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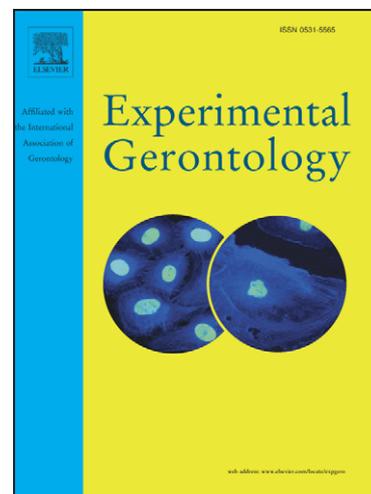
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Gender specific effects of heat induced hormesis in Hsf-deficient *Drosophila melanogaster*

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Abstract

In insects mild heat stress early in life has been reported to increase life span and heat resistance later in life, a phenomenon termed hormesis. Here, we test if the induction of the heat shock response by mild heat stress is mediating hormesis in longevity and heat resistance at older age. To test this hypothesis we used two heat shock transcription factor (Hsf) mutant stocks. One stock harbours a mutation giving rise to a heat sensitive Hsf which inactivates the heat shock response at high temperature and the other is a rescued mutant giving rise to a wildtype phenotype. We measured longevity, heat resistance and expression level of a heat shock protein, Hsp70, in controls and mildly heat treated flies. We found a marked difference between males and females with males showing a beneficial effect of the early heat treatment on longevity and heat resistance later in life in the rescued line, seemingly mediated by the production of heat shock proteins (Hsps). The results indicate that heat inducible Hsps are important for heat induced hormesis in longevity and heat stress resistance. However, the results also suggest that other processes are involved and that different mechanisms might have marked gender specific impact.

Introduction

The hormesis phenomenon where a mild exposure to an otherwise detrimental stress factor becomes beneficial is well known for many organisms and life history traits (Le Bourg and Minois, 1997; Minois, 2000; Parsons, 2000; Cypser et al., 2006). Mild stress treatments that have been shown to induce hormesis include heat, radiation, hypergravity and starvation (Rattan, 1998; Minois, 2000; Le Bourg et al., 2001; Hercus et al., 2003). Exposure to mild stress at a young age has been shown to increase resistance to severe stress exposure later in life, delay behavioural aging and increase longevity (Le Bourg and Minois, 1998; Hercus et al., 2003). This shows that a mild stress exposure has effects, which last much longer than immediate stress induced plastic changes in protein and gene expression suggest (Dahlggaard et al., 1998; Sørensen et al., 2005). The hormetic effects on longevity have received special attention as these may have implications in medical research. The relative contribution of different mechanisms believed to promote hormesis under different environmental stresses conditions is not well understood. However, house keeping systems induced by environmental stress, including oxidative stress scavengers, DNA repair systems, the heat stress response and their regulators are among the prime candidates (Rattan, 2004; Cypser et al., 2006).

Some results suggest an important role for Hsps for heat induced hormesis (Tatar et al., 1997; Kristensen et al., 2003). Heat shock proteins (Hsps) are induced by a wide variety of stress types and are involved in repair of cellular damage (Feder and Hofmann, 1999; Sørensen et al., 2003) and might thus provide a general mechanism

of hormesis. Several studies have associated induction of Hsps and Hsp overexpression in transgenic lines with increased stress resistance and life span extension. For example, expression level of the heat shock protein, Hsp70, has been positively correlated with life span extension in transgenic overexpressing *Drosophila* (Tatar et al., 1997). Also, overexpression of Hsp26 and Hsp27 increased life span in *Drosophila* (Wang et al., 2004). However, another study has failed to find a hormetic induced life span extension due to Hsp70 overexpression using the same transgenic lines as Tatar *et al.* (1997) leaving doubt about the general role of inducible Hsps for hormesis (Minois et al., 2001). Thus, hormesis seems to be a complex phenomenon that probably is the sum of multiple mechanisms, with partially beneficial and partially detrimental effects but with an overall net positive effect with respect to the trait studied.

Here, we tested the effect of the heat shock response for mild heat induced hormesis in *Drosophila melanogaster*. To isolate and investigate the effect of inducible Hsps we used a set of mutant stocks where one stock harbours a mutation giving rise to a heat sensitive heat shock transcription factor (Hsf) that becomes inactivated when flies are exposed to temperatures above 30°C. The other stock is derived from the previous but has been modified with an additional functional *hsf* gene (Jedlicka et al., 1997). We measured longevity, heat resistance and Hsp70 expression level in controls and mild heat treated flies from the two mutant stocks described above. The hypothesis tested was that induction of Hsps is mediating hormesis. Thus, the Hsf deficient stock would benefit only little, if at all, from the early mild heat treatments

as this treatment induces some damage but no or only little Hsps (net benefit small or even negative). The rescued mutant stock would be able to induce Hsps upon heat shock and thus benefits from Hsp expression may clearly outweigh the damage (net benefit presumed positive and possibly large). Hsp70 was measured, partly as a control for the Hsf sensitive and rescued mutant phenotypes with regard to induction of Hsps and partly to examine if the early mild heat treatment manifested itself as changes in Hsp70 expression later in life.

Materials and Methods

Origin and maintenance of experimental flies

The experiments were performed on two lines of *D. melanogaster* originally created by Jedlicka et al. (1997) and obtained from the Bloomington stock Center. One line (Hsf⁰, stock number 5489) is a heat sensitive mutant line where the heat shock factor (Hsf) becomes non-functional at temperatures above 30°C (Hsf⁴ in Jedlicka et al. 1997) and is additionally marked with *cn*¹ and *bw*¹. This line has a very limited induction of heat shock proteins (Hsps) associated with the heat shock response (Nielsen et al., 2005). The other line (Hsf⁺, stock number 5490) was derived from the Hsf heat sensitive line but has a functional *hsf* gene inserted and these flies are able to induce heat shock proteins upon heat shock exposure. The insertion is maintained against a balancer chromosome, therefore the genetic background of the lines is slightly different for the third chromosome (see Jedlicka et al., 1997 for details). Both lines were maintained under standard laboratory conditions at 25°C on agar, sugar,

yeast and oatmeal medium, 12/12 h light/dark cycle. Flies were bred in 200 ml culture bottles with 36 ml of medium and 30 pairs per bottle laying eggs for 24 h.

Experimental protocols

Flies (0-24 hrs) were collected from the bottles and separated into males and females under CO₂ anaesthesia (hereafter referred to as 'age 0' of the experiment). The flies were transferred to vials with 10 flies in each vial. The flies were maintained at standard laboratory conditions and were transferred to fresh vials three times weekly (every second or third day).

Early stress treatment

Half the collected flies were exposed to mild heat stress three times during early life, on days 3, 6 and 9 of the experiment. This group of flies was denoted 'early stress'. During the treatment flies were transferred to new vials with fresh food and exposed to 34°C for 3h in a pre-heated waterbath. Hereafter, vials were returned to 25°C. This treatment did not lead to mortality and is adequate to induce the stress response in wildtype *D. melanogaster* (around 30% of maximal Hsp70 expression (Hercus et al., 2003)). The other half of the flies denoted 'no stress flies' were kept at standard laboratory conditions.

Longevity

We measured longevity in 10 replicate vials of 10 flies per line, sex and treatment (no stress or early stress). At each transfer the number of dead flies in each vial was

recorded until all flies had died. The hormetic effect of early mild stress on life span was re-investigated following the original experiment to verify the results. This second experiment followed the same protocol as the original experiment with a few exceptions. For the replication of the longevity part of the experiment flies were bred on instant *Drosophila* medium for 2 generations (Carolina Biological Supply, Burlington, NC, USA) and scored for mortality and changed to fresh vials strictly every 2 days (in the first run flies were not scored during weekends).

Heat resistance

A heat coma assay (see Kellett et al., 2005 for details) was used when assessing flies for heat resistance. Flies were placed individually in capped empty 10 ml vials. All vials were immersed in a $38.3 \pm 0.1^\circ\text{C}$ circulating waterbath with a glass front to allow flies to be scored as they were inactivated. The time for the fly to be rendered completely inactive by heat stress was recorded as its 'heat coma time'. Heat resistance was measured at age 12 and 24 days. We assayed 20 flies from each line, sex and treatment on each of the 2 ages.

Hsp70 expression level

Two sets of flies were prepared for analysis of Hsp70 expression level at intermediate and older age (12 and 24 days, respectively). One set was exposed to a heat shock of 36°C for 1 h and the other set was taken directly from 25°C . Both sets were snap frozen in liquid nitrogen and stored at -80°C in centrifuge tubes until analysis. For the measurement of inducible Hsp70, flies were homogenized and the Hsp70 level was

determined by the ELISA technique utilizing the antibody 7FB, which is specific for the inducible Hsp70 in *D. melanogaster* (Welte et al., 1993). Each sample consisted of a vial of flies (7-10 flies). For samples heat shocked prior to freezing we measured the Hsp70 expression level in three replicate samples. Due to a higher than expected mortality during the experiment we were left with fewer flies than expected. Thus, we prioritized to use the available flies for a full design of heat resistance and heat induced Hsp70. Therefore, we only had 2 replicates for testing Hsp70 levels in flies taken directly from 25°C (where no inducible Hsp70 was expected). The assays were run on 96 micro-well ELISA plates with one replicate of all treatments measured in quadruplicate on each plate. The grand mean of each replicate (plate) was normalized to the grand mean of replicate (plate) 1 in each set. The ELISA protocol used is described in detail in Sørensen et al. (1999).

Statistics

Data for heat resistance, longevity and Hsp70 expression level were analyzed with ANOVA with Line, Sex and Treatment as fixed factors. All interactions were included in the model. Individual contrasts between early heat treated and control groups were done by t-tests. The software package SPSS 13.0 was used for all analyses (SPSS, 2004).

Results

Longevity

Mean longevity (\pm SEM) for the two replications of the longevity experiments are presented in Table 1 and mortality curves in Figure 1 (second experiment). ANOVA analysis of the first longevity experiment showed significant effects of Line, Sex, the Line x Sex and the Sex x Treatment interactions (Table 2). Males generally had lower longevity than females. In males, both groups of Hsf⁰ flies and the untreated group of Hsf⁺ flies all had similar longevity, while mildly heat stress treated Hsf⁺ flies had increased longevity. In the individual comparisons of the treatment effect only Hsf⁺ males showed an effect of treatment ($t=3.65$, $P<0.01$). In females, the Hsf⁰ flies seemed to live a little longer than Hsf⁺ flies after mild heat stress treatment. When tested individually only Hsf⁺ males showed a significant difference between treatments.

The repeat of the longevity experiment verified the result from the first experiment. ANOVA analysis showed results very similar to the original experiment with significant effects of Line, Sex, and the Sex x Treatment interactions (Table 2). Again, females of both lines showed a tendency towards decreased longevity when stressed as young adults and only males of the Hsf⁺ line showed increased longevity. Like in the first replicate of the longevity experiment the effect of early mild stress was only significant in Hsf⁺ males in an individual comparison ($t=2.14$, $P<0.05$).

Heat resistance

We measured heat resistance in 12 and 24 day old flies (Figure 2). In 12 day old flies the ANOVA showed significant effects of Sex, Line, Treatment and the Line x

Treatment interaction (see Table 2). Males had higher heat resistance relative to females and, in untreated flies, the Hsf⁺ line had higher heat resistance than the Hsf⁰ line in females only. Hsf⁺ males treated with mild heat stress as young had nearly twice as high heat resistance compared to non-treated males. Hsf⁺ and Hsf⁰ females did not differ in their level of heat resistance. Heat resistance of early mild stressed and non-stressed males and females from the Hsf⁰ line was not significantly different. The treatment effect (mild stress vs. no stress) in males was the only significant contrast ($t=2.83$, $P<0.01$). For flies tested at age 24 days the ANOVA showed significant effects on Line and Line x Sex interaction (see Table 2). In non-treated flies, the Hsf⁺ flies had higher heat resistance than the Hsf⁰ flies, and as found on day 12 especially so in females. Mildly heat treated Hsf⁺ females seemed to have slightly lower heat resistance than non treated flies giving rise to the significant interaction term as Hsf⁺ males still had higher heat resistance at this age. The relative higher heat resistance in mildly heat treated Hsf⁺ males was lower at day 24 than at day 12 but still significant in a t-test, as the only contrast ($t=2.28$, $P<0.05$).

Hsp70 expression

The expression level of Hsp70 was measured on days 12 and 24 of the experiment. One set of flies was exposed to an Hsp70 inducing heat stress, while another set was taken from control conditions and directly frozen for analysis of basal expression level of Hsp70 (Table 2). Without stress there was a slightly higher expression level in males giving rise to a significant effect of Sex on day 12, however, this was not significant on day 24 and there were no other significant effects on day 24. Treatment

with mild heat stress in early life did therefore not manifest itself in any differences in basal expression level of Hsp70.

Hsp70 expression after heat induction at 36°C for 1h on day 12 showed significant effects of Line, Sex and the Line x Sex interaction (Table 2, Figure 3a). Both sexes of the Hsf⁰ line showed expression levels similar to the level of non heat treated flies regardless of treatment. In the Hsf⁺ line females had increased expression levels compared to males. In males, the expression level of heat induced Hsp70 was little affected by a mild heat treatment in early life. Females seemed to show a slight, but non-significant, increase in expression after heat induction of Hsp70 expression. However, no individual t-tests showed significant differences. On day 24 the overall pattern was the same as on day 12 (Table 2, Figure 3b). Here, the ANOVA showed significant effects of Line and Treatment, with low expression level in Hsf⁰ flies and no differences between sexes or treatments. Females from the Hsf⁺ line expressed more than males but the sex effect was not significant on day 24. In both sexes the mildly heat treated flies expressed more Hsp70 leading to the significant treatment term and this difference was also significant in males when tested directly using a t-test ($t=4.44$, $P<0.05$), while the other comparisons were not significant.

Discussion

We found a marked interaction between gender and treatment, with the hormetic effect on longevity being restricted to males of the rescued line (Hsf⁺). Several studies

reported a marked sex difference with the hormetic effect of e.g. exposure to heat and hypergravity restricted to the male gender (Le Bourg et al., 2000; Minois, 2000; Le Bourg et al., 2002). However, Hercus *et al.* (2003) did find heat induced hormesis for life span in female *D. melanogaster* suggesting that the phenomenon exists in both sexes, but that females have a different sensitivity and/or that hormesis is induced by partly different mechanisms in the two sexes. Here, females showed a reduced longevity after early stress treatment in both lines suggesting that females indeed are more sensitive (i.e. the net effect of the early stress exposures was deleterious) and that this higher sensitivity is unrelated to the ability to induce Hsps.

Heat resistance was increased in males when exposed to mild heat stress early in life, if they were able to express a functional Hsf at high temperature, i.e. in the Hsf⁺ line. The effect of early stress treatment was long lasting and found still in 24 day old male flies, although the hormetic increase in heat resistance was more pronounced in 12 day old male flies. Females only showed a slight increase in resistance at day 12. This clearly shows the importance of the stress response for long lasting inducible heat resistance, at least in males. Hsf⁰ flies showed no benefit from mild heat stress. Another interesting sex specific pattern was observed. Early mild stress induced increased heat resistance in males but with no apparent difference between the two lines when untreated. Conversely, in females the Hsf⁺ line had increased heat resistance but no indication of an effect of early mild stress. Thus, the dynamics of the induced effects are very different in the two sexes, and might shed light on the numerous sex effects found in *Drosophila*.

Several studies indicate that long lived individuals are also often more stress resistant (Vermeulen and Loeschcke, 2007). Our results support this idea of a correlation between stress resistance and longevity. Heat stress resistance and resistance to other types of environmental stress have been shown to be correlated with longevity in a number of studies (e.g. Hoffmann and Parsons, 1993; Norry and Loeschcke, 2003) and could be caused by the existence of a general stress resistance mechanism (see Vermeulen and Loeschcke, 2007 for review). The expression of Hsps might be one such system that upon activation can increase both longevity and resistance to several types of stress. However, the correlation between life span and resistance is not always detected (Phelan et al., 2003) and lines selected for long life are not invariably more stress resistant (Bubliy and Loeschcke, 2005). Therefore, it seems likely that the genetic architecture of longevity is complex and that the Hsf/Hsp system is one among several systems that can lead to a long life.

The basal expression level of Hsp70 was not affected by heat stress exposure early in life. At the age of 12 days no difference was observed between early and no stress treatments. However, when re-exposed to heat at the age of 24 days Hsp70 level was higher in early stressed male flies compared to controls. Similar effects of early mild stress on later responses to heat was found by Kristensen et al. (2003). Thus, males might show hormesis in longevity and heat stress resistance because they are able to respond faster to the stress by inducing Hsps (Kristensen et al., 2003). However, a difference in the rate of ageing between the two treatments might be the primary

effect leading to these results. This could be interpreted as a higher flexibility in plastic responses when a stress early in life prepares for responding to stress later in life. However, at 12 days of age when no difference in Hsp level was observed the difference in heat resistance was at the highest. Thus, no simple relationship is found between Hsp70 expression level, heat resistance and performance at old age (hormesis). Longevity was shorter for males relative to females and therefore we cannot rule out that selection influenced on the results. However, this does not affect the results of the within gender comparisons.

Hormesis has been found across species and is induced by multiple mild stress factors. Still, some studies fail to detect hormesis even when using similar stress factors and treatments as the studies reporting hormesis. Even though inducible Hsps are prime candidates for contributing to the phenomenon of heat induced hormesis, the experimental evidence is scarce. Moreover, disparate results have been obtained in the studies performed (see e.g. Tatar et al., 1997; Minois et al., 2001; Wang et al., 2004). These inconsistent results might be explained if hormetic effects are only seen when the treatment is strong enough to induce stress repair systems and at the same time only leads to relatively little damage. The balance between beneficial and detrimental effects should be in favour of the beneficial ones in order to obtain a hormetic effect, and the net balance is expected to be genotype and environment dependent. The parameter width (e.g. temperature) giving rise to a net positive effect in a specific environment for a given genotype may be quite narrow and no or even negative net effects could be expected to occur frequently. Furthermore, the

expression level of Hsps is thought to be tightly regulated to avoid the associated costs of expression (Sørensen et al., 2003). Thus, the approach used here by taking advantage of deficiency instead of overexpression of candidate genes and proteins is a good supplement to other studies. Moreover, this study, by targeting the heat shock factor, investigated a suit of inducible chaperones instead of single proteins. As this study was investigating only one genetic background more studies are needed to verify the generality of the results. The life span observed in the longevity experiments here is short compared to common life span of wild type *D. melanogaster*. This probably reflects that the lines examined are inbred and furthermore genetically engineered, which might affect their general performance across many traits. In the second longevity experiment we raised the flies for two generation on a more nutritious medium (Carolina instant medium) prior to the experiment. While no effect was observed in the Hsf⁰ line it might explain the increased longevity of the Hsf⁺ line in experiment two.

However, even if absolute life spans were different among the two repeats of the longevity experiment, the effects of early stress treatment was qualitatively similar and the results seem to be robust. Thus, the results indicate that heat inducible Hsps, being the transcriptional targets of Hsf, are indeed important for heat induced hormesis in longevity and heat stress resistance, however, the results also support that other factors are involved and that the involved mechanisms might contribute differently in the two sexes. The dissimilar hormetic effects in males and females probably is explained partly by the difference in response to Hsp deficiency.

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Legends

Figure 1. Survival curves for the second longevity experiment of males (a) and females (b). Circles represent flies from the Hsf^0 line, which has a heat sensitive Hsf mutation and the corresponding rescued control, Hsf^+ , is represented by triangles. Filled symbols represent flies that were not mildly heat treated as young adults while open symbols represent flies that were mildly heat treated at days 3, 6 and 9 after emergence.

Figure 2. Heat resistance (\pm SEM) of 12 days old flies (a) or 24 days old flies (b). Flies were exposed to 38.3°C and time until total immobilization was recorded. Hsf^0 is the heat sensitive Hsf mutant and Hsf^+ is the rescued control. Light grey bars represent males (M) and dark grey bars females (F). Striped bars represent flies that did not receive a mild heat treatment as young adults (-) while open bars represent flies that were mildly heat treated at age 3, 6 and 9 days after emergence (+). Stars represent significant differences between non- and mildly heat treated flies (t-test, *: $P < 0.05$; **: $P < 0.01$).

Figure 3. Expression level of Hsp70 after a 1 h exposure to 36°C (\pm SEM) in Hsf^0 flies, which has a heat sensitive Hsf mutation and the corresponding rescued control, Hsf^+ . Samples were taken of 12 (a) or 24 (b) days old flies. Light grey bars represent males (M) and dark grey bars females (F). Striped bars represent flies that were not mildly heat treated as young adults (-) while open bars represent flies that were mildly heat treated at days 3, 6 and 9 after emergence (+). The star represents significant differences between non- and mildly heat treated flies (t-test, *: $P < 0.05$).

Table 1. Longevity in days (\pm SEM) in the two longevity experiments. Hsf^0 is the heat sensitive Hsf mutant and Hsf^+ is the rescued control. F denotes females and M denotes males. Flies that did not receive a mild heat treatment as young adults are marked “-“ while flies that were mildly heat treated at days 3, 6 and 9 after emergence are marked with “+”.

| | Hsf^0 M- | Hsf^0 M+ | Hsf^+ M- | Hsf^+ M+ | Hsf^0 F- | Hsf^0 F+ | Hsf^+ F- | Hsf^+ F+ |
|--------------|------------|------------|------------|------------|------------|------------|------------|------------|
| Experiment 1 | 21.5 (1.1) | 22.0 (1.2) | 21.1 (1.0) | 25.8 (0.7) | 38.4 (1.5) | 36.7 (1.4) | 30.1 (1.7) | 26.3 (1.4) |
| Experiment 2 | 21.7 (1.3) | 22.0 (0.6) | 30.6 (1.0) | 34.0 (1.2) | 28.7 (1.8) | 25.9 (2.2) | 38.7(1.7) | 36.2 (1.9) |

Table 2. Results of ANOVA analysis of longevity (two experiments), heat resistance at days 12 and 24 (HS 12, HS 24), Hsp70 expression after heat exposure at 36°C at days 12 and 24 (Hsp70 D12+, Hsp70 D24+) and Hsp70 expression without heat exposure at days 12 and 24 (Hsp70 D12-, Hsp70 D24-).

| Source | Mean squares (df) | | | | | | | |
|----------------------------|-------------------|-------------|----------|---------|------------|------------|-------------|-------------|
| | Longevity 1 | Longevity 2 | HS 12 | HS24 | Hsp70 D12+ | Hsp70 D24+ | Hsp70 D12- | Hsp70 D24- |
| Line (1) | 190*** | 2129*** | 1499 *** | 4316*** | 4.763 *** | 5.425 *** | 4.1E-05 | 2.1E-06 |
| Sex (1) | 2440*** | 566*** | 598 ** | 85 | 0.063 * | 0.045 | 0.0001 * | 2.4E-05 |
| Treatment (1) | 9 | 4 | 452* | 53 | 0.003 | 0.068 * | 7.1E-08 | 0.0001 |
| Line x Sex (1) | 459 *** | 1 | 11 | 1081*** | 0.082 * | 0.052 | 2.4E-08 | 8.2E-07 |
| Line x Treatment (1) | 32 | 15 | 699 ** | 1 | 0.013 | 0.055 | 1.38E-06 | 4.2E-05 |
| Sex x Treatment (1) | 74 * | 99* | 282 | 129 | 0.031 | 0.005 | 1.39E-05 | 4.1E-05 |
| Line x Sex x Treatment (1) | 13 | 10 | 128 | 353 | 0.016 | 0.003 | 2.2E-06 | 9.4E-05 |
| Error | 17 (72) | 24 (72) | 73 (152) | 68(152) | 0.014 (16) | 0.013 (16) | 1.2E-05 (8) | 9.6E-05 (8) |

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

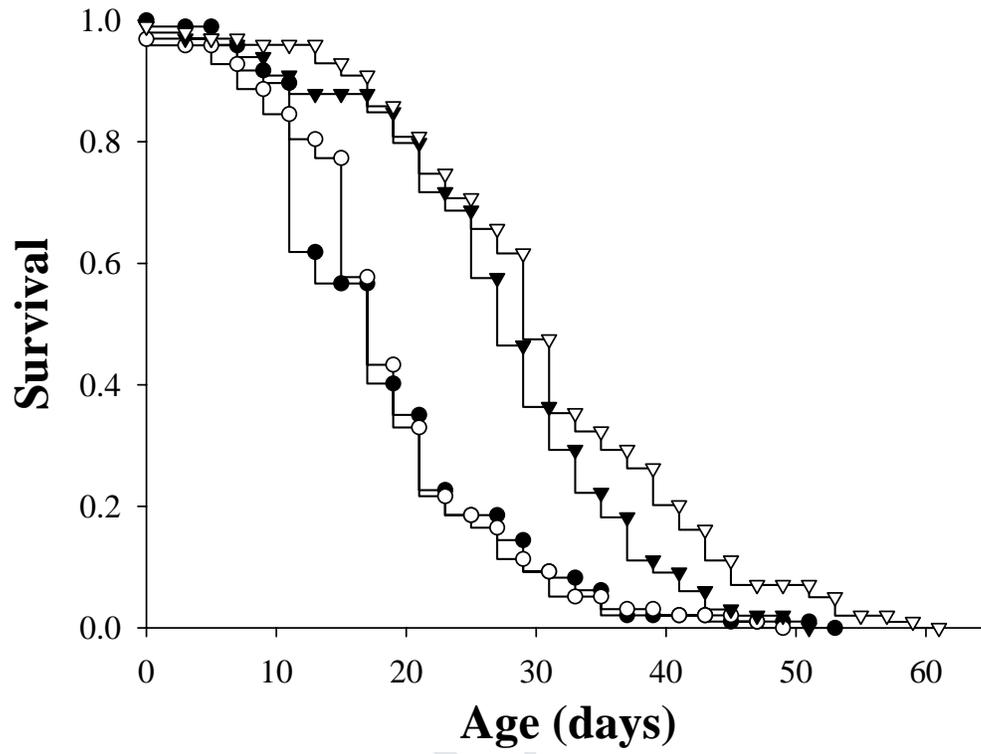


Figure 1a.

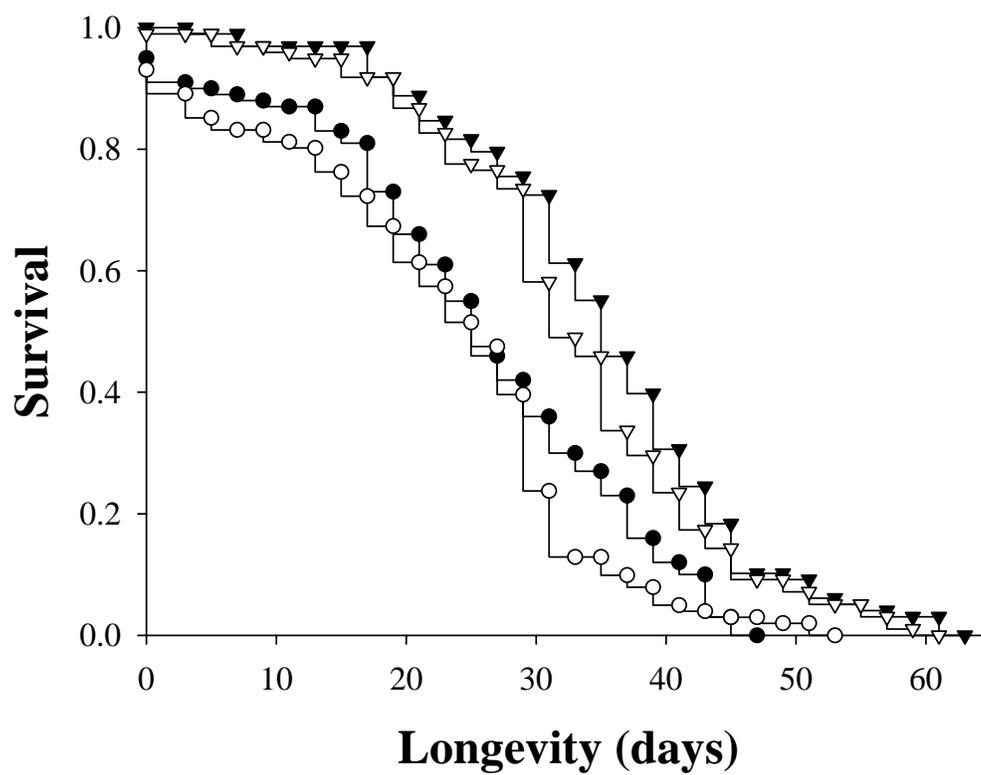


Figure 1b.

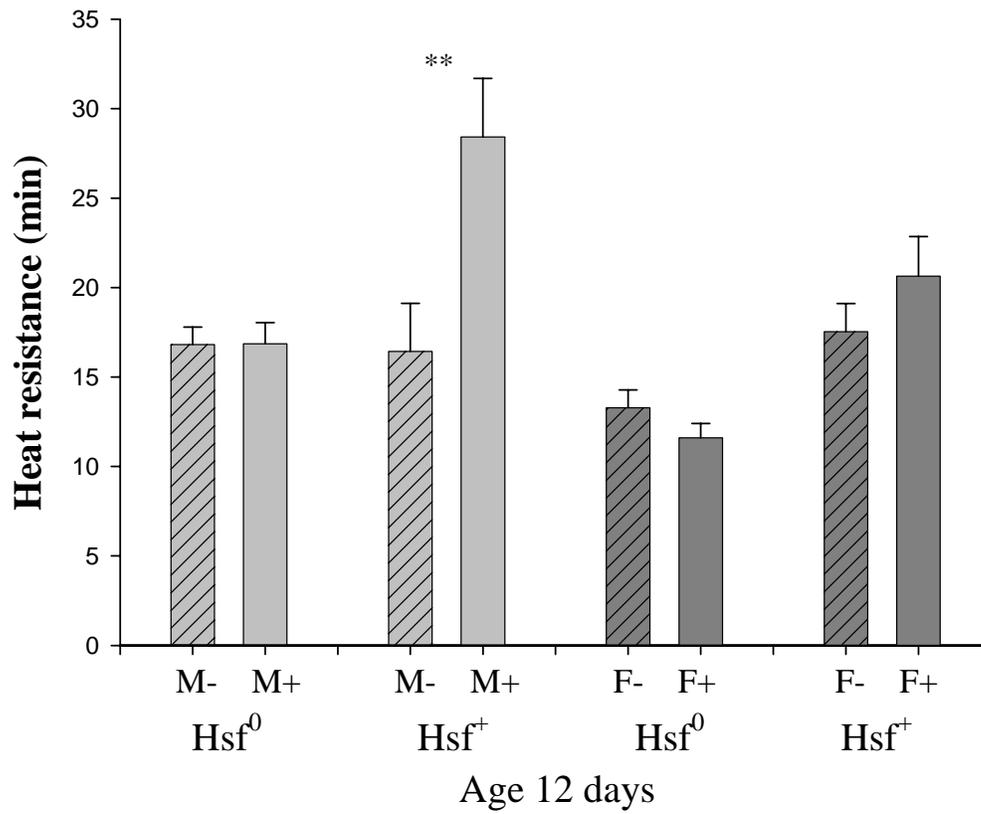


Figure 2a.

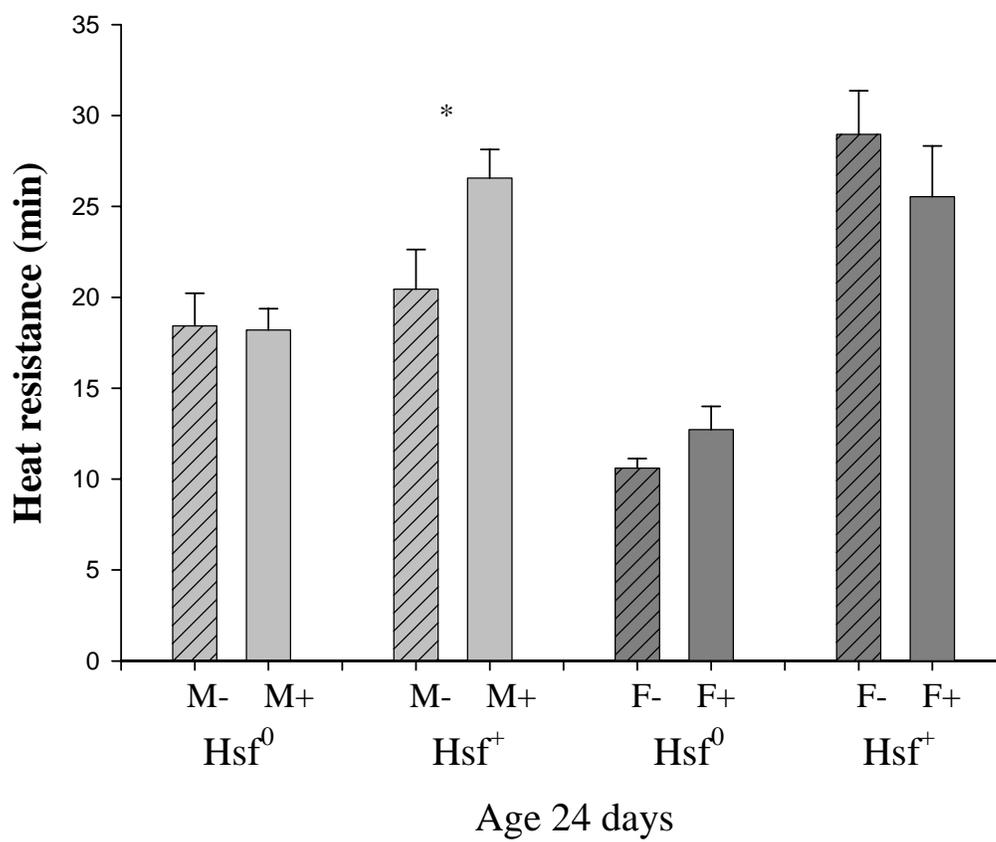


Figure 2b.

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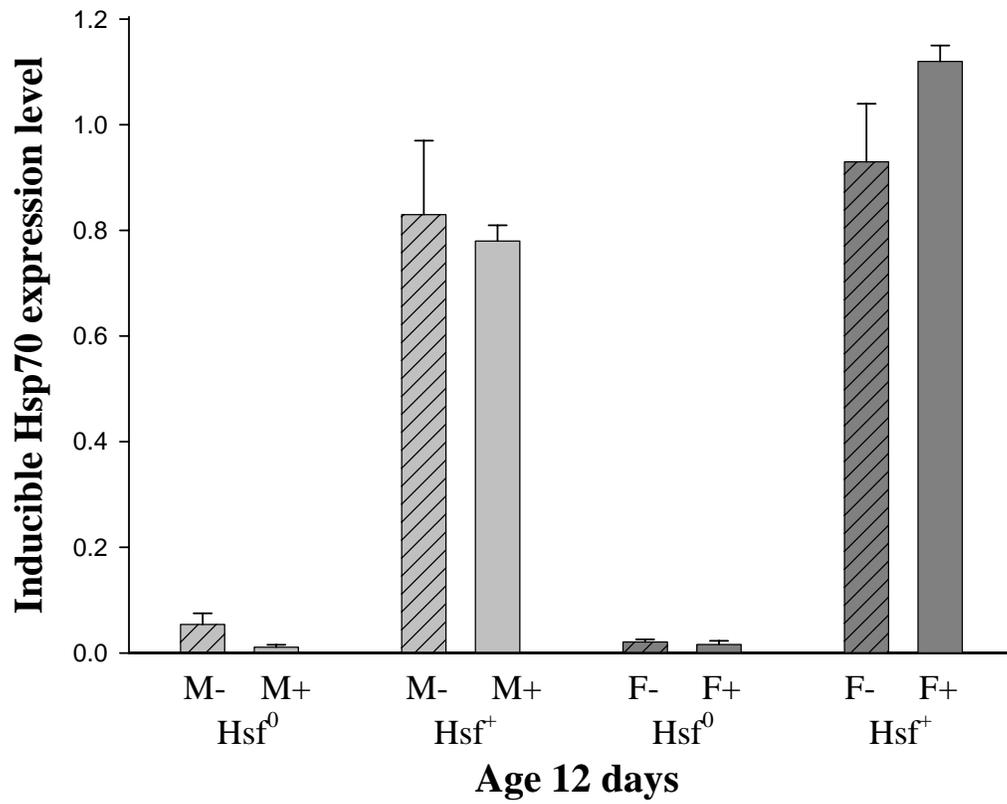


Figure 3a.

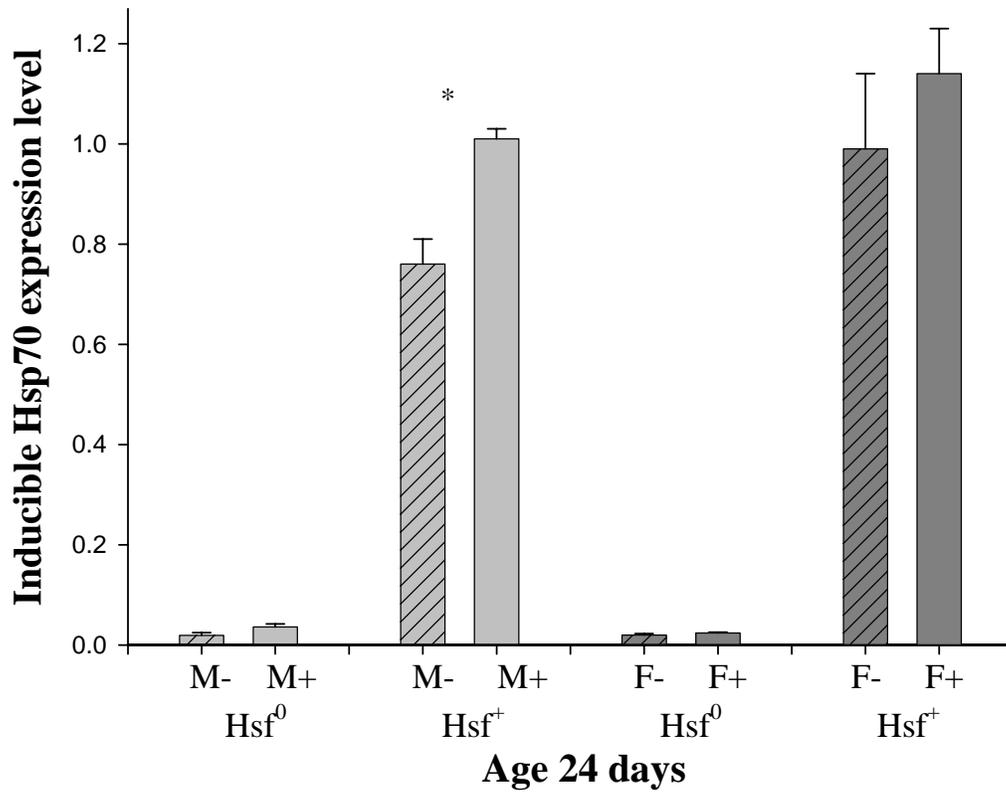


Figure 3b.

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