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Research article

Lifespan extension of *Drosophila melanogaster* through hormesis by repeated mild heat stress

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Abstract

We assessed the impact of repeated episodes of a mild heat stress on lifespan, fecundity, heat stress resistance and Hsp70 expression in *Drosophila melanogaster*. There was a significant increase in lifespan of females repeatedly exposed to a mild heat stress when measured in both a pair and a group situation. There was no effect on fecundity when the flies were first exposed to the mild heat stress at an age later than 3 days old, but when it did occur on day 3, there was a significant effect on cumulative fecundity levels over 18 days. The negative fitness effect appears to be the result of a direct cessation or reduction of oviposition during the first bout of stress exposure, and is influenced by the age at which this first exposure occurs. The mild heat stress had no impact on egg viability. The mild heat stress exposures increased resistance to potentially lethal heat stress and levels of Hsp70 expression in heat-exposed flies were higher than those in controls.

Introduction

Stress resistance can be increased if individuals are pre-exposed to a sub-lethal level of stress and allowed to recover before exposure to a higher level of stress. This process, termed 'acclimation' or 'hardening', involves the induction of the so-called heat shock proteins (hsp) or stress proteins (Lindquist 1981). Negative effects or costs of the process of acclimation have been seen in various organisms (Hoffmann 1995) and in female *D. melanogaster*, the consequence of this increased resistance and/or protein production is manifested as a reduction in fecundity (Krebs and Loeschcke 1994a). Hsp70 is one of the large stress proteins that has recently received a lot of attention in the fields of stress resistance and evolution. For example, work using Hsp70 extra-copy lines of *D. melanogaster* has shown that too much Hsp70 or the expression of Hsp70 in the absence of stress can be detrimental to fitness (Feder et al. 1996; Krebs 1999).

Maynard Smith (1958) was one of the first to report that an exposure to a short period of heat stress increased lifespan in female *D. melanogaster*. Recent years have seen much research into the observation that lifespan in *Drosophila* and other organisms can be increased through brief exposure to conditions of environmental stress (e.g., Khazaeli et al. 1997; Le Bourg and Minois 1997; Lithgow et al. 1995; Rattan 1998; Minois 2000; Le Bourg et al. 2000, 2001). Such longevity extending and other anti-aging effects observed after exposure to mild stress are representative of the phenomenon of hormesis (Parsons 1990; Rattan 2001). Using transgenic lines, Tatar (1999) and Silberman and Tatar (2000) found no effects on the number of eggs laid by females exposed to a heat level that leads to an extension of mean lifespan, but the productivity of these females was affected as a result of decreased egg hatching.

Since, in the past, the heat-hardening process and induction of hsps to high levels were shown to have

a fitness effect, yet could also lead to an increased lifespan and/or decreased rate of aging, we set out to determine what would happen to lifespan and other traits if a low level of Hsp induction occurred at repeated intervals, resulting in the (assumed) upregulation of the stress response system over a longer period of time than has been previously used (e.g., Khazaeli et al. 1997; Silberman and Tatar 2000). The experiments presented here involved a stress level inducing approximately 30% of maximal Hsp70 response in young *D. melanogaster*. The aim of this regime revolved around the question of whether what could be considered a more ecologically relevant stress scenario can lead to an increased lifespan, and whether it can do so without any negative fitness effects.

Materials and methods

Experimental design

A mass-bred *D. melanogaster* population was generated by combining equal numbers of F_7 adults from 30 isofemale lines collected in October 1999 near Leiden, The Netherlands. This base stock was maintained at 25 °C (12:12 D:L). Pilot studies were performed to investigate the temperature and exposure time required (using controlled-temperature cabinets) to induce approx. 30% of standard Hsp70 expression in young (three days old) female flies. ELISA was used following methods of Dahlgaard et al. (1998) and the standard for Hsp70 expression levels created by exposing flies for 60 min to a water bath at 36 °C and allowing recovery at 25 °C for 1 h. This standard is regarded as inducing close to maximal levels of Hsp70 expression in adults of this species (Krebs 1999). Exposure to 34 °C for 3 h resulted in 25–37% of standard Hsp70 expression.

The impact of the timing of repeated episodes of mild heat stress (i.e., 30% Hsp70 expression) on traits was investigated. Experimental flies either received no exposure, or four bouts of exposure to the mild heat stress outlined above. Exposure of adults occurred at three-day intervals in order to minimize any potentially direct cumulative damage and/or negative hardening effects (see Krebs and Loeschcke 1994a). Initial exposure occurred on age day 3 (i.e., heat hardening occurred on days 3, 6, 9, 12). The maximum number of exposures was kept at 4, since we considered the stress would become more severe with age. The experiment was performed with experimental flies from the F_5 generation of the base population reared at a controlled larval density of 35 larvae per vial. Flies assessed for levels of heat resistance or Hsp70 expression were exposed to the mild heat stress in groups of 12 per vial simultaneously with flies measured for fecundity and longevity.

Trait measurements

Fecundity. The number of eggs laid daily at 25 °C was measured for 80 females per treatment. Females were paired with two males upon eclosion and provided with a plastic spoon coated with a layer of agar for oviposition. The agar had a small amount of charcoal powder added for easier detection of eggs and a 10% yeast solution painted across the agar surface. Spoons were changed and eggs scored daily.

Longevity. Longevity was measured at 25 °C. Lifespan of females was measured using two methods: in a pair and in a group situation. This was done because previous experiments using *Drosophila* show that the lifespan achieved depends on the way in which the flies are maintained (e.g., Partridge and Farquhar 1981). Any effects of exposure to mild heat stress on longevity may differ with methodology. The first measure of longevity for each treatment involved the pairs set up for fecundity measurements and the second involved groups of 10 females and 3 males in a vial. For both methods, longevity was assessed for 80 females per treatment.

Temperature stress resistance

Survival after a high (potentially lethal) temperature stress was measured in females on day 17. Sixteen replicates of ten females per treatment were exposed in empty glass vials to 39 °C for 27 min using a water bath. This length of time led to ca. 80% death in flies that had not been previously exposed to heat. Flies were left to recover at 25 °C for 24 h and the number of survivors per replicate vial was scored.

Hsp70 expression levels

Eight replicate vials of 10 females per treatment were assessed for levels of Hsp70 expression after heat shock at 39 °C for 27 min. Flies were frozen for analysis after 1 h recovery at 25 °C. Levels of Hsp70 expression were assessed using ELISA, with the antibody 7.FB (provided by S. Lindquist).

This antibody is used to determine the amount of inducible Hsp70 protein, and levels in the samples were expressed relative to the same standard used in the pilot study. Data collected were proportional and an arcsine transformation was performed before analysis.

Results

Trait values are presented in Figure 1 and Table 1. Mean longevity measures revealed significant effects of methodology (pair *versus* group: $F_{(1,312)} = 12.282$, $P < 0.001$) and heat treatment ($F_{(1,312)} = 9{,}791$, $P = 0.002$), with no interaction between the two factors. Since there was a significant methodology effect, data were analyzed separately for each method. For both methods, there was a significant effect of exposure to the mild heat on lifespan (pairs: $F_{(1,158)} = 5.2196$, $P = 0.024$, groups: $F_{(1,158)} = 4.551$, $P = 0.034$). In both instances, mean lifespan was increased by approximately 6 days, equivalent to 10% for this population under these conditions. Lifespan curves are presented in Figures 2a and b.

Daily fecundity data are presented in Figure 2c. There was a significant effect of treatment only on day 3 ($F_{(1,158)} = 13.712$, $P < 0.001$), where the females from the treatment receiving no stress laid a greater mean number of eggs than those from the treatment receiving mild heat exposure. Cumulative fecundity (days 2–18) showed a significant effect of treatment ($F_{(1,158)} = 5.049$, $P = 0.026$). Flies exposed on four occasions to the mild heat stress laid fewer eggs than those not exposed.

The level of survival after exposure to a potentially lethal heat stress on day 17 (5 days after the heat hardening treatment) showed a significant effect of treatment ($F_{(1,14)} = 7.124$, $P = 0.018$). Hsp70 expression level differed significantly between treatments ($F_{(1,14)} = 6.362$, $P = 0.027$), with the treatment receiving exposure having higher levels of expression of Hsp70 at day 17.

Exposure to mild heat stress affected fecundity on day 3. This result was investigated further by performing another experiment to monitor levels of fecundity and egg viability across smaller time intervals during this day. The number of eggs laid was assessed during specific time intervals starting from the time of exposure to the stress ($N = 20$/treatment).

Consistent with the initial experiment, heat exposure had an effect on the number of eggs laid

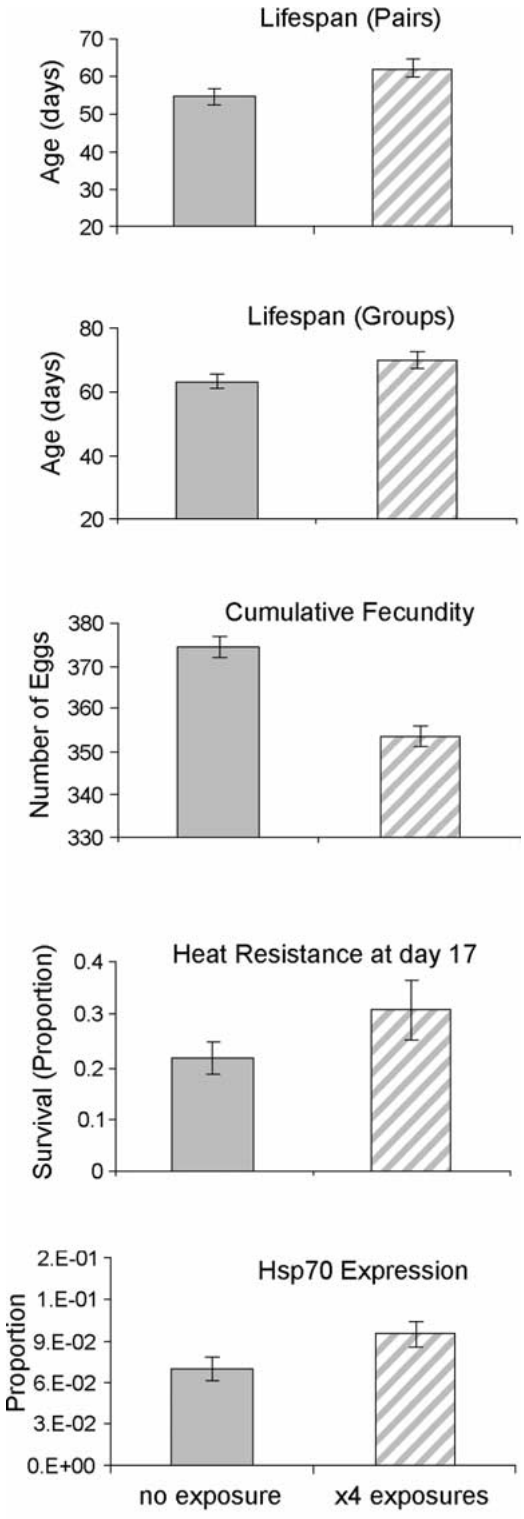

Figure 1. Mean trait values for the treatment receiving no stress exposure (solid bars) and the treatment receiving four bouts of exposure (striped bars) to the mild heat stress. Error bars represent the standard error of the mean.

Table 1. Means for traits and time (days) until specific percentages of the population had died. There were five experimental groups in the second experiment. Flies either received no exposure at all to the mild heat stress or received a treatment ranging from 4 exposures (days 3, 6, 9, 12), 3 exposures (days 6, 9, 12), 2 exposures (days 9, 12) to 1 exposure (day 12).

	Mean trait value (S.E.)					Days until percentage dead					
	Longevity		Cumulative	Heat (prop.	Hsp70	25% dead		50% dead		75% dead	
	Pair	Group	fecundity	survival)		Pair	Group	Pair	Group	Pair	Group
Experiment I	*	*	*	*	*						
0 exposures	54.73 (2.3)	63.29 (2.0)	374.54 (6.6)	0.217 (0.02)	0.07 (8.7E-03)	40	56	52	62.5	65	73
4 exposures	62.26 (2.4)	69.96 (2.4)	353.54 (6.6)	0.31 (0.01)	0.09 (9.1E-03)	46	54.5	61	72.5	76.5	81
Experiment II		*									
0 exposures	55.20 (3.5)	64.58 (2.8)	378.45 (8.7)	0.200 (0.02)	0.13 (1.8E-02)	36.5	53	49.5	66.5	73	72.5
4 exposures	61.15 (3.8)	74.55 (3.2)	358.73 (7.9)	0.313 (0.09)	0.17 (9.7E-03)	43	60	52	79.5	84.5	90.5
3 exposures	52.50 (3.5)	68.32 (3.5)	382.88 (11.2)	0.200 (0.03)	0.16 (1.1E-02)	36.5	54	50	73	69	87.5
2 exposures	49.70 (3.9)	61.78 (4.2)	355.63 (10.1)	0.262 (0.07)	0.13 (1.8E-02)	23	39	50	61	70	84.5
1 exposure	55.50 (4.2)	60.40 (3.6)	387.28 (8.7)	0.163 (0.04)	0.16 (5.9E-03)	30	41	58	64	72	76

*$P < 0.05$.

during day 3 ($F_{(1,39)} = 4.644$, $P = 0.038$). Figure 3 shows the mean proportion of eggs laid for the smaller specific time intervals measured. Analysis indicates that the proportion of eggs laid differed only for the period of time the stress exposure occurred (stress period: $F_{(1,39)} = 12.333$, $P = 0.001$; since data from this time period were not normally distributed for flies receiving the stress, a Wilcoxon signed rank test was used to confirm the ANOVA results: $Z = -2.689$, $P = 0.007$). No other time interval for the remainder of the day showed significant differences in egg numbers between treatments. Thus, it appears that the effect of this mild heat stress on fecundity is largely a direct result of a cessation of or reduction in egg laying during the period of exposure to the stress, and that there are no delayed effects of the heat stress on fecundity. Levels of egg hatchability/viability over the first four days of egg laying were assessed and no significant effect of the heat treatment was seen on this trait at any stage (data not presented).

Given the results of the experiment comparing four bouts of stress to no stress exposure, another experiment was performed to investigate the effect of repeated mild heat stress exposure on traits when flies were exposed to the stress for the first time at an age older than day 3. The same traits were measured on flies divided into five heat treatment regimes that differed with respect to the day of initial stress application and the number of exposures (e.g. days 3, 6, 9, 12; days 6, 9, 12; days 9, 12; day 12; no stress). Results of this investigation are presented in Table 1.

Daily fecundity measurements again showed a significant effect of heat treatment regime only on day 3 ($F_{(4,195)} = 3.321$, $P = 0.014$) and *post-hoc* tests (Tukey) indicated that the treatment receiving four exposures laid fewer eggs on this day than the other four treatments. When the initial heat exposure occurred on a day later than day 3, there was no significant effect of the heat treatment on fecundity, Cumulative levels of fecundity did not differ between treatments, although the treatment receiving four bouts of mild heat stress exposure had a lower mean number of eggs compared to controls.

For measures of mean lifespan, there was a significant effect of method ($F_{(1,390)} = 23.115$, $P < 0.001$) and treatment ($F_{(4,390)} = 3.125$, $P = 0.015$) with no interaction. For mean lifespan in pairs, there was no significant difference between treatments, although mean values for the treatment receiving four bouts of exposure were the same degree higher than those of the control, as in the previous experiment. There was a significant treatment effect ($F_{(4,195)} = 2.684$, $P = 0.033$) for measurements made in a group situation. *Post-hoc* tests (Tukey) indicated that those receiving the treatment four bouts of exposure had a mean lifespan significantly longer than those receiving one exposure that occurring on day 12; the other 3 groups were intermediate. There appears to be no cumulative effect of the mild heat exposures on mean lifespan. If lifespan data are expressed in terms of the day on which specific percentages of the population died (see Table 1), the time at which the last 25% of the popula-

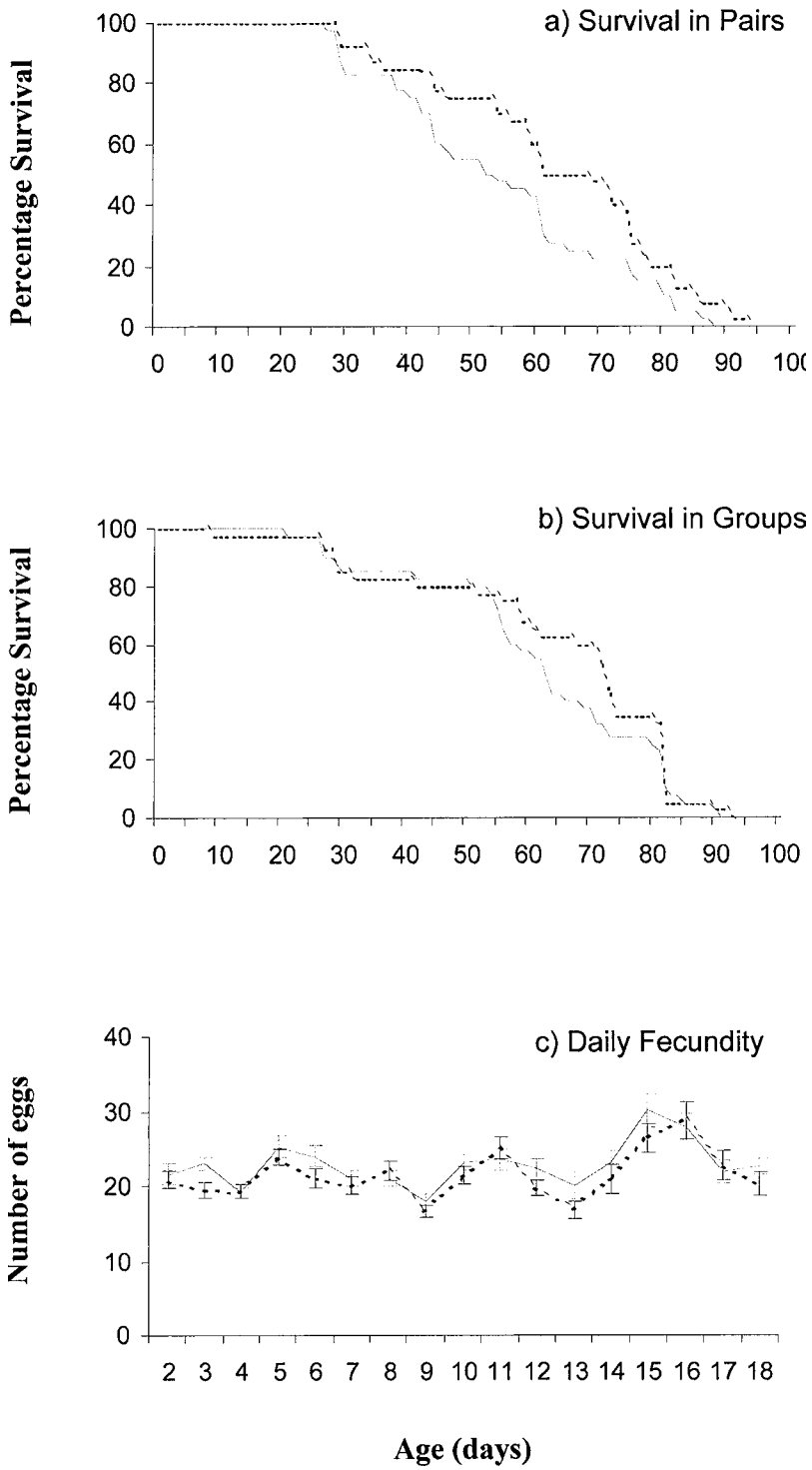

Figure 2. Survival curves for female flies in a pair situation (a), survival measures of female flies in a group situation (b) and measures of daily fecundity (c) made at 25 °C. The solid line indicates the control treatment and the dotted line indicates the treatment receiving four bouts of mild heat exposure. Error bars represent the standard error of the mean.

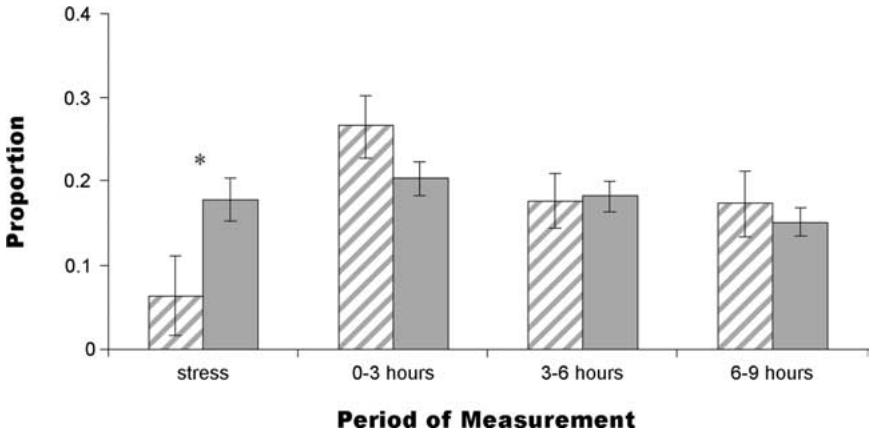

Figure 3. Breakdown of fecundity as a proportion of the total eggs laid on day 3 into specific time intervals. Striped bars represent the individuals exposed to the mild heat stress and solid bars indicate control. Error bars represent the standard error of the mean.

tion remained alive (i.e., 75% dead) is where the effect is most seen.

The level of survival after exposure to a potentially lethal heat stress and level of Hsp70 expression at day 17 showed no significance in an ANOVA, although means suggested a trend for the treatment receiving four bouts of exposure to mild heat stress to have higher levels of survival and expression.

Discussion

Work on *D. melanogaster* indicates a cost of the extension seen in lifespan when individuals are exposed to a stressful environment. Females exposed to stress had lower levels of fecundity (Maynard Smith 1958), and Silberman and Tatar (2000) found that a level of stress exposure known to increase lifespan and decrease the rate of aging had a cost in terms of egg viability. In the study presented here, repeated exposure to a lower level of stress (in terms of the level of Hsp70 expression) led to increases in mean lifespan to about the same extent as studies using higher levels of stress exposure (Khazaeli et al. 1997). We also found a cost of this extension, not in the viability of the eggs but in the number of eggs laid. This appears to be the result of the direct cessation of oviposition during the period of stress, with no fecundity effects thereafter.

The effects seen in terms of lifespan are similar to those seen previously in *Drosophila* using a higher level of stress (heat – Khazaeli et al. 1997; hypergravity – Le Bourg and Minois 1997) and presumably higher levels of Hsp induction and expression (Krebs 1999). Our data indicate that high levels of stress exposure are not necessarily needed to impact on lifespan in *Drosophila*. Using transgenic lines, Tatar et al. (1997) suggested that this might be the case. Correspondingly, studies showing that high levels of stress proteins are not needed to confer high levels of heat resistance. Indeed, maximum resistance is not seen until Hsp70 levels are reduced to almost baseline levels (Krebs and Feder 1998; Dahlgaard et al. 1998). Hsp70 expression levels affect other Hsp proteins (Parsell and Lindquist 1993) and it may be that exposure to the mild level of stress used here which instigates a flow of proteins (see Dahlgaard et al. 1998) which in turn, affects traits.

The aim of the experiments described in this article was to assess the impact of repeated exposure of a much milder level of stress than was used in the past. One bout of mild stress exposure had no apparent effect on mean lifespan in this study when it was applied on day 12, whilst Khazaeli et al. (1997) found that one bout of heat exposure impacted on both lifespan and mortality rate. This difference may be due to the timing of the stress exposure and the level of stress used (note also that a single bout of exposure to the stress level used here only on day 3 had no impact on lifespan; M.J. Hercus, unpublished data). It is interesting that the flies not receiving exposure to the mild heat stress on day 3 did not show the same degree of impact on mean lifespan. Such treatments did not show an obvious influence on fecundity either, which suggests that a change in one trait may be unable to occur without affecting another. This may be expected from life-history theory (Roff 1992), and the presence of trade-offs or pleiotropy effects

are the basis for some evolutionary theories of aging (see Rose 1991). We feel it important to test for any associations between traits rather than to assess the impact of a treatment on one trait (such as lifespan) alone (Hercus and Loeschcke 2001). Costs associated with heat acclimation in *D. melanogaster* are reduced fecundity (Krebs and Loeschcke 1994a) and territoriality (Zamudio et al. 1995), and other *Drosophila* species have shown costs associated with the acclimation response (e.g., Berrigan and Hoffmann 1998). While other organisms show that acclimation can occur without any costs (e.g., Hoffmann and Hewa-Kapuge 2000), we know of no examples in *Drosophila* where this occurs.

There was consistency with respect to control treatment measurement across methods and experiments. While no differences were seen in the effect that repeated episodes of mild heat stress had on mean lifespan across methods, methodology affected the lifespan achieved. Lifespan is influenced by environmental factors and population dynamics, such as temperature (Partridge et al. 1995), mating activity (Partridge and Farquhar 1981) and crowding (Joshi and Mueller 1997). We found that flies maintained in a group situation lived longer, which could be considered contrary to what might be expected from previous work. This difference may be due to yeast availability and/or mating activity. For example, females in a pair situation were exposed to yeast in a paste form and those in a group situation to live yeast grains sprinkled on top of the medium. Perhaps flies in the group situation were able to utilize the yeast provided less readily and this impacted on lifespan.

Young flies appear to be more affected by a mild heat stress (in terms of fecundity) than flies subjected to the same stress at a later age. Perhaps initial stress protein induction is more costly at a young age when other mechanisms of stress resistance may be used. Krebs and Loeschcke (1994a) found that exposure to a heat stress inducing Hsp to a high level affected levels of productivity and that multiple exposures to the stress resulted in further reduced fecundity. In our experiments there were no apparent cumulative effects of exposure on fecundity.

Higher levels of heat stress exposure may have impacted more on the level of resistance to a potentially lethal level of heat stress. We do not consider the increased level of heat stress resistance in flies experiencing repeated exposure to the mild heat stress to be the result of a direct acclimation event. Krebs and Loeschcke (1994a, b) suggest that acclimation effects in *D. melanogaster* exposed to a higher level of stress than that used in our experiments last for up to three days. We are confident that both the potential for negative effects and for direct acclimation effects of the mild heat stress exposure(s) were already overcome by the time heat stress resistance was measured.

Levels of Hsp70 expressed after exposure to a potentially lethal heat stress to flies at day 17 were low in this study. Changes in levels of expression with increasing age have previously been seen in *Drosophila*. Both induced and constitutive levels of Hsp70 have been shown to decrease with age (Minois et al. 1999; Neidzwiecki, Kongpachith and Fleming 1991; Sørensen and Loeschcke 2002). Four bouts of mild heat exposures had a significant effect on resistance to potentially lethal heat stress later in life, and levels of expression of Hsp70 were higher than in the control treatment. Perhaps repeated exposure to a mild level of heat stress early in life leads to an upregulation of Hsp expression that may last throughout life. This would be interesting to investigate further.

To extrapolate to the field situation, repeated exposure to a mild level of stress may be considered to occur more readily than a single bout of a higher level of stress. This was our main motivation behind the level of stress exposure and regime used. Whether one could further lower the stress level (and therefore the assumed induction/expression levels of stress proteins) and still see an impact on lifespan and associated impact on fitness, remains to be seen. Other questions also interesting to follow up include whether there is an effect of repeated exposure during other life stages on adult longevity and/or fitness and stress resistance. Preliminary experiments (M.J. Hercus, unpublished data) looking at the effects of repeated mild heat stress (equivalent to the one used here, in terms of Hsp induction) during larval stages indicate that there is potential to increase adult lifespan by exposure to stress at this life stage, where the effect of stress in terms of adult lifespan and life history traits is dependent on exposure severity and number.

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