses.

189 *Resistance to heat at 4 weeks of age*

190 Resistance to heat (90 min at 37° C) was observed at 27 days of age in flies not subjected  
191 to starvation and in flies starved for 16 h, either at the end of starvation (no delay) or after a 2,  
192 4, or 6 h delay (group 39/2012).

193 *Resistance to heat at 6 weeks of age*

194 Resistance to heat (60, 75 or 90 min at 37° C, respectively groups 45/2012, 3/2013 and  
195 42/2012) was observed at 41 days of age in flies not subjected to starvation and in flies starved  
196 for 16 h, either at the end of starvation (no delay) or after a 2, 4, or 6 h delay.

197

198 **Resistance to hydrogen peroxide**

199 *Resistance to hydrogen peroxide at one week of age*

200 Flies not subjected to starvation or starved for 24 h were transferred at 6 days of age  
201 (group 35/2012), either at the end of starvation (no delay) or after a 2, 4, or 6 h delay, to  
202 polystyrene vials (diameter: 17 mm, length: 63 mm) closed by a polypropylene plug, as in a  
203 previous article (Le Bourg 2008). This plug was cut with a razor blade in order to insert into it a  
204 strip of chromatography paper (Whatman, 3MM Chr, ca. 10 by 30 mm). One hundred µl of a M/2  
205 saccharose solution (Prolabo 27478.296) were deposited on the strip with hydrogen peroxide (3.3%  
206 (w/v), i.e. 979 mM) diluted from 30% (w/w) hydrogen peroxide (Prolabo 23622.298). New  
207 solutions of saccharose were prepared each week and solutions were stored at 4°C. In order to  
208 prevent desiccation, the vials containing the flies were stored in closed wet boxes. Every day and up  
209 to the death of the last fly, the number of dead flies was recorded, the plug and the strip were  
210 replaced by new ones and 100 µl of the solution were deposited on the new strip. As the plug was

211 tightly inserted into the vial, the old strips were still wet when they were discarded, i.e. flies were  
212 not subjected to desiccation. The survival times were analyzed with a factorial ANOVA testing for  
213 the effect of sex, starvation group, and their interaction.

214 *Resistance to hydrogen peroxide at 4 weeks of age*

215 Resistance to hydrogen peroxide was observed at 27 days of age in flies not subjected to  
216 starvation and in flies starved for 16 h, either at the end of starvation (no delay) or after a 2, 4,  
217 or 6 h delay (group 50/2012). The survival times were log-transformed before computing a  
218 factorial ANOVA testing for the effect of sex, starvation group, and their interaction.

219 *Resistance to hydrogen peroxide at 6 weeks of age*

220 Resistance to hydrogen peroxide was observed at 41 days of age (group 52/2012), the very  
221 same procedure as that used with 4 week-old flies being used.

222

223 **Longevity of infected flies**

224 *Infection procedure*

225 The spores of the fungus *Beauveria bassiana* kept at  $-80^{\circ}$  C in 20% glycerol were  
226 incubated at  $25^{\circ}$  C in 90 mm Petri dishes containing the appropriate medium (for one liter of  
227 distilled water, the autoclaved medium contained: peptone (Sigma P463): 1 g, glucose (Fluka  
228 49159): 20 g, malt extract (Fluka 70167): 20 g, agar: 15 g). After sporulation, which occurs ca 4  
229 weeks after spreading spores on the medium, flies were infected. Flies were very slightly  
230 anesthetized with ether and shaken for ca one minute in a Petri dish containing a sporulating  
231 fungal culture. After having checked under stereomicroscope that all flies were well covered with  
232 spores, flies were transferred to new vials.

233 *Longevity after infection at one week of age*

234 Flies of the group 21/2012 not subjected to starvation or starved for 24 h were infected at  
235 6 days of age, either at the end of starvation (no delay) or after a 2, 4, or 6 h delay. Longevity  
236 was recorded daily from the day following infection until the death of the last fly. Longevity data  
237 were log-transformed before to be analyzed with a factorial ANOVA testing for the effect of sex,  
238 starvation group, and their interaction.

239 *Longevity after infection at 4 weeks of age*

240 Flies of the group 43/2012 were infected at 27 days of age, the same procedure as that

241 used with one week-old flies being used, except that flies were starved for 16 hours.

242 *Longevity after infection at 6 weeks of age*

243 Flies of the group 44/2012 were infected at 41 days of age, the very same procedure as  
244 that used with 4 week-old flies being used.

245

246 **Learning**

247 Individual flies were trained into a T-maze to suppress their natural positive phototactic  
248 tendency (Le Bourg and Buecher 2002). Flies had to choose between a lighted arm, leading to a  
249 lighted vial containing a filter paper wetted with an aversive quinine solution, and a darkened arm  
250 leading to a dry darkened vial (no aversive stimulus). Flies not choosing the lighted vial at the first  
251 trial were discarded because they are considered as photonegative. Most of young flies of both  
252 sexes have an increased tendency during a 16-trials training session to choose the darkened vial  
253 when the lighted vial is associated with aversive stimuli (humidity and quinine, Le Bourg 2005),  
254 while most of flies tested with a dry lighted vial repeatedly choose this vial. No effect of age has  
255 been observed on this learning task (Le Bourg 2004) and material and methods have been  
256 previously described in detail (Le Bourg and Buecher 2002). In the experiments reported below the  
257 darkened arm was dry and contained no paper, and the lighted vial contained either a dry paper (Dry  
258 group) or a paper wetted with a  $10^{-1}$  M quinine hydrochloride solution (QCl group). The present  
259 experiments tested the effect of starvation on learning (QCl group) and phototaxis (Dry group)  
260 scores. The 16 trials were divided in 4 blocks of 4 successive trials: choosing the lighted vial was a  
261 photopositive choice (score: 1) and choosing the darkened vial was a photonegative choice (score:  
262 0). Thus, flies always choosing the lighted vial got a score of 16.

263 *Various starvation lengths*

264 In a first experiment, one week-old flies were starved or not for various lengths (ca 17.5 to 26  
265 h) before to be trained to test whether starvation could modify learning and phototaxis scores. This  
266 experiment was carried out up to obtain 15 flies with intact legs completing the 16 trials for each  
267 combination of sex (male or female), starvation (starvation or control) and reinforcement (QCl or  
268 Dry) groups (n = 120). Data were analyzed with 4-way repeated measures ANOVAs testing for the  
269 effect of sex, starvation and reinforcement groups, and blocks of trials (repeated factor).

270 *Various recovery lengths after starvation*

271 In a second experiment, one week-old flies were starved or not for 24 h before to be  
272 transferred to their rearing vials containing the usual rearing medium. They were trained after  
273 various delays (ca 0 to 6.5 h) to test whether a delay after starvation could modify the effect of

274 starvation. This experiment was carried out up to obtain 10 flies with intact legs completing the 16  
275 trials for each combination of sex, starvation and reinforcement groups ( $n = 80$ ). Data were  
276 analyzed with 4-way repeated measures ANOVAs testing for the effect of sex, starvation and  
277 reinforcement groups, and blocks of trials (repeated factor).

278

## 279 **Results**

280 The results of resistance to stress experiments are summarized in Table 1.

### 281 **Resistance to cold**

#### 282 *Resistance to a cold stress at one week of age*

283 These experiments tested whether a 24 h starvation could increase resistance to a cold  
284 stress, and also if the length of this stress (16, 20, 24, 32, 48, or 72 h at  $0^{\circ}$  C) or the delay  
285 after starvation (0, 2, 4, 6, 8, 24, 48 h) had some effect.

286 Being subjected to starvation increased resistance to a 16 h cold stress (Fig. 1A,  $F(2, 433)$   
287  $= 5.91$ ,  $p = 0.0029$ ) and males better resisted than females ( $F(1, 433) = 4.98$ ,  $p = 0.0262$ ). All  
288 male groups and starved females had a ca 90% survival, while not starved (control) females had a  
289 ca 50% survival (sex by starvation group interaction:  $F(2, 433) = 9.75$ ,  $p < 0.0001$ ). Thus,  
290 starvation increased resistance to a 16 h cold stress in females but this cold stress had nearly no  
291 deleterious effect in males. Therefore, a longer starvation was used in the next experiment.

292 Starvation with a delay before cold stress increased survival to a 20 h cold stress (Fig. 1B,  
293  $F(4, 696) = 24.26$ ,  $p < 0.0001$ ) and no sex effect was observed ( $F$  close to 1). The sex by  
294 starvation group interaction ( $F(4, 696) = 3.24$ ,  $p = 0.0120$ ) showed that all male groups had a ca  
295 90% survival, except in the no delay group (50% survival). Starved females with a delay before the  
296 cold stress had also a ca 90% survival, while control females and starved females with no delay  
297 after starvation had a 50% survival. Therefore, starvation increased survival of females, provided  
298 there was a delay after starvation, and males with no delay after starvation had a lower survival  
299 than the other groups of males, contrarily to what was observed with the 16 h cold stress.  
300 Therefore, the 20 h cold stress had more negative effects on survival than the 16 h one,  
301 particularly because starvation with no delay before a cold stress decreased survival of males.  
302 Starvation with a delay erased these negative effects (males) or increased survival to a cold  
303 stress (females).

304 A 24 h cold stress (Fig. 1C) strongly decreased survival (compare Fig. 1B and C).  
305 Starvation increased resistance to cold (Fig. 1C,  $F(4, 560) = 17.79$ ,  $p < 0.0001$ ) and females  
306 better resisted than males ( $F(1, 560) = 22.81$ ,  $p < 0.0001$ ). However, the sex by starvation group  
307 interaction ( $F(4, 560) = 9.52$ ,  $p < 0.0001$ ) showed that ca 85% of females with a delay before the  
308 cold stress but only 40% of females with no delay and nearly no control female survived to cold.  
309 In males, starvation had a positive effect if there was a 6 h delay before the cold stress, and the  
310 percentage of survivors increased with the length of the delay. As for the 20 h cold stress,  
311 starved males with no delay survived less than control males. Thus, a 24 h cold stress is  
312 detrimental but flies can be protected if there is a delay after starvation (only with a 6 h delay in  
313 males), females with no delay surviving better than control ones, but less than those with a  
314 delay. A replicate experiment (group 07/2013) confirmed the positive effect of starvation in  
315 females (percentages of survivors  $\pm$  confidence interval at  $p = 0.05$  of control, no delay, 2, 4 and 6 h  
316 delays groups:  $52.70 \pm 11.37$ ,  $82.67 \pm 8.57$ , 100%, 100%,  $94.59 \pm 5.15$ ,  $\chi^2 = 96.99$ , 4 df,  $p <$   
317  $0.0001$ ). In this experiment, the cold stress had nearly no effect in control males and in starved  
318 ones with a delay before the cold stress, but it decreased survival if there was no delay  
319 (percentages of survivors  $\pm$  confidence interval at  $p = 0.05$  of control, no delay, 2, 4 and 6 h delays  
320 groups:  $89.33 \pm 6.99$ ,  $67.61 \pm 10.89$ ,  $88.73 \pm 7.36$ ,  $91.89 \pm 6.22$ ,  $92.86 \pm 6.03$ ,  $\chi^2 = 26.18$ , 4 df,  $p <$   
321  $0.0001$ ). The higher resistance to a 24 h cold stress in this replicate experiment prohibits a clear  
322 effect of cold to be observed in males, but the effects of starvation in females are similar to those  
323 observed in the previous experiment.

324 A 32 h cold stress (Fig. 1D) strongly decreased survival (compare Fig. 1B-D). Starvation  
325 increased resistance to cold (Fig. 1D,  $F(4, 720) = 20.11$ ,  $p < 0.0001$ ) and females better resisted  
326 than males ( $F(1, 720) = 133.23$ ,  $p < 0.0001$ ). The sex by starvation group interaction ( $F(4, 720)$   
327  $= 5.68$ ,  $p = 0.0002$ ) showed that no sex effect was observed in control flies but that starved  
328 females better resisted than males. In males, starvation had a positive effect only if there was a 4  
329 h delay before the cold stress (post-hoc test).

330 The 48 h cold stress killed most of the flies (in each starvation and sex group,  $70 \leq n \leq$   
331  $75$ ). Only a few males survived in the 2 and 6 h delay groups (percentages of survivors  $\pm$   
332 confidence interval at  $p = 0.05$  of control, no delay, 2, 4 and 6 h delays groups: 0, 0,  $1.37 \pm 2.67$ , 0,  
333  $11.11 \pm 7.26$ ,  $\chi^2 = 27.90$ , 4 df,  $p < 0.0001$ ). In females, a few flies of the 4 h delay group and ca

334 one third of the 6 h delay group survived (percentages of survivors  $\pm$  confidence interval at  $p =$   
335 0.05 of control, no delay, 2, 4 and 6 h delays groups: 0, 0, 0,  $5.56 \pm 5.29$ ,  $31.08 \pm 10.55$ ,  $\chi^2 = 78.72$ ,  
336 4 df,  $p < 0.0001$ ). Thus, starved flies survived to a 48 h cold stress only if there was a long delay  
337 between starvation and the cold stress and all control flies died. However, a 72 h cold stress  
338 killed all flies, even if they were starved before this cold stress (in each starvation and sex group,  
339  $67 \leq n \leq 75$ , total  $n = 719$ ).

340 The effect of long delays after starvation (24 and 48 h) was tested in the next experiment.  
341 Survival after a 24 h cold stress differed among the starvation groups (Fig. 1E,  $F(4, 539) =$   
342  $17.45$ ,  $p < 0.0001$ ). The results of the control, no delay and 6 h delay groups were similar to  
343 those previously observed (compare Fig. 1C and E) and survival decreased in the 24 and 48 h  
344 groups. The percentages of survival in the 24 h delay groups were similar to those of the control  
345 groups, but the 48 h groups had the lowest survival. No sex effect was observed ( $F$  close to 1)  
346 but the sex by starvation group interaction ( $F(4, 539) = 3.06$ ,  $p = 0.0165$ ) showed that control  
347 males survived better than no delay ones, while the contrary was observed in females, as  
348 previously observed (compare Fig. 1C and E).

349 The main conclusion of all these experiments is that a 24 h starvation can increase survival  
350 to a severe cold stress in young flies. Survival is the highest if there is a few hours delay between  
351 starvation and cold stress, but males with no delay before this cold stress have a lower survival  
352 than control males, while females can exhibit the opposite pattern.

#### 353 *Resistance to a cold stress at 4 weeks of age*

354 About one third of the starved 27 day-old males died during the 24 h starvation and,  
355 among the survivors, no one survived to the 24 h cold stress. Only two females died during the  
356 starvation period (which could be due to natural mortality at this age) and starvation increased  
357 their survival (Fig. 2A,  $\chi^2 = 13.37$ , 4 df,  $p = 0.0096$ ), the highest survival being observed with the  
358 longest delay between starvation and cold stress.

359 Shorter starvation (20 h) and cold stress (20 h) were used in the hope to increase the  
360 percentage of survivors. However, ca 23% of the starved males died during the starvation period.  
361 No one survived to the cold stress in the no delay and 2 h delay groups, and only a few ones in the  
362 other groups (Fig. 2B). Only 2 starved females died during the starvation period and starvation  
363 slightly increased survival, provided the delay between starvation and cold stress was 4 or 6 h (Fig.  
364 2B,  $\chi^2 = 9.56$ , 4 df,  $p = 0.0485$ ).

365 Therefore, as the percentage of male survivors was still very low, shorter starvation (16 h) and  
366 cold stress (16 h) conditions were used in a new experiment. Less than 9% of males and only one  
367 female died during the starvation period. Females better resisted than males ( $F(1, 556) = 60.74, p <$   
368  $0.0001$ ) and starvation increased survival, particularly if there was a delay after the starvation period  
369 (Fig. 2C,  $F(1, 556) = 8.09, p < 0.0001$ ), the interaction between **sex and starvation treatment**  
370 being not significant ( $F$  close to 1).

371 On the whole, it can be concluded that starvation at 4 weeks of age had a positive effect on  
372 survival to a strong cold stress, provided the starvation and cold periods are shorter than in young  
373 flies.

#### 374 *Resistance to a cold stress at 6 weeks of age*

375 Only a few 41 day-old flies died during the 20 h starvation, which could also be due to natural  
376 mortality at this age. No male fly survived among the 200 ones subjected to the 20 h cold stress and  
377 3 females survived among the 53 ones subjected to this cold stress. Therefore, the length of  
378 starvation is appropriate but the length of the cold stress is too long and it was reduced in the next  
379 experiment.

380 Thus, a 20 h starvation and a 16 h cold stress were used. Less than 10% of males or of  
381 females died during the starvation period. Nearly no males survived to the cold stress, except in the  
382 groups with a delay after starvation. Starvation increased survival of females, provided there was a  
383 delay after the starvation period (Fig. 3A,  $\chi^2 = 18.53, 4 \text{ df}, p = 0.0010$ ).

384 In a next experiment, a 16 h starvation and a 16 h cold stress were used in the hope to increase  
385 survival in males. All females survived starvation but ca 9% of males died. Only one male survived  
386 to the cold stress ( $n = 239$ ) and starvation failed to increase survival of females (Fig. 3B,  $\chi^2 = 6.72,$   
387  $4 \text{ df}, \text{n.s.}$ ), even if there was a tendency for a positive effect to be observed if there was a long delay  
388 after starvation.

389 Therefore, a new experiment used a 16 h starvation and a 8 h cold stress. Only one male and  
390 one female died during starvation. Males survived less to cold than females (Fig. 3C,  $F(1, 303) =$   
391  $18.40, p < 0.0001$ ) and, due to a low number of females, both the starvation effect and its interaction  
392 with sex were not significant ( $F$ s close to 1), even if starved females tended to better survive than  
393 control ones. However, it is clear that there was not any tendency for a positive effect in males.

394 A new experiment then used a shorter cold stress (6 h) and the same starvation duration (16  
395 h), in the hope to increase survival in males. About 9% of males and 5% of females died during  
396 starvation and, as expected, a higher percentage of flies survived to this cold stress, females better  
397 surviving than males (66.67 vs 39.91%,  $F(1, 420) = 28.64, p < 0.0001$ ). However, starvation and its

398 interaction with sex had no effect on survival ( $F_s < 1$ ).

399 A last experiment then used a still shorter cold stress (4 h) and the same starvation duration  
400 (16 h). Only two males died during starvation and a high percentage of flies survived to the cold  
401 stress, females better surviving than males (72.50 vs 60.55%,  $F(1, 297) = 4.13$ ,  $p = 0.0432$ ).  
402 However, starvation and its interaction with sex had no effect on survival ( $F_s$  close to 1).

403 To sum up all these experiments involving old flies, starvation had a significant positive effect  
404 on survival of females to a strong cold stress in one experiment only (Fig. 3A), the same tendency  
405 albeit not significant being observed in other experiments. Whatever the strength of the cold stress  
406 could be, no positive effect was ever observed in males.

#### 407 *Conclusion*

408 Starvation increased resistance to cold stress at young and middle ages, but no clear effect was  
409 observed at old age.

410

#### 411 **Resistance to heat**

##### 412 *Resistance to heat at one week of age*

413 Starvation had some effect in flies heat-stressed for 90 minutes, (Fig. 4A,  $F(4, 710) = 5.02$ ,  
414  $p = 0.0005$ ), flies being subjected to starvation with no delay or a 2 h delay before the heat  
415 stress surviving less than the other groups. Males better resisted than **females** ( $F(1, 710) =$   
416  $51.56$ ,  $p < 0.0001$ ) and the sex by starvation interaction ( $F(4, 710) = 3.12$ ,  $p = 0.0147$ ) showed  
417 that starved males with no delay before the heat stress better resisted than females.

418 Sex, starvation and their interaction had no effect in flies heat-stressed for 120 minutes  
419 ( $F_s$  close to 1), because only 41 of the 728 flies survived (6%).

420 Therefore, the main conclusion is that starvation had no positive effect on survival to a 90  
421 min heat stress, because survival of starved groups never exceeded that of control flies. In  
422 addition, being subjected to starvation with no delay or a short delay before a heat stress was  
423 detrimental.

##### 424 *Resistance to heat at 4 weeks of age*

425 Females better resisted than males to a 90 min heat stress (Fig. 4B,  $F(1, 392) = 35.23$ ,  $p <$   
426  $0.0001$ ). The starvation effect was significant ( $F(4, 392) = 7.76$ ,  $p < 0.0001$ ) as well as its  
427 interaction with sex ( $F(1, 392) = 6.50$ ,  $p < 0.0001$ ). Figure 4B shows that starvation had no  
428 effect in females while starved males had a lower resistance than control males, this effect being



429 less important when the delay between the end of the starvation period and the heat stress  
430 increased. Thus, no positive effect of starvation was observed and, to the contrary, starvation  
431 decreased resistance to heat in males.

#### 432 *Resistance to heat at 6 weeks of age*

433 Only 5 moribund flies survived among the 171 subjected to a 90 min 37° C heat shock.  
434 Since starvation did not help old flies to survive this very strong stress, a second experiment  
435 used a 60 min 37° C shock. In this experiment, the starvation effect was significant ( $F(4, 329) =$   
436  $13.07, p < 0.0001$ ) as well as its interaction with sex ( $F(1, 329) = 4.54, p = 0.0014$ ), but the  
437 effect of sex was not significant ( $F$  close to 1). Figure 4C shows that starvation had no effect or  
438 decreased survival in females while starved males with no delay before heat shock had a lower  
439 resistance to heat than control males. By contrast, starved males with a 4 or 6 h delay had a  
440 slightly improved survival, a not significant effect however (post-hoc tests) which was mainly due  
441 to moribund flies. When these moribund flies had died, 3 days after the heat shock, the  
442 percentages of survivors in the control, 4 and 6 h delays male groups were similar (respectively,  
443 ca 42, 46 and 47%, these percentages being 42% for the 2 h group and 0% for the no delay  
444 group). Thus, starvation decreased resistance to heat if there was no delay between starvation  
445 and heat shock and had no effect if there was a delay.

446 A slightly longer heat stress (75 min) was used in a third experiment. Flies had a slightly  
447 lower resistance to heat than in the previous experiment using a 60 min heat shock, but the  
448 results were very similar (Fig. 4D; starvation effect:  $F(1, 514) = 12.00, p < 0.0001$ ; sex effect:  
449  $F < 1$ ; interaction:  $F(1, 512) = 3.30, p 0.0110$ ). Thus, as for the previous experiment, starvation  
450 decreased resistance to heat if there was no delay after starvation and had no effect if there was  
451 a delay.

#### 452 *Conclusion*

453 Starvation did not increase or decreased resistance to heat stress at all ages.

454

#### 455 **Resistance to hydrogen peroxide**

##### 456 *Resistance to hydrogen peroxide at one week of age*

457 Hydrogen peroxide killed young flies in ca 4 days and males survived very slightly longer  
458 than females (means  $\pm$  SEM:  $3.84 \pm 0.05$  vs  $3.47 \pm 0.05$  days,  $F(1, 728) = 28.61, p < 0.0001$ ).  
459 Starvation slightly decreased survival time (means  $\pm$  SEM of control, no delay, 2, 4 and 6 h delays

460 groups:  $3.93 \pm 0.07$ ,  $3.36 \pm 0.08$ ,  $3.43 \pm 0.08$ ,  $3.68 \pm 0.08$ ,  $3.86 \pm 0.08$  days,  $F(4, 728) = 10.79$ ,  $p <$   
461  $0.0001$ ), the interaction with sex being not significant ( $F$  close to 1). Therefore, starvation did not  
462 help young flies to resist oxidative stress and, to the contrary, slightly decreased resistance.

#### 463 *Resistance to hydrogen peroxide at 4 weeks of age*

464 Females survived one day longer than males (means  $\pm$  SEM:  $3.66 \pm 0.07$  vs  $2.76 \pm 0.06$  days,  
465  $F(1, 505) = 108.96$ ,  $p < 0.0001$ ). Starvation decreased survival time (means  $\pm$  SEM of control, no  
466 delay, 2, 4 and 6 h delays groups:  $3.51 \pm 0.11$ ,  $3.02 \pm 0.14$ ,  $2.98 \pm 0.11$ ,  $3.23 \pm 0.11$ ,  $3.28 \pm 0.10$   
467 days,  $F(4, 505) = 7.26$ ,  $p < 0.0001$ ), and the interaction with sex showed that this effect was mainly  
468 due to males ( $F(4, 505) = 9.72$ ,  $p < 0.0001$ ). The means of females were in the range 3.31-3.94 days  
469 while control males survived for  $3.33 \pm 0.13$  days and the means of starved males were in the range  
470 2.14-2.87 days. Therefore, starvation did not help middle-aged flies to resist oxidative stress and, to  
471 the contrary, decreased survival time, mainly in males.

#### 472 *Resistance to hydrogen peroxide at 6 weeks of age*

473 Females survived slightly longer than males (means  $\pm$  SEM:  $3.07 \pm 0.09$  vs  $2.54 \pm 0.05$  days,  
474  $F(1, 316) = 32.79$ ,  $p < 0.0001$ ). Starvation decreased survival time (means  $\pm$  SEM of control, no  
475 delay, 2, 4 and 6 h delays groups:  $3.07 \pm 0.11$ ,  $2.66 \pm 0.11$ ,  $2.78 \pm 0.11$ ,  $2.70 \pm 0.10$ ,  $2.41 \pm 0.09$   
476 days,  $F(4, 316) = 5.03$ ,  $p = 0.0001$ ) and the interaction between starvation and sex was not  
477 significant ( $F < 1$ ). Therefore, starvation did not help old flies to resist oxidative stress but  
478 decreased survival time.

#### 479 *Conclusion*

480 Starvation decreased resistance to hydrogen peroxide at all ages.

#### 482 *Longevity of infected flies*

##### 483 *Longevity after infection at one week of age*

484 Males survived longer than females (means  $\pm$  SEM:  $17.76 \pm 0.84$  vs  $9.93 \pm 0.26$  days,  
485  $F(1, 516) = 107.62$ ,  $p < 0.0001$ ). Most of females died in a narrow range, while some males had a  
486 normal longevity, the last one dying more than 60 days after infection. Starvation had no effect  
487 on survival time ( $F$  close to 1) and the sex by starvation group interaction ( $F(4, 516) = 4.17$ ,  $p =$   
488  $0.0025$ ) showed that all female groups had similar survival times while males of the no delay and  
489 2 h delay groups survived for a shorter time than the other groups (means  $\pm$  SEM of the  
490 control, no delay, 2, 4 and 6 h delays male groups:  $20.43 \pm 2.07$ ,  $14.63 \pm 1.51$ ,  $14.61 \pm 1.34$ ,  
491  $19.02 \pm 1.96$ ,  $19.96 \pm 2.14$  days). Therefore, starvation had no positive effect on resistance to

492 fungal infection in females and decreased survival time of males, this effect being erased if they  
493 had a 4 or 6 h delay after starvation before to be infected. However, starved flies did not outlive  
494 control ones.

495 *Longevity after infection at 4 weeks of age*

496 Starved flies lived for a shorter time than control ones ( $F(4, 556) = 7.50, p < 0.0001$ ,  
497 means  $\pm$  SEM of the control, no delay, 2, 4 and 6 h delays groups:  $11.24 \pm 0.65, 7.58 \pm 0.28,$   
498  $8.43 \pm 0.43, 8.83 \pm 0.48, 8.62 \pm 0.37$  days). The sex factor had no effect on survival time ( $F$   
499 close to 1) but its interaction with the starvation factor ( $F(4, 556) = 3.98, p = 0.0034$ ) showed  
500 that males lived slightly shorter than females in the no delay and 6 h delay groups while they  
501 lived slightly longer in the other groups. Therefore, starvation had a negative effect on  
502 resistance to fungal infection in middle-aged flies.

503 *Longevity after infection at 6 weeks of age*

504 Females survived longer than **males** (means  $\pm$  SEM:  $6.35 \pm 0.23$  and  $5.80 \pm 0.24$  days,  
505  $F(1, 346) = 4.48, p = 0.0349$ ). Starvation had no effect on survival time and the sex by starvation  
506 group interaction was also not significant ( $F$ s close to 1). Therefore, starvation had no positive  
507 effect on resistance to fungal infection in old flies.

508 **Conclusion**

509 Starvation did not increase resistance to fungal infection at all ages, but could decrease it.

510

511 **Learning**

512 *Various starvation lengths*

513 As expected, flies trained with quinine made a higher number of photonegative choices than  
514 those trained with a dry vial (Fig. 5,  $F(1, 112) = 302.34, p < 0.0001$ ). Starved flies got higher scores  
515 than control ones ( $F(1, 112) = 10.71, p = 0.0014$ ). The number of photonegative choices increased  
516 with the order of blocks ( $F(3, 336) = 36.18, p < 0.0001$ ) and the interaction between reinforcement  
517 and the order of blocks ( $F(3, 336) = 2.67, p = 0.0477$ ) showed that scores of flies trained with  
518 quinine reached a plateau (means of the 4 successive blocks: 1.55, 2.35, 2.75, 2.78 photonegative  
519 choices), while scores of flies trained with no quinine slightly increased along blocks (0.33, 0.82,  
520 0.95, 1.13 photonegative choices). The sex factor and all the other interactions were not significant,  
521 particularly the one between starvation and reinforcement. The starvation effect was thus similar in  
522 flies trained with or without quinine, as confirmed by separate ANOVAs showing significant effects

523 of starvation in each of these two groups (data not shown). The effect of starvation on learning  
524 scores is thus linked to a higher tendency to make photonegative choices in the absence of an  
525 aversive reinforcer, which prohibits to conclude that starvation simply improved learning scores.  
526 Separate analyses also showed that there was no significant correlation between the length of the  
527 starvation period and the scores in any sex or reinforcement group (data not shown). The effect of a  
528 delay after starvation was studied in the next experiment.

#### 529 *Various recovery lengths after starvation*

530 Flies trained with quinine made a higher number of photonegative choices than those trained  
531 with a dry vial ( $F(1, 72) = 134.44, p < 0.0001$ ). The number of photonegative choices increased  
532 with the order of blocks ( $F(3, 216) = 20.01, p < 0.0001$ ) and the interaction between reinforcement  
533 and the order of blocks ( $F(3, 216) = 6.62, p = 0.0003$ ) showed that scores of flies trained with  
534 quinine reached a plateau (means of the 4 blocks: 1.50, 2.53, 2.78, 2.98 photonegative choices),  
535 while scores of flies trained with no quinine slightly varied **among** blocks (0.60, 1.03, 0.55, 1.05  
536 photonegative choices). The second-order interaction between sex, reinforcement and blocks was  
537 also significant ( $F(3, 216) = 2.97, p = 0.0328$ ), mainly because scores of females trained with  
538 quinine plateaued while those of males increased with the order of blocks. The sex and starvation  
539 factors were not significant, as well as all the other interactions. Separate analyses also showed that  
540 there was no significant correlation between the length of the recovery period and the scores in any  
541 sex or reinforcement group (data not shown). In summary, when there was a recovery period after  
542 starvation, no effect of a 24 h starvation on learning or phototaxis scores was observed.

#### 543 *Conclusion*

544 Starvation seemed to increase learning scores but this effect was due to increased  
545 photonegative tendencies. No effect was observed if there was a delay between starvation and  
546 training.

547

#### 548 **Discussion**

549 There is now a large interest for the possible positive effects of dietary restriction on  
550 healthspan and lifespan (e.g. Everitt et al. 2010), even if there is a debate on its use in human beings  
551 (e.g. Le Bourg and Rattan 2006; Dirks and Leeuwenburgh 2006; Le Bourg 2010b; Gavrilova and  
552 Gavrilov 2012). Beside studies on dietary restriction, some results indicate that fasting, i.e. a  
553 complete starvation for a short period, can be of therapeutic value (see the introduction and Anton  
554 and Leeuwenburgh 2013).

555 While the results of the very few studies on the effects of fasting in *D. melanogaster* are

556 promising, because starvation increased resistance to an anoxia-reperfusion injury (Vigne et al.  
557 2009) or protected *relish* flies against gram-negative bacteria (Brown et al. 2009), more studies are  
558 needed to know the effects of starvation on resistance to various severe stresses. The present study  
559 thus observed resistance to heat, cold, fungal infection, and hydrogen peroxide in wild-type flies.  
560 Starvation, with a delay or no delay before the severe stress, did not increase or even decreased  
561 resistance to these severe stresses if we except the cold stress. Therefore, starvation increased frailty  
562 even if flies had some time to recover after starvation.

563 Nevertheless, young (Fig. 1) and middle-aged flies (Fig. 2) better resisted to a long 0°C cold  
564 stress if, in most of the cases, there was a delay between starvation and the cold stress, but  
565 starvation did not clearly increase resistance at 6 weeks of age, except in females of one experiment  
566 (Fig. 3A). As starvation often decreased resistance to cold if there was no delay between starvation  
567 and the cold stress, it seems that starvation had both positive and negative effects: starvation  
568 increased frailty and thus could decrease resistance to cold if this stress occurred with no delay after  
569 starvation but, at the same time, starvation induced unknown mechanisms to resist this cold stress.  
570 If there was a delay between starvation and the cold stress flies could recover from starvation and  
571 take advantage of these mechanisms, which could explain their higher resistance to the cold stress.  
572 This higher resistance is maximal 2-6 h after starvation and decreases thereafter (Fig. 1E).

573 **In *D. melanogaster*, not** all mild stresses are equally efficient against severe stresses, because  
574 hypergravity exposure increases resistance to heat, but has no effect on resistance to cold, hydrogen  
575 peroxide or fungal infection, while a cold stress increases resistance to these stresses but marginally  
576 to hydrogen peroxide (see table 1 in Le Bourg 2009). A heat stress also increased resistance to cold  
577 (Minois 2001), even if a recent meta-analysis showed that it does not increase lifespan (Lagisz et al.  
578 2013), contrarily to pretreatments by cold or hypergravity (Le Bourg 2009). The present results  
579 show that starvation is more similar to hypergravity or heat than to cold, because it only increases  
580 cold resistance.

581 What could explain the better resistance of starved flies against cold stress? Starving wild-  
582 type flies for 24 h induces the expression of the anti-gram-negative-bacterial gene *Diptericin* and of  
583 the antifungal gene *Drosomycin* (Brown et al. 2009). Starving larvae for 6 h also induces the  
584 expression of antimicrobial peptide genes, particularly *Drosomycin*, but not in dFOXO mutants  
585 (Becker et al. 2010). Therefore, if dFOXO is at play, as expected if starvation occurs (Puig and  
586 Mattila 2011), it could be expected that *dFOXO* mutants would not survive better to cold after  
587 starvation, contrarily to wild-type flies. In the same way, could it be that *Dif* flies, which are unable  
588 to synthesize drosomycin after fungal infection (Rutschmann et al. 2000), could not better survive  
589 to a cold stress after starvation? Testing *dFOXO* and *Dif* mutants would be of interest in future

590 studies. However, it is known that a pretreatment by cold increases resistance to a severe cold stress  
591 in *Dif* flies as in wild-type ones (Le Bourg et al. 2012). **It has been shown that a cold-sensitive**  
592 **transient receptor potential channel could partly explain the increased longevity of**  
593 ***Caneorhabditis elegans* nematodes living at colder temperatures, and thus that this increased**  
594 **longevity was not only explained by slower chemical reactions at colder temperatures (Xiao et**  
595 **al. 2013). Could such a phenomenon partly explain the better resistance of flies to cold after**  
596 **being subjected to starvation?**

597 Learning ability in a T-maze was also studied: a 17-26 h starvation increased learning scores  
598 and decreased positive phototaxis tendencies. By contrast, Thimgan et al. (2010), using the same  
599 task, did not observe any effect of 7 or 12 h starvations on learning and phototaxis scores. As flies  
600 crossing the maze learn to prefer the dark arm of the maze because the lighted one they initially  
601 prefer is associated with a punishment, a decreased preference for lighted areas could explain why  
602 learning scores increase. Thus, starved flies could choose the darkened arm because starvation  
603 decreased their photopositive tendencies, and not because they have a better learning ability or  
604 short-term memory. Flies cold-stressed before a learning session using the same task also displayed  
605 increased learning scores and decreased positive phototaxis tendencies (Le Bourg 2007). It thus  
606 seems that mild stresses, like starvation and cold, can slightly decrease positive phototaxis  
607 tendencies. Positive phototaxis tendencies also slightly decrease with age (Le Bourg and Badia  
608 1995) but, as the effect of starvation is reversible, because no effect on phototaxis tendencies and  
609 learning scores is observed when there is a delay between starvation and learning (see above),  
610 starvation probably does not induce a precocious aging. In the same way, the effects of starvation  
611 are probably not explained by an improved memory because a 24 h starvation has no effect on 1-h  
612 memory in a pavlovian olfactory conditioning test (Li et al. 2009). Similarly, a 21 h starvation  
613 before conditioning with the same olfactory procedure had no effect on memory measured 24 h  
614 after conditioning in flies also starved between conditioning and memory testing, thus for a total of  
615 45 h before memory testing (Plaçais and Pr at 2013).

616 In summary, this study shows that fasting can increase resistance to a severe cold stress in *D.*  
617 *melanogaster*, particularly if there is a delay between starvation and the cold stress, but not to  
618 several other strong stresses. The positive effect of fasting is thus limited to a few stresses, cold  
619 (this study), anoxia-reoxygenation (Vigne et al. 2009) and gram-negative bacterial infection of  
620 *relish* flies (Brown et al. 2009), and it can decrease resistance to several severe stresses (heat, fungal  
621 infection, hydrogen peroxide: this study), particularly if the stress is applied immediately after  
622 starvation.

623 Yet, fasting has several beneficial effects in mammals (e.g. in the event of cardiac, renal or

624 brain ischemia: see the introduction), and it has been suggested that a longer period of fasting than  
625 the current “one-night fast” before surgery could help to protect against post-operative hazards  
626 (Mitchell et al. 2010). As fasting for a short period is non-invasive, easy to implement, and a not  
627 risky procedure, one could use it before surgery and maybe as an adjuvant to chemotherapy against  
628 cancer (Lee and Longo 2011) if it proves to be efficient. Knowing the mechanisms of the protection  
629 offered by starvation against cold stress in flies could pave the way for studies in mammals, and  
630 maybe in human beings.

631

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635

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743 **Figure captions**

744 Figure 1. Percentage of survivors ( $\pm$  confidence interval at  $p = 0.05$ ) three days after a  
745 long cold stress ( $0^{\circ}$  C) as a function of sex, starvation group, and length of cold stress in 6 day-  
746 old flies. The starvation length was always 24 h. On all figures “control” is the no starvation  
747 group, and the cold stress was applied at the end of starvation (“no delay” group) or after a  
748 delay (2, 4, 6, 8, 24, 48 h groups). A. 16 h cold stress, each bar is the mean of 58–86 flies. B.  
749 20 h cold stress, each bar is the mean of 67–74 flies. C. 24 h cold stress, each bar is the  
750 percentage of 54–60 flies. D. 32 h cold stress, each bar is the percentage of 70–75 flies. E. 24 h  
751 cold stress, each bar is the percentage of 44–59 flies.

752  
753 Figure 2. Percentage of survivors ( $\pm$  confidence interval at  $p = 0.05$ ) three days after a  
754 long cold stress ( $0^{\circ}$  C) as a function of sex, starvation group, and length of cold stress in 27  
755 day-old flies. On all figures “control” is the no starvation group, and the cold stress was applied  
756 at the end of starvation (“no delay” group) or after a delay (2, 4, 6 h groups). A. 24 h starvation  
757 and 24 h cold stress, for each bar  $n$  is 44–66 females, 37–72 males were observed in each group  
758 but no one survived to the cold stress (ca one third of males died during starvation). B. 20 h  
759 starvation and 20 h cold stress, for each bar  $n$  is 48–68 females, 26–67 males were observed in  
760 each group but no one survived to the cold stress in the no delay and 2 h groups (ca one quarter  
761 of males died during starvation). C. 16 h starvation and 16 h cold stress, for each bar  $n$  is 35–68  
762 flies.

763  
764 Figure 3. Percentage of survivors ( $\pm$  confidence interval at  $p = 0.05$ ) three days after a  
765 long cold stress ( $0^{\circ}$  C) as a function of sex, starvation group, and length of cold stress in 41  
766 day-old flies. On all figures “control” is the no starvation group, and the cold stress was applied  
767 at the end of starvation (“no delay” group) or after a delay (2, 4, 6 h groups). A. 20 h starvation  
768 and 16 h cold stress, for each bar  $n$  is 33–44 females, 46–65 males were observed in each group  
769 but no one survived to the cold stress in the no delay and 2 h groups (ca 9% of flies of each sex  
770 died during starvation). B. 16 h starvation and 16 h cold stress, for each bar  $n$  is 19–25 females,  
771 44–57 males were observed in each group but only one survived to the cold stress in the 4 h  
772 group (ca 9% of males died during starvation). C. 16 h starvation and 8 h cold stress, for each

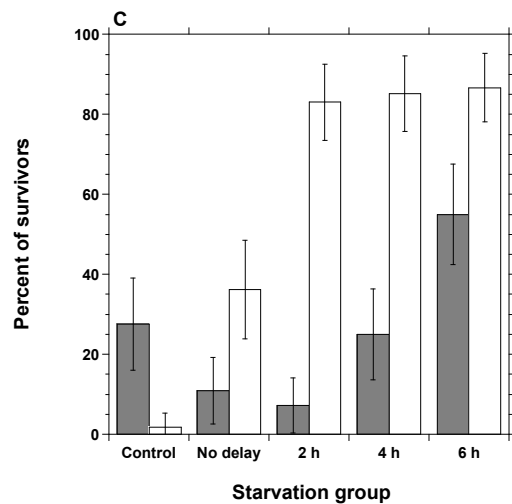
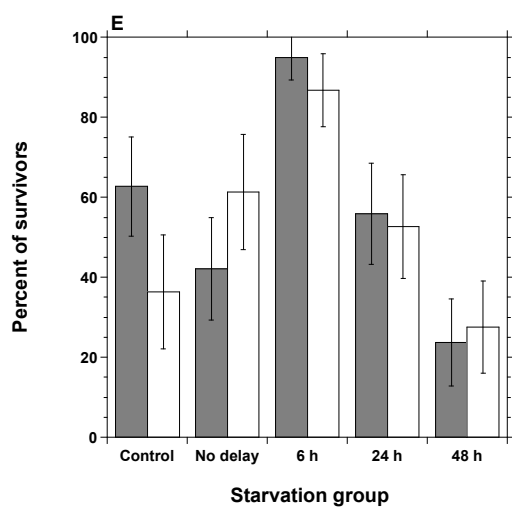
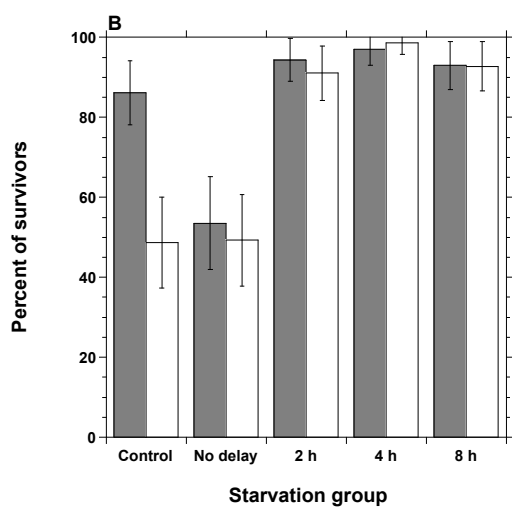
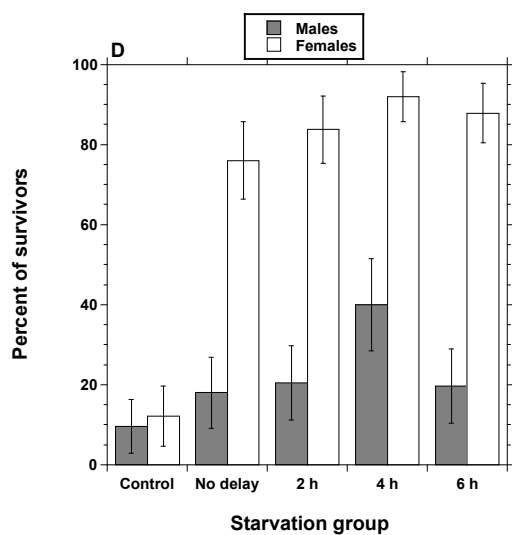
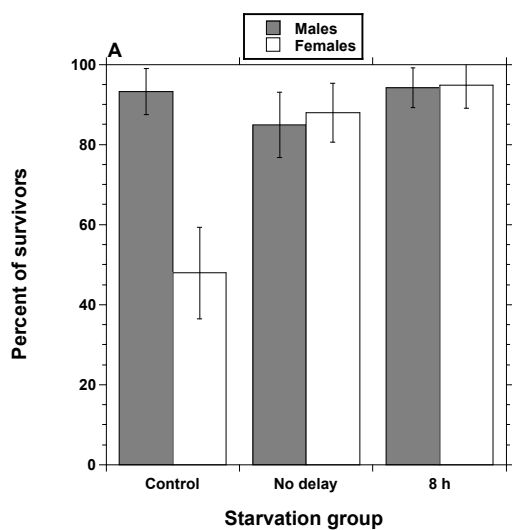
773 bar n is 15–23 females or 36–54 males (only one male and one female died during starvation).

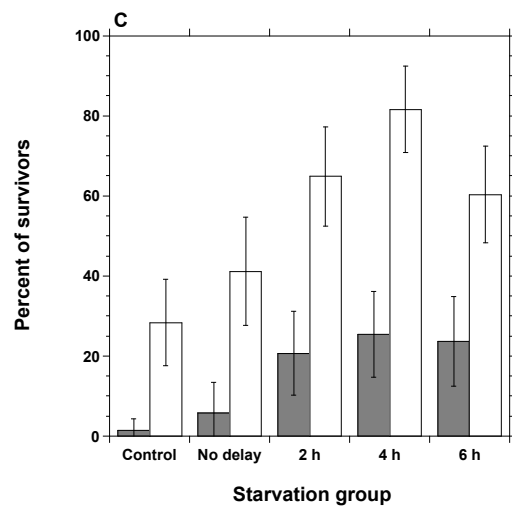
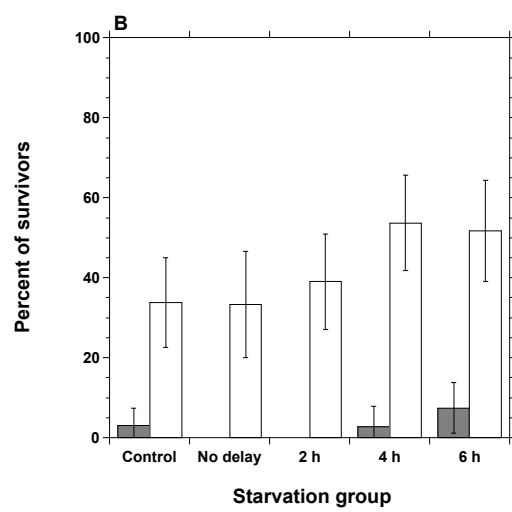
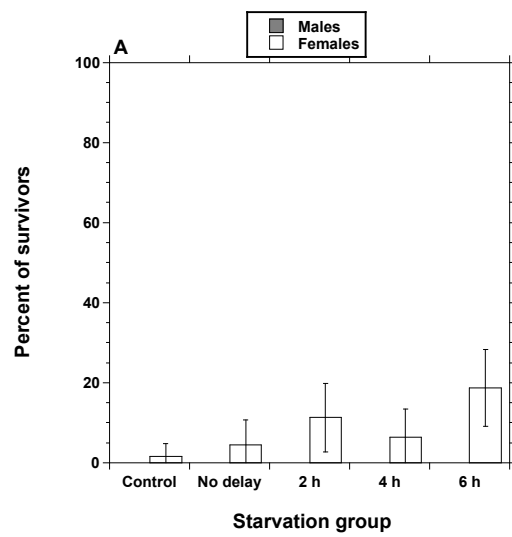
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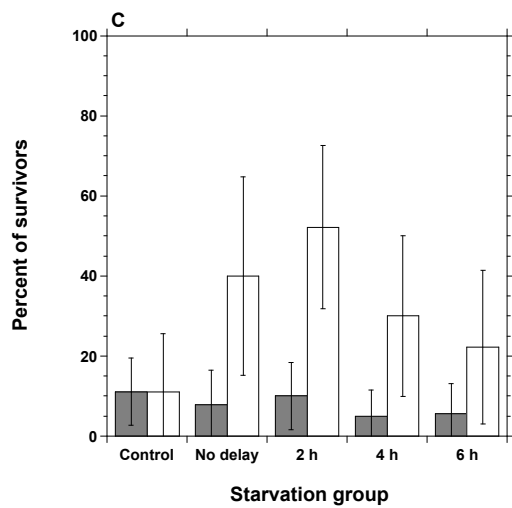
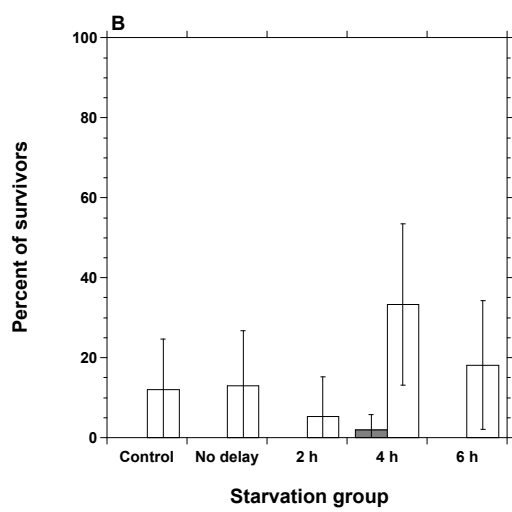
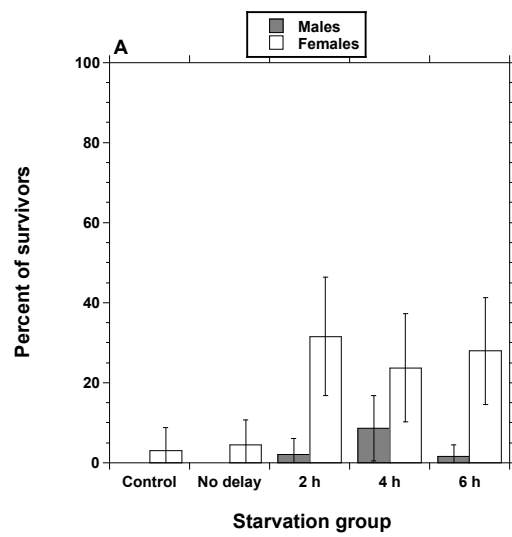
775 Figure 4. Percentage of survivors ( $\pm$  confidence interval at  $p = 0.05$ ) one day after a heat  
776 shock ( $37^{\circ}$  C) as a function of sex and starvation group. A. 90 min heat stress at 6 days of age,  
777 each bar is the percentage of 63–74 flies. B. 90 min heat stress at 27 days of age, each bar is  
778 the percentage of 31–51 flies. C. 60 min heat stress at 41 days of age, each bar is the  
779 percentage of 25–41 flies. D. 75 min heat stress at 41 days of age, each bar is the percentage of  
780 46–59 flies.

781

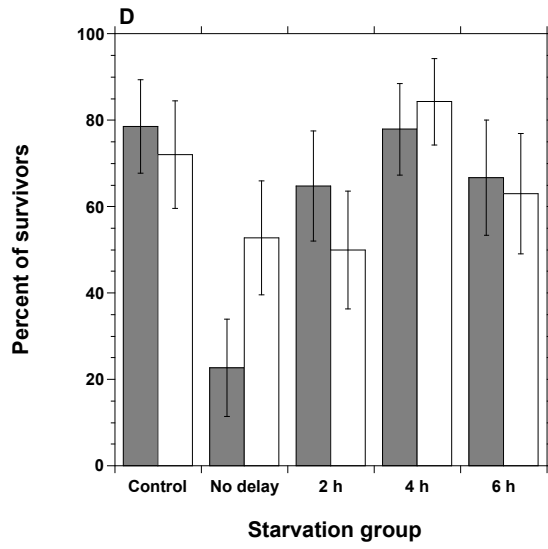
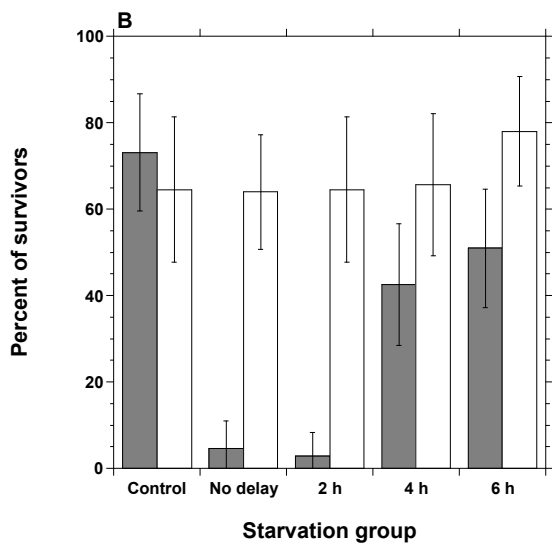
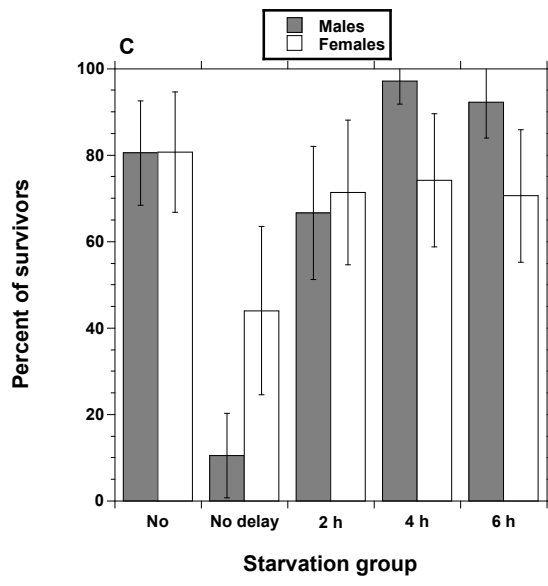
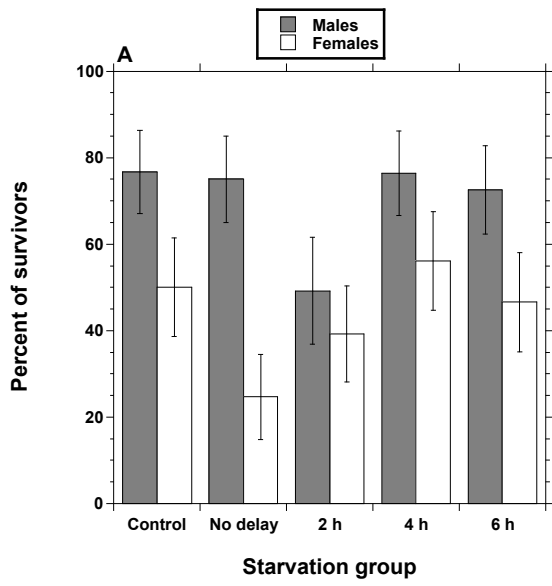
782 Figure 5. Mean learning (quinine groups) or photonegative (dry groups) scores  $\pm$  SEM of  
783 flies as a function of sex and starvation. The score is the mean number of photonegative choices  
784 during 16 trials. Flies were starved or not (ca 17.5 to 26 h). Flies were either trained in a learning  
785 procedure (quinine groups) or tested for their photopositive tendency (dry groups). Each bar is the  
786 mean of 15 males or females.

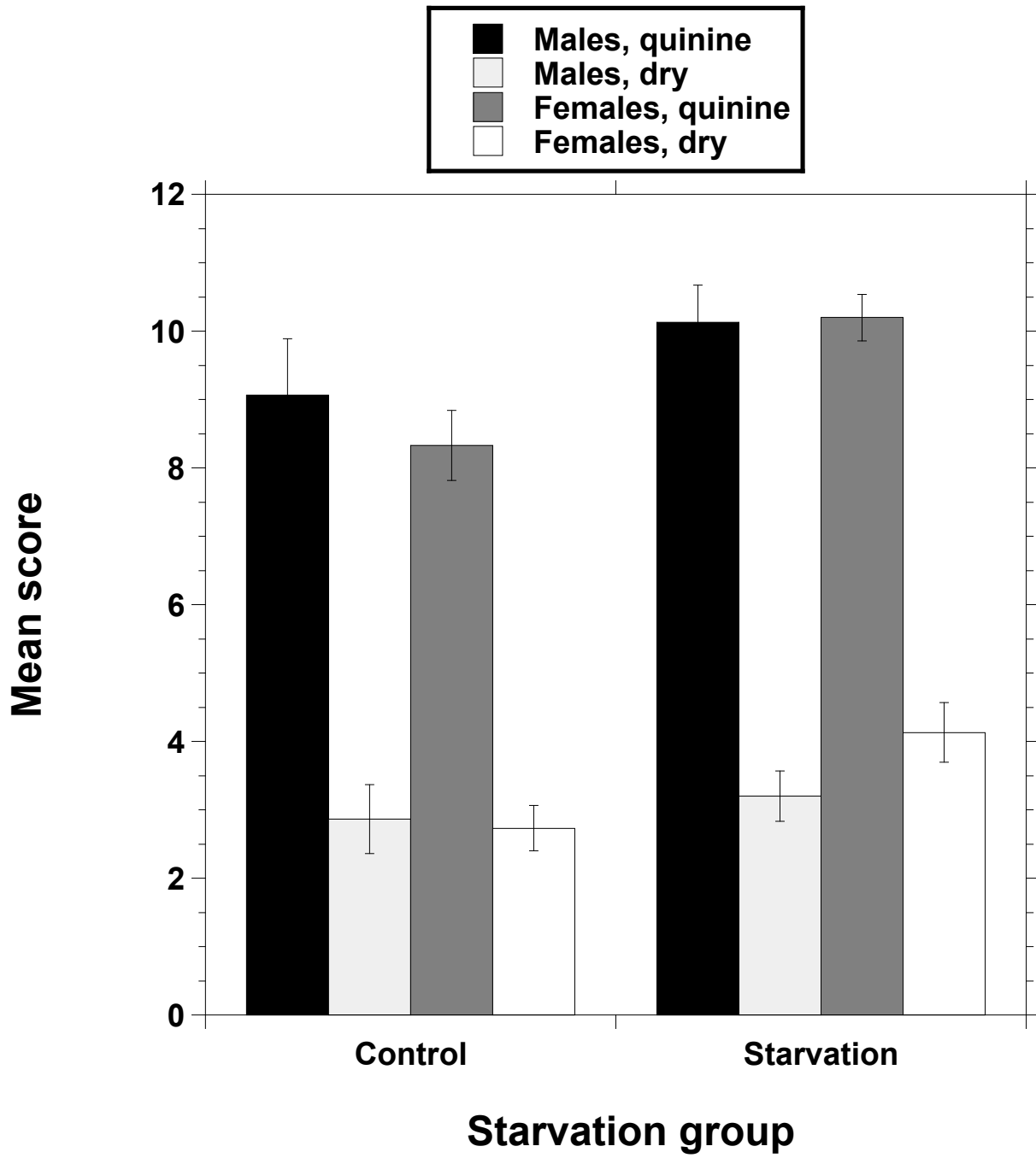












## Table

Table 1. Summary of the effects of starvation on resistance to severe stresses in males and females of various ages. The effect of starvation is shown as 0 (no effect), – (deleterious effect) or + (better resistance to stress). When nearly no fly survived the starvation or stress treatments, this is indicated as “dead”. The starvation duration is indicated in parentheses for middle-aged and old flies; it was always 24 h in young flies, but 16, 20, or 24 h in middle-aged and old flies.

	Males			Females		
	1 week	4 weeks	6 weeks	1 week	4 weeks	6 weeks
<b>Length of cold stress</b>						
4 h			0 (16)			0 (16)
6 h			0 (16)			0 (16)
8 h			0 (16)			0 (16)
16 h	0	+ (16)	dead (20)	+	+ (16)	+ (20)
16 h			dead (16)			0 (16)
20 h	+	dead (20)	dead (20)	+	+ (20)	dead (20)
24 h	+	dead (24)		+	+ (24)	
32 h	+			+		
48 h	+			+		
<b>Length of heat stress</b>						
60 min			0 (16)			0 (16)
75 min			0 (16)			0 (16)
90 min	–	– (16)	dead (16)	–	0 (16)	dead (16)
120 min	0			0		
<b>Hydrogen peroxide</b>	–	– (16)	– (16)	–	0 (16)	– (16)
<b>Fungal infection</b>	–	– (16)	0 (16)	0	– (16)	0 (16)

Answers to referees

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