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**Characterisation of the positive effects of mild stress on
ageing and resistance to stress**

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Abstract

The positive effects of mild stress on ageing, lifespan and resistance to stress have been studied mainly in *Drosophila melanogaster* flies and in the nematode *Caenorhabditis elegans*. These studies now allow to know the effects of the strength of the mild stress and of the number of exposures, the duration of the positive effects, if mild stress is effective when applied at any age, and whether combining two or three mild stresses is more efficient than a single one. This article summarises these results.

Key-words

Mild stress — Heat stress — Lifespan — Hormesis — *Drosophila melanogaster* — *Caenorhabditis elegans*

Introduction

In 1977, the gerontologist George Sacher wrote in a review article dealing partly with hormesis, i.e. the positive effects of mild stress, that “hormetic effects are unlikely to occur in the healthy active individual, and are more likely to be significant in the ill or depressed animal”. He concluded that “hormesis is in one sense an obstacle in the path of gerobiological research, and efforts to understand and annul it would be well justified”, which could be done by developing “living environments that are optimal for... behavioral, physiological, and immunological health” of animal models. Clearly speaking, this article could not encourage other scientists to publish results showing hormetic effects and one can bet that such results have been kept in the bottom drawer of the desk. At the same time, Vladimir Frolkis (1982, p.9), in the Soviet Institute of Gerontology in Kiev, Ukraine, reported that 100-day-old rats subjected to “repeated brief stress” had longer mean (+19%) and maximal (+12%) lifespans. Later on, he indicated that “cold, sound, electrical stimulation, physical load, and a temporary restriction of motor activity” increased mean (+18%) and maximal lifespans (+11%), while the “rough stress” decreased lifespan (Frolkis 1993). Unfortunately, this author was stingy with details and these results were simply ignored by other scientists. Thus, on the one hand, a US author denigrated hormesis, while, on the other hand, a Soviet author did not publish in international journals his results showing such effects. In such conditions, there was only a little chance to deepen the knowledge on hormesis.

Times have changed, and numerous results have now been published by various teams in international journals, showing clearly that hormetic effects do exist and that we should wonder whether and how they can be used in therapy (Rattan and Kyriazis 2019). Since the review article of Neafsey (1990), mainly dealing with changes of mortality rates because of hormesis, and the review

on hormesis and ageing by Minois (2000), many new results have been published and hormesis has been discussed in books (Le Bourg and Rattan 2008; Mattson and Calabrese 2010, Rattan and Le Bourg 2014, Rattan and Kyriazis 2019) or in special issues of Ageing Research Reviews (see e.g. Mattson 2008) or Dose-Response (see Le Bourg and Rattan 2010).

One could suppose that observing hormetic effects could depend on various experimental conditions, such as the duration and/or strength of the mild stress, the observed phenotype (e.g. lifespan or resistance to a severe stress), the age of application of the mild stress, and so on. Thanks to many studies performed since the turn of the century, it is now possible to characterise the hormetic effects by relying on numerous experimental results, and not only on theoretical thinking. Therefore, this article does not describe hormetic effects in various species but focuses on their features, which have been widely studied in the fly *Drosophila melanogaster*, but not only.

1. Strength of the mild stress and number of exposures to it

The definition of hormesis is “a dose-response phenomenon which is characterized with a low dose stimulation and a high dose inhibition” (Calabrese 2008). Thus, a first problem is to determine the range of the low doses inducing a hormetic effect, before the no observed adverse effect level is reached (Fig. 1A). A simple procedure is to vary the duration of exposure to a mild stress.

A clear example of a perfect hormetic curve is that of Cypser and Johnson (2002) showing that *Caenorhabditis elegans* nematodes live longer if exposed for a short time to a mild heat stress (35 °C), this mild stress also increasing the survival time at 35 °C. However, deleterious effects are observed when the time at 35 °C increases (Fig. 2, see also Wu et al. 2008, Pickering et al. 2013). Similar results are observed for *D. melanogaster* male flies kept in hypergravity (HG) for various durations, i.e. at gravity levels higher than that of the Earth (1-5 g, Fig. 3). HG increased lifespan if lasting for less than ca 30 days (Le Bourg et al. 2000), in accordance with previous experiments showing that keeping flies in HG for life decreased lifespan (Le Bourg and Lints 1989, Lints et al. 1993). However, in most of experiments, HG had no positive effect on lifespan of females (Le Bourg and Minois 1997) or slightly decreased it (Le Bourg et al. 2000). This last study also showed that 14, 19 or 24 days in HG (1-7.38 g) had a similar positive effect in males and that the HG level was of a minor importance. Therefore, these experiments in flies and nematodes show that a mild stress may have positive effects if not lasting for a too long time but also that, once a positive effect is observed, the level of the mild stress (strength or duration) may be of a minor importance. Similarly, even if repeating a mild stress up to five times (35 °C at 1, 4, 7, 10, and 13 days of age for respectively 60, 55, 50, 40 and 30 min) increases lifespan in *C. elegans*, the size of the positive effect decreases with the number of repeats (Wu et al. 2009).

By contrast, if the duration of the mild stress is too short, it can have no effect even if it occurs several times. Figure 3 shows that fractionating 12 days of HG in 3 periods of 4 days separated by 3 days at 1 g did not increase lifespan of males, probably because a 4 days exposure is a too mild stress. Similarly, female flies live longer than control ones only if they are subjected to 4 successive heat shocks (1 hour at 36 °C) and kept in groups of 10 females with 3 males, but 1, 2, or 3 heat shocks have no effect (Hercus et al. 2003, Fig. 4A). However, it has also been observed that a too mild dose can nevertheless have a positive effect if it is repeated. In *C. elegans*, 4 hours at 30 or 33 °C do not increase lifespan (Olsen et al., 2006, Fig. 4B, compare with 2 hours at 35 °C in Fig. 2) but repeating this 4 hours exposure at successive ages increased it, this result being observed in a wild-type line (N2) and in the mutant TJ1060. Similarly, subjecting male flies to daily one-hour exposures at 0 °C had no effect on lifespan if done for 5 days from the age of 5 days or that of 12 days, but increased it in flies exposed from 5 days of age during two periods of 5 days separated by 2 days (Le Bourg 2007).

All these results modify the shape of the theoretical hormetic curve because it may happen that very low doses have no effect and that, once a dose has a positive effect, a plateau is observed (Fig. 1B). This proposal does not substitute to the usual hormetic curve but is a possible refinement of it.

2. Is mild stress effective when applied at any age?

It has been reported in *C. elegans*, either that a single heat shock had no positive effect on lifespan at 4, 8, 12, or 16 days of age (mutant TJ1060), or that a positive effect was observed at 8 and 12 days of age only in the N2 wild-type line (Olsen et al. 2006, Fig. 5A). In flies, applying a mild cold stress at increasing ages during two weeks (weeks 1 and 2, weeks 2 and 3, weeks 3 and 4, or weeks 4 and 5) increased survival time at 37 °C at 6 weeks of age of both sexes (Le Bourg 2011, Fig. 5B). This mild cold stress also increased lifespan of males and their lifespan after infection with a fungus at 6 weeks of age (Fig. 5B), except if it was applied at the oldest ages (weeks 4 and 5).

Therefore, a mild stress applied at various ages can have hormetic effects at old age, but these effects can be less observed when the mild stress is applied at very old ages (weeks 4 and 5).

3. Is mild stress effective for life?

The previous results (Fig. 5B) indicate that a mild cold stress applied during the first two weeks of age can increase resistance to heat at 6 weeks of age in these flies living at 25 °C, which have a mean lifespan of ca 7-8 weeks. One can thus wonder whether the mild stress has positive

130 effects for a duration of 4 weeks or rather for life, whatever its length can be. Because decreasing
131 the temperature increases lifespan, this hypothesis was tested by transferring flies, after the last cold
132 pretreatment at 2 weeks of age, at 19 or 22 °C, a last group of flies being kept at 25 °C. Survival
133 time at 37 °C was observed at 6 or 8 weeks of age in flies kept at 25 °C, at 6 or 10 weeks of age in
134 flies kept at 22 °C, and at 7 or 10 weeks of age in flies kept at 19 °C. In males, the cold pretreatment
135 increased survival time at 37 °C at any age and in any temperature group, no clear effect being
136 observed in females. Therefore, the mild cold stress applied at young age was efficient not only for
137 4 weeks but for life whatever its length could be, and at least for 8 weeks after the last cold
138 pretreatment (Le Bourg 2016).

139

140 **4. The effects of combining a mild stress and other stresses**

141 *4.1. Combining a mild stress with a factor having positive, neutral, or negative effects*

142 In real life, various events happen at the same time and one could wonder what would happen
143 when a mild stress co-occurs with a stimulus having deleterious, neutral or positive effects on the
144 trait under study, particularly because Sacher (1977) wrote that “hormetic effects are unlikely to
145 occur in the healthy active individual, and are more likely to be significant in the ill or depressed
146 animal”.

147 This has been studied by combining in the same flies a mild cold stress with hormetic effects
148 and the removal of live yeast from the feeding medium, which has positive, neutral or negative
149 effects, depending on the studied trait. When this removal had no effect, as on survival time at
150 37 °C, the positive effect of the mild cold stress was still observed. When removal had a slight
151 deleterious effect, as on lifespan after infection with a fungus, the positive effect of the mild stress
152 was still observed but to a lesser extent. Finally, when removal was very deleterious, as on survival
153 after a severe cold stress, the mild cold stress was unable to mitigate this effect (Fig. 6). By contrast,
154 when removal of live yeast had positive effects, as on the ability to climb on a vertical surface, these
155 effects were additive with the positive effects of the mild cold stress (Le Bourg 2010).

156 Similarly, the positive effect of HG on lifespan of virgin males is abolished in mated flies
157 which live less than virgin ones, and the same result is observed if virgin males live at 30 °C rather
158 than at 25 °C (Le Bourg et al. 2004). By contrast, when virgin flies live in individual vials, which
159 increases lifespan, the positive effect of HG on lifespan is more important (Le Bourg et al. 2000).
160 These results showing that hormetic effects can also be observed in longevous flies, and not in
161 short-lived ones, are in accordance with those of Sarup and Loeschcke (2011) reporting hormetic
162 effects in long-lived strains of flies.

163 These results indicate, in sharp contrast with Sacher (1977), that hormetic effects are more

164 prone to be observed in flies in “living environments that are optimal for... behavioral,
165 physiological, and immunological health” than in those decreasing lifespan, such as mating and
166 high temperature.

167 One could summarise all these results by saying that when a factor has a positive effect this
168 effect adds to the positive effect of the mild stress and that, when a factor has increasing negative
169 effects, the positive effect of the mild stress is erased up to be nullified.

170 4.2. Combining mild stresses

171 Previous studies have shown that mild cold stress (e.g. Le Bourg 2007), HG (e.g. Le Bourg et
172 al. 2000), and short heat shocks (e.g. Le Bourg et al. 2001, Hercus et al 2003) may have positive
173 effects in flies, even if the effects of short heat shocks are often modest in various invertebrate
174 species (Lagisz et al. 2013). Combining HG and mild cold stress had additive effects on survival at
175 37 °C or on male lifespan (Le Bourg 2012). Similarly, combining heat shock (31 or 35 °C) and
176 desiccation (0 % relative humidity) mild stresses in *Drosophila simulans* female flies can increase
177 survival time at 38 °C and/or to desiccation more than each mild stress alone, but with a slight cost
178 on lifespan that was similar when only one mild stress or their combination are used (Bubliy et al.
179 2013).

180 When a mild stress has positive effects in one sex and negative ones in the other one, its
181 combination with another mild stress with the same effects is thus expected to induce more positive
182 effects in the former sex and more negative ones in the second sex. This can be observed in flies
183 subjected to a mild cold stress and HG, which each can decrease female lifespan and increase that
184 of males: while males show additive positive effects of the two mild stresses, females display
185 additive negative effects (Le Bourg 2012, Fig. 7).

186 The survival of flies to a severe cold stress can be increased by a mild cold stress, HG or
187 fasting. However, while combining two stresses among these three ones was more efficient than a
188 single stress, combining the three stresses was not more or even less efficient than combining only
189 two stresses (Le Bourg 2017).

190 4.3. Conclusions

191 On the one hand, it can be concluded that combining two mild stresses with positive effects
192 can be more efficient than using a single mild stress. However, combining three mild stresses is not
193 more efficient than combining two ones. Similar results can be observed in humans: combining
194 physical exercise and thermal therapy (6 hours at 40 °C daily on the painful knee) has additive
195 positive effects on knee pain in elderly women (Kim et al. 2013).

196 On the other hand, combining a mild stress with positive effects with another one with
197 negative effects can decrease the positive effects of the former mild stress.

5. Conclusions

These studies have allowed to better characterise the hormetic effects, mainly in flies but also in nematodes. It is unfortunate that studies on vertebrates are scarce, particularly because the hormetic effects could be used in therapy. For instance, carbon monoxide could be used in cardiology, as studies on pigs have shown that it can minimise the consequences of ischemia-reperfusion (e.g. Lavitrano et al. 2004; Goebel et al. 2009), and clinical studies have used remote ischemic conditioning in humans, but with not still significant effects (Hausenloy et al. 2015, 2019, Meybohm et al. 2015, see Garratt et al. 2016 and Vanezis 2019 for a discussion of these results). Similarly, local heat has been used to decrease inflammation of knees (e.g. Forestier et al. 2010), and sauna seems to decrease cardio-vascular and all-cause mortality and protect from stroke (Laukkanen et al. 2015, Kunutsor et al. 2018, review in Laukkanen et al. 2018). At variance with the Sacher's opinion (1977) that "hormetic effects are unlikely to occur in the healthy active individual", it seems that mild stress can be either used by healthy people, for relaxation and pleasure purposes (sauna), and by unhealthy ones for therapy purposes (e.g. Forestier et al. 2010, Kim et al. 2013, Scapagnini et al. 2014)).

The previous studies, mainly performed in nematodes and flies, can allow to conclude:

1: Mild stresses can have hormetic effects if their strength/duration is not too low/short and not too strong/long. In such conditions, the hormetic curve can be different from the traditional one (Fig. 1A) and is rather a succession of two plateaus, before deleterious effects linked to a too strong stress are observed (Fig. 1B).

2: Mild stresses can have hormetic effects not only at young age, but also at older ages. However, this could depend on the trait under study as applying the mild stress at old age can also have no effect. It could be that in some cases a mild stress at a young age is becoming a stronger stress at old age.

3: A mild stress applied at young age can have long-lasting effects (like a vaccine, in a way). This is obviously shown as an increased lifespan, but not only, as survival to a severe stress can be increased at old age. In addition, the positive effects can be observed for life, whatever its length could be, and not only for a fixed duration. This could be of a particular importance for human therapy issues if this would also be observed in clinical studies.

4: The positive effects of two different mild stresses can be additive, which means that the addition of two mild stresses is not inducing a strong stress with deleterious effects, provided these two mild stresses have positive effects. By contrast, if one of these mild stresses has negative effects on a given trait, the positive effects of the other stress can be minimised and even nullified. Turning

232 to therapy issues, it would mean that a risk-benefit assessment is needed if combining two mild
233 stresses (e.g. sauna and physical activity) to be sure that their effects are additive in all cases. To
234 consider a fictitious example, if sauna and physical activity could add their positive effects on
235 cardio-vascular mortality but increase the risk for stroke, while each mild stress has a positive effect
236 on the occurrence of stroke, combining the two mild stresses would be an issue despite the positive
237 effects on cardio-vascular mortality. Finally, it seems that combining three mild stresses does not
238 give better positive effects than combining two ones, maybe because their combination brings the
239 strength of the combined stresses near the end of the hormetic zone (Fig. 1B).

240 All these studies point in the same direction: studying the effects of mild stress is useful and
241 better knowing their features could pave the way for new tools in therapy and prevention of age-
242 related issues. However, translating the results of experimental studies in clinics is always a
243 challenge and one cannot conclude that the results observed in animal models will be observed,
244 *mutatis mutandis*, in human beings.

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Figures

Figure 1. A. Theoretical hormetic curve showing, as the dose is increasing (abscissae), the point of maximal hormetic effect and the no observed adverse effect level. The plus sign, on the ordinates, indicates a positive effect (e.g. a longer lifespan) and the minus one a deleterious effect.

B. Proposal for a new theoretical hormetic curve, taking into account experimental results, the maximal hormetic effect point being replaced with a maximal hormetic zone.

370 Figure 2. Relative thermotolerance and lifespan of *C. elegans* nematodes subjected to a
371 pretreatment at 35 °C. Thermotolerance is the survival time at 35 °C (hours) and lifespan is
372 observed at 20 °C (days). Each point is the mean of 33-55 worms for thermotolerance and of 283-
373 584 ones for lifespan (redrawn from Cypser and Johnson 2002).

375 Figure 3. Relative lifespan of *D. melanogaster* males flies subjected to hypergravity (HG: 3 or
376 5 g, 3 or 5 times the Earth's gravity level) for various durations (3 to 46 days). A positive effect of
377 HG was never observed in females. Flies were either kept in vials of 15 virgin males ("group") or in
378 individual vials ("individual"). In the experiments with a continuous line and in the "fractionated"
379 groups, lifespan was observed daily from emergence. In the other experiments, lifespan was
380 observed from the day of transfer to 1 g (only some flies died except if transfer from HG to 1 g
381 occurred after the age of 46 days). Each point is the mean of ca 60 flies (redrawn from Table 1 in Le
382 Bourg et al. 2000), except in the fractionated experiment for which it is 75. In this last experiment,
383 flies were kept for 12 days in HG in 3 periods of 4 days separated by 3 days at 1 g.

384 Figure 4. A. Mean lifespan $\pm$ SEM of *D. melanogaster* female flies subjected to one or up to 4
385 heat shocks at 36 °C at successive ages (3, 6, 9, 12 days of age, total n is ca 400, redrawn from table
386 1 in Hercus et al. 2003). B. Mean lifespan $\pm$ SEM of *C. elegans* nematodes subjected to one or up to
387 4 heat shocks at 30 °C (TJ1060) or 33 °C (N2) at successive ages (4, 8, 12, 16 days of age, for each
388 point n = 76-137, redrawn from table 1 in Olsen et al. 2006).

389 Figure 5. A. Mean lifespan $\pm$ SEM of *C. elegans* nematodes subjected to one heat shock at
390 30 °C (TJ1060) or 33 °C (N2) at various ages (n = 41-137, redrawn from table 1 in Olsen et al.
391 2006). The two left points are the same as the two left points in Fig. 4B. B. Mean lifespan or
392 survival time at 37 °C $\pm$ SEM of *D. melanogaster* flies. Flies were cold-pretreated daily (0 °C for 60
393 min) or not during two periods of 5 days separated by 2 days, starting at 5, 12, 19, or 26 days of age
394 (weeks 1-2, weeks 2-3, weeks 3-4, and weeks 4-5). Their lifespan from emergence (days, n is ca
395 100 for each bar), survival time at 37 °C observed at 6 weeks of age (minutes, n = 33-35), and
396 lifespan after infection at 41 days of age with the fungus *Beauveria bassiana* (days, n = 25-37) were
397 observed. Non-significant results in females are not shown (redrawn from Le Bourg 2011).

399 Figure 6. Combination of a mild stress with a factor, the presence or absence of live yeast at
400 the surface of the medium, having either neutral or negative effects, depending on the trait under
401 study. Flies were cold-pretreated daily (0 °C for 60 min) or not during two periods of 5 days
402 separated by 2 days, starting at 5 days of age. Mean survival time $\pm$ SEM at 37 °C at 3 weeks of age
403 (minutes, $n = 20$ for each bar), mean lifespan $\pm$ SEM after infection at 26 days of age with the
404 fungus *B. bassiana* (days, $n = 25-55$), and percentage of survivors after 16 hours at 0 °C at 19 days
405 of age (n is ca 35) were observed. Non-significant results in females (infection) are not shown
406 (redrawn from Le Bourg 2010) and the results on survival to cold are only displayed to show that
407 removing live yeast made that flies could not survive to a cold shock (other results in the same
408 article showed that the mild cold stress could increase survival in flies with live yeast).

409 Figure 7. Combination of two mild stresses, HG and mild cold stress, with opposite effects on
410 mean lifespan $\pm$ SEM of males and females. Flies were cold-pretreated daily (0 °C for 60 min) or
411 not during two periods of 5 days separated by 2 days, starting at 5 days of age, and lived either at 1 g
412 or 5 g. Each bar is the mean of 86-101 flies (redrawn from Le Bourg 2012).