Mild heat stress at a young age in *Drosophila melanogaster* leads to increased Hsp70 synthesis after stress exposure later in life

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Abstract

In a number of animal species it has been shown that exposure to low levels of stress at a young age has a positive effect on stress resistance later in life, and on longevity. The positive effects have been attributed to the activation of defence/cleaning systems (heat shock proteins (Hsps), antioxidases, DNA repair) or to effects of a changed metabolic rate, or both. We investigated the effect of mild stress exposures early in life on Hsp70 synthesis after a harder stress exposure later in life in five isofemale lines of *Drosophila melanogaster*. Female flies were either exposed to repeated bouts of mild heat stress (3 h at 34°C) at a young age (days 2, 4 and 6 post-eclosion) or held under standard laboratory conditions. At 16 and 32 days of adult age, respectively, flies were exposed to a high temperature treatment known to induce Hsp70 in the investigated species (1 h at 37ºC). Thereafter, the inducible Hsp70 levels were measured. Our data show a tendency towards increased Hsp70 synthesis with increased age for both 'mild stress' and 'no stress' flies. Moreover, the results show that flies exposed to mild stress at a young age synthesized more Hsp70 upon induction, compared to control flies, and that this difference was accentuated at 32 days compared to 16 days of age. Thus, bouts of mild heat stress at a young age impact on the physiological stress response system later in life. This may be caused by an increased ability to react to future stresses. Alternatively, the mild stress exposure at a young age may actually have caused cellular damages increasing the need for Hsp70 levels after stress exposure later in life. The importance of an Hsp70 upregulation (throughout life) in explaining the phenomenon of hormesis is discussed, together with alternative hypotheses, and suggestions for further studies.

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Introduction

Hormesis describes the phenomenon that an exposure early in life to a low level of stress that in higher doses would be deleterious can have beneficial effects later in life. Hormesis is well known in several organisms including humans and *Drosophila* and for several types of stress including radiation, heat and chemicals (Mine *et al*. 1990; Khazaeli *et al*. 1997; Rattan 1998; Parsons 2000; Verbeke *et al*. 2002; Hercus *et al*. 2003). The beneficial effects of a mild stress exposure in early life include increased subsequent stress resistance and, particularly, increased longevity and postponed ageing. Hormesis is, therefore, not only a transient effect of the exposure, but may also be of long-term importance (Forbes 2000; Minois 2000). It is usually attributed to stimulation and activation of 'housekeeping' systems (heat shock proteins, antioxidases, DNA repair), or to effects of a changed metabolic rate and investment in maintenance, or both (Tatar *et al*. 1997; Minois 2000;

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Fonager *et al*. 2002). As all natural populations are expected to encounter stressful environmental conditions at least occasionally, hormesis has been suggested to be a general mechanism conserved by evolution, enabling organisms to maintain high performance when exposed to suboptimal conditions (Forbes 2000; Parsons 2000).

Inducible expression of genes that encode heat shock proteins (Hsps) is essential for surviving severe stress exposure. Hsps function as molecular chaperones involved in 'housekeeping' functions in the cell, including prevention of aggregation of damaged proteins, transportation, folding and unfolding, assembly and disassembly of multistructured units, and in degradation of misfolded or aggregated proteins (Gething and Sambrook 1992; Parsell and Lindquist 1993). These functions are important in the cell even under nonstressful conditions, but are accentuated after intrinsic and extrinsic stress exposures (Feder and Hofmann 1999; Fonager *et al*. 2002; Kristensen *et al*. 2002; Sørensen *et al*. 2003). Thus, increased synthesis of the major stress-inducible Hsp in *Drosophila* (Hsp70), as well as other Hsps, seems to be an important part of a mechanism for maintaining the structural and functional ability (homoeostasis) of the cell under and after stress exposure, thereby allowing the organism to survive periods of stressful environmental conditions.

The association between Hsps and stress resistance has been shown in a number of studies (see Feder and Hofmann 1999; Sørensen *et al*. 2003 for reviews). It has been suggested that Hsps also play a direct role in the extended longevity and stress resistance of flies exposed to nonlethal stress at a young age (Tatar *et al*. 1997; Minois 2000). This hypothesis is based on the potential of Hsps to act as regulators of the durability of the soma in the face of chronic internal and external stress (Jazwinski 1996; Lithgow and Kirkwood 1996). However, induced synthesis of Hsp70 in *Drosophila* is short-lived and the protein returns to nondetectable levels within hours of exposure to stress (Feder *et al*. 1995; Dahlgaard *et al*. 1998). Therefore it has also been argued that Hsps only play a minor role in the hormetic effects that are observed to persist for weeks after stress exposure.

The well-established association between exposure to mild stress and the subsequent increase in longevity and stress resistance (Khazaeli *et al*. 1997; Le Bourg and Minois 1997; Sørensen and Loeschcke 2001; Hercus *et al*. 2003) does not seem to be an acclimation effect as it lasts for many days or weeks (Loeschcke *et al*. 1994; Hercus *et al*. 2003). Moreover, the increased longevity in such cases has been shown not to be attributable to lower energetic costs caused by suppression of reproductive activity in stressed individuals only (Khazaeli *et al*. 1997). However, the mechanism involved in this phenomenon is still to a large extent unresolved.

In this paper we examine how an exposure to repeated (Hsp70-inducing) mild heat stress in early adult life affects the level of Hsp70 later in life. The mild stress level used in this study has earlier been shown to positively affect longevity and heat stress resistance in the same *D. melanogaster* populations (Hercus *et al*. 2003). It is not known how mild stress treatments at a young age affect the stress response and Hsp70 synthesis after stress exposure later in life. However, if Hsp70 is involved in the late-life effects of early stress exposure, we expect that Hsp70 synthesis levels should be increased in later life. If stress-induced Hsp70 synthesis is indeed upregulated in later life two alternative causes can be suggested: First, stressed flies may have a higher 'need' due to a negative cellular impact of the mild stress exposure experienced at a young age. Second, flies stressed in early life may have a more readily evoked Hsp response. A higher level of Hsp70 synthesis in flies according to those hypotheses might, in turn, partly explain the increased longevity and resistance, which could be due to the beneficial effects on these traits of higher Hsp70 synthesis after stress exposures, probably due to an improved 'housekeeping'.

Materials and methods

***Experimental flies*:** Five independent isofemale lines of *Drosophila melanogaster* were used in this study. The founder females were caught near Leiden, The Netherlands, in September 1999. The lines were kept in the laboratory at 20ºC (on agar, sugar, yeast, oatmeal medium) in high numbers prior to this experiment for 25 generations in a 12 h : 12 h light : dark cycle. Before setting up the experiment lines were transferred to 25ºC and the population sizes were increased to obtain sufficient numbers of flies for the experiment. The parental density in the bottles from which the flies were collected was approximately 40 pairs per bottle (egg laying for 24 h).

***Heat treatments*:** Females were collected as virgins under CO_2 anaesthesia (20 vials with 20 females for each line), and transferred to vials that were divided at random into the two experimental groups. Vials belonging to experimental group 1 received three bouts of mild heat stress at 2, 4 and 6 days post-eclosion, each treatment consisting of a 3-h exposure to 34ºC which is known to induce Hsp70 to approximately 33% of the maximum induction level (Hercus *et al*. 2003). Experimental flies were placed in empty glass vials with moistened stoppers to avoid desiccation stress, and the vials were submerged in a preheated water bath. After the exposure flies were transferred back to 25ºC. Experimental group 2 served as control and were maintained at 25ºC. All flies were transferred to vials with fresh medium every second day throughout the experiment. When the flies reached 16 days of age, half the vials of each experimental group (five vials of each

treatment) were exposed to 37ºC for 1 h in a preheated waterbath in empty vials with moistened stoppers. The remaining vials (five vials per line from each experimental group) received the same heat treatment at 32 days of age (figure 1). After the heat treatments at 16 or 32 days of age the vials were transferred back to 25ºC for 1 h before being frozen and stored at – 80ºC until protein analysis. The mortality during the investigated lifespan and after stress exposures (3 h at 34ºC and 1 h at 37°C) was very low and occurred randomly between experimental groups (data not shown). If, occasionally, dead flies were observed after heat treatment at day 16 or 32 those flies were removed from samples before freezing.

***Hsp70 level*:** Hsp70 level after induction at 37ºC for 1 h at days 16 and 32 was measured by the ELISA technique. The analysis followed the protocol by Sørensen *et al*. (1999). The flies were taken from – 80ºC and homogenized on ice. Samples were adjusted to equal amounts of total protein content by BCA analysis, and subsequently the Hsp70 expression level was determined in the samples using a monoclonal antibody (7.FB) specific for the inducible Hsp70 in *Drosophila* (Velazquez and Lindquist 1984; Welte *et al*. 1993). In total 100 samples (each sample constituted of 20 homogenized flies) were investigated (2 experimental groups × 2 age classes × 5 lines × 5 replicates per line). Samples from each isofemale line were analysed separately on the same microwell ELISA plate (20 samples each represented by four replicate microwells). The level of Hsp70 in each sample was estimated from the intensity of an enzymatic reaction and measured as absorbance with a spectrophotometer. The grand mean of each plate was standardized to the grand mean of plate one thus making between-plate comparisons possible.

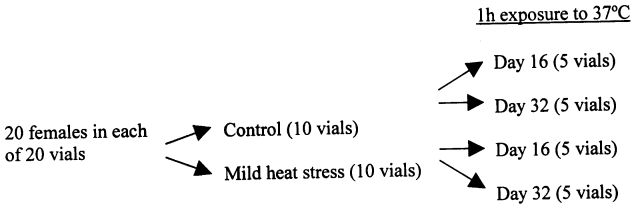

Figure 1. Experimental design, which was replicated in five isofemale lines (see text for details).

***Statistical analysis*:** All statistical analyses were performed with the program package JMP (JMP 1997). To improve normality and homogeneity of variances, the data were arcsine-square-root transformed. Normality and homogeneity of variances on the transformed data were confirmed by Shapiro–Wilk tests and by graphical inspection of data (data not shown).

Results

A full factorial ANOVA was performed testing the effects of isofemale line (fixed factor), treatment (experimental group one or two, fixed factor), age (16 or 32 days, fixed factor), and their interactions on Hsp70 level. The effect of isofemale line and age was highly significant, whereas the effect of treatment was nonsignificant (table 1). All interactions were significant. Owing to the highly significant effect of isofemale line, the five independent lines were also tested individually. The effect of treatment (mild stress or no stress exposure at a young age) was significant or at the border of significance (isofemale line 3: $P = 0.055$) in four of the five isofemale lines, whereas the effect of age was significant in all five isofemale lines. The interaction between treatment and age was significant in three isofemale lines (table 2).

Flies exposed to repeated mild stress at a young age generally synthesized more Hsp70 after a hardening treatment later in life compared to flies that were not exposed to stress at a young age. This difference in synthesis level between the two experimental groups ('stress' and 'no stress' flies) was generally more pronounced at 32 days of age than at 16 days of age (figure 2) as seen from the significant age × treatment interaction term in the overall ANOVA model (table 1).

Table 1. ANOVA on Hsp70 levels in isofemale lines exposed to either mild heat stress (three bouts of 34ºC for 3 h at 2, 4 and 6 days post-eclosion) or no stress at a young age, and heat stress (37°C for 1 h) at 16 or 32 days of age.

Source of variation	d.f.	MS	*F* ratio	*P*
Isofemale line	4	0.0056	8.7	< 0.0001
Treatment	1	0.00078	1.2	0.28
Age	1	0.1658	257.8	< 0.0001
Isofemale line × treatment	4	0.00567	8.8	< 0.0001
Isofemale line × age	4	0.00584	9.1	< 0.0001
Treatment × age	1	0.00298	4.6	0.034
Isofemale line × treatment × age	4	0.00483	7.5	< 0.0001
Error	80	0.00064		

d.f., Degrees of freedom; MS, mean square.

Discussion

In this study we found that a repeated mild heat stress in early adult life of *D. melanogaster* leads to increased Hsp70 synthesis after stress exposure later in life and that this increase was larger at 32 days compared to that at 16 days of age. Furthermore, flies on average (independent of experimental group) expressed more Hsp70 at an older age (32 days versus 16 days). This result shows that Hsp70 may be involved in the positive effects of mild stress on increased longevity and stress resistance. A chaperone system that is upregulated in a cost-efficient manner seems to be a potential candidate for explaining hormetic effects where mild stress is assumed to activate housekeeping functions. Alternatively, flies might actually have become damaged by the repeated mild stress exposure at a young age. Therefore, under this scenario, the higher Hsp70 level of early mildly stressed flies observed after stress exposure later in life may be the result of flies sensing this stress as being more severe. This may be due to accumulation of denatured aggregated proteins and other cellular damage as a result of the early mild stress exposures. This idea may not be in conflict with the knowledge that the mild stress exposure regimes used in this study have been shown to increase longevity and stress resistance (Hercus *et al*. 2003) as different traits can be affected differently by stress. In such cases, the net effect on the organism would be determined by a balance between benefits resulting from the induction of the stress response system and the deleterious effects of the stress.

Furthermore, the upregulation of the inducible Hsp70 in 'mildly stressed flies' may actually help in repairing both accumulated and newly induced damages to the cellular system, implying that flies having been exposed to mild stress actually live longer and are more resistant.

Our data, however, do not allow for a conclusion on whether the result is due to a higher 'need' rather than a 'higher capacity' of the Hsp70 system in flies exposed to mild stress at a young age. Investigating whether flies exposed to stress at a young age actually express Hsp70 even under benign environmental conditions would give more insight into this issue.

The isofemale lines varied in their pattern of Hsp70 level across age and experimental groups. This variation is expected when considering isofemale lines as they are likely to be genetically differentiated. Whether these differences between lines are correlated with variation in longevity and stress resistance across lines needs to be studied. In isofemale lines 1–3 the effect of the stress exposure on Hsp70 level is accentuated at 32 days of age compared to that at 16 days of age. This indicates that the effect of early mild stress exposure on Hsp70 expression level after stress exposure later in life increases with age,

Table 2. ANOVA table showing the effects of treatment, age and the interaction between the two sources of variation on Hsp70 level in each of five individual isofemale lines. The *F* values equal MS as the degrees of freedom are one in all cases.

Isofemale line	Source of variation	d.f.	MS	*P*
1	Treatment	1	7.9	0.013
	Age	1	16.0	0.001
	Treatment × age	1	13.0	0.024
	Error	16	0.00054	
2	Treatment	1	1.8	0.19
	Age	1	45.8	< 0.0001
	Treatment × age	1	10.2	0.057
	Error	16	0.00045	
3	Treatment	1	4.3	0.055
	Age	1	78.1	< 0.0001
	Treatment × age	1	23.9	0.0002
	Error	16	0.00034	
4	Treatment	1	5.7	0.03
	Age	1	113.5	< 0.0001
	Treatment × age	1	3.2	0.09
	Error	16	0.00071	
5	Treatment	1	6.0	0.027
	Age	1	44.4	< 0.0001
	Treatment × age	1	0.5	0.48
	Error	16	0.00119	

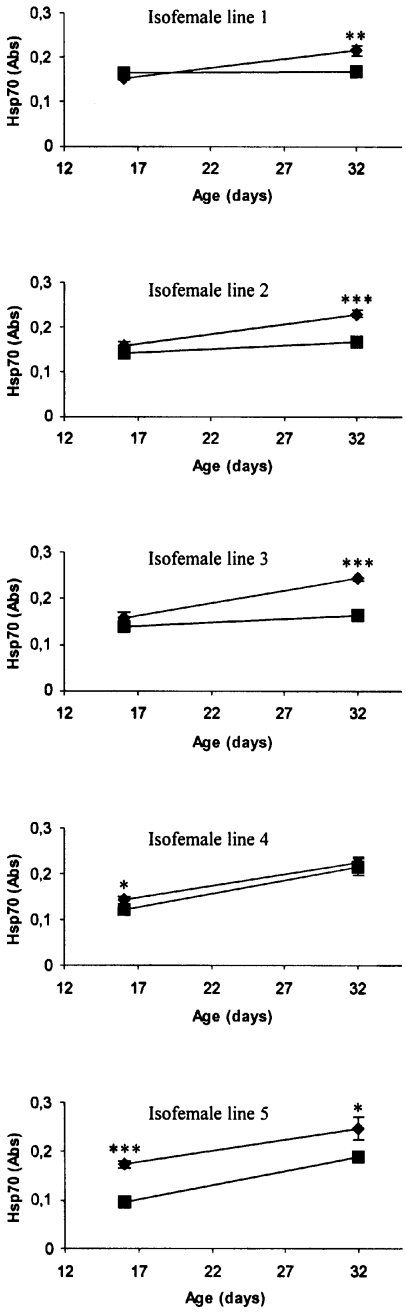

Figure 2. Mean absorbance ± SE for female flies exposed to 37°C for one hour at 16 or 32 days of age, after being exposed to either no stress (■) or a repeated mild heat stress (◆) at a young age. For each isofemale line the effect of treatment at 16 and 32 days were tested by Student's *t* test (isofemale line 1: 16 days: $t = 1.27$, $F_{(1,8)} = 1.61$; 32 days: $t = -3.66$, $F_{(1,8)} = 13.4$; isofemale line 2: 16 days: $t = -1.27$, $F_{(1,8)} = 1.61$; 32 days: $t = -7.61$, $F_{(1,8)} = 57.84$; isofemale line 3: 16 days: $t = 1.9$, $F_{(1,8)} = -1.89$; 32 days: $t = -12.39$, $F_{(1,8)} = 153.56$; isofemale line 4: 16 days: $t = -2.45$, $F_{(1,8)} = 5.99$; 32 days: $t = -0.31$, $F_{(1,8)} = 0.095$; isofemale line 5: 16 days: $t = -6.49$, $F_{(1,8)} = 42.05$; 32 days: $t = -2.85$, $F_{(1,8)} = 8.15$). Significant differences between 'stress' and 'no stress' flies at 16 and 32 days of age are indicated on the figures ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

at least during early senescence. In the remaining two isofemale lines the pattern is less clear.

In this study there was an overall tendency towards increased Hsp70 synthesis with increase in age. In the literature there are some controversies on whether Hsp70 is downregulated or upregulated with age (Wheeler *et al*. 1999; Sørensen and Loeschcke 2002). Changes in age-dependent Hsp70 synthesis may be due to different causes (Sørensen and Loeschcke 2002). The first is the inducible synthesis after and in response to stress exposure. The second (as suggested by Wheeler *et al*. 1999) is synthesis that occurs in aged individuals without heat or other stress exposures. The age-induced synthesis of Hsp70 is probably caused by genomic injuries (Wheeler *et al*. 1999) in a manner similar to the one observed in highly inbred *Drosophila* (Kristensen *et al*. 2002). Because of accumulation of age-related genomic injuries it makes sense that the difference between 'stress' and 'no stress' flies in Hsp70 levels in this study in the majority of lines is reinforced with higher age.

In conclusion, this study shows that in female *D. melanogaster* repeated mild heat stress exposure at a young age affects Hsp70 level after stress exposure later in life, and that this effect is accentuated at 32 days compared to that at 16 days of age. This shows that the Hsp70 system may be involved in the increased longevity and stress resistance observed in individuals exposed to mild stress at a young age. For flies in natural populations continually being exposed to Hsp-inducing mild stress throughout life, such a response may be adaptive. However, we cannot distinguish at this point whether our results are due to a higher 'need' rather than a 'higher capacity' of the Hsp70 system in flies exposed to mild stress at a young age.

To increase understanding of the complex association between Hsp70 level and hormesis some additional studies should be performed. We suggest that lines should be investigated, in a similar design as the one used here, for longevity, stress resistance and synthesis of Hsp70 (and other stress-inducible as well as constitutively expressed heat shock cognates) with and without Hsp-inducing stress treatments later in life.

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