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Deleterious consequences of Hsp70 overexpression in *Drosophila melanogaster* larvae

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Abstract We compared transgenic *Drosophila* larvae varying in *hsp70* copy number to assess the consequences of Hsp70 overexpression for growth and development after heat shock. Exposure to a mildly elevated temperature (36°C) induced expression of Hsp70 (and presumably other heat shock proteins) and improved tolerance of more severe heat stress, 38.5–39.5°C. We examined this pattern in two independently derived pairs of extra-copy and excision strains that differed primarily in *hsp70* copy number (with 22 and 10 copies, respectively). Extra-copy larvae produced more Hsp70 in response to high temperature than did excision larvae, but surpassed the excision strain in survival only immediately after thermal stress. Excision larvae survived to adulthood at higher proportions than did extra-copy larvae and grew more rapidly after thermal stress. Furthermore, multiple pretreatment reduced survival of 1st-instar extra-copy larvae, but did not affect the corresponding excision strain. While extra Hsp70 provides additional protection against the immediate damage from heat stress, abnormally high concentrations can decrease growth, development and survival to adulthood.

INTRODUCTION

Most recent biochemical and physiological studies of heat shock proteins and molecular chaperones have focused on the benefits these proteins confer in their interaction with unfolded proteins (Morimoto et al 1994; Hartl 1996). Heat shock proteins (Hsps), however, could be deleterious to the cell in at least three ways. First, during or after stress Hsps abound within the cell, and their synthesis, maintenance, and degradation could consume substantial fractions of the cell's energy and amino acid stores and preoccupy its protein synthetic machinery (Coleman et al 1995). In support of this mechanism, Heckathorne et al (1996a,b) reported that at least two enzymes normally in high concentrations in plants, ribulose 1–5 bisphosphate carboxylase/oxygenase and phosphoenolpyruvate carboxylase, were depleted to supply nitrogen for Hsp synthesis, and that this catabolism was enhanced in plants grown in nitrogen-poor soil. Second, in some species, such as *Drosophila melanogaster*, the heat-shock response is associated with the depression of background protein synthesis (DiDomenico et al 1982), which could deprive the cell of critical proteins. Finally, Hsps could directly interfere with the synthesis, folding, and degradation of other cellular proteins by binding them inappropriately (Lindquist 1993).

Several lines of evidence are consistent with deleterious consequences of Hsp expression. The heat shock response is tightly autoregulated and is strongly repressed in the absence of stress, as if to avoid overexpression or ectopic expression (Lindquist 1993). Similarly, the heat shock response is reduced or absent early in ontogeny (Dura 1981; Heikkila et al 1985; Muller et al 1985). Experimental demonstrations of disadvantages of Hsp expression are few: Conditions known to induce Hsp synthesis reduce fecundity in female *Drosophila* (Krebs and Loeschcke 1994), specific induction of Hsp70 reduces the proliferation of *Drosophila* cells in culture (Feder et al 1992), and an *hsp104* deletion mutant of yeast (*Saccharomyces cerevisiae*) grows faster than wild type on some media (Sanchez et al 1992). Within the cell,

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overexpression of the Hsp70 family member, *grp78*, specifically inhibits the secretion of proteins in the *grp78*-dependent secretory pathway (but not other secreted proteins), and reduction in *grp78* enhances secretion (Dorner et al 1988, 1992). Hsc70 actually promotes protein aggregation when present in excess (M. Borrelli and J. Lepock, personal communication).

Ordinarily, normal regulatory mechanisms may prevent Hsps from reaching levels at which deleterious effects become obvious, and thereby hinder investigations of these effects. To overcome this impediment, we have investigated whole *Drosophila* transformed with 12 extra copies of the *hsp70* gene at the 87C7 locus (Welte et al 1993), which supplements the 10–12 natural copies of the gene (Ish-Horowicz et al 1979). The same process that creates this 'extra-copy' strain simultaneously creates an 'excision' strain, which has an identical site of transgene integration, but lacks the extra copies of *hsp70,* and produces normal levels of Hsp70 (Welte et al 1993). By examining two such pairs of strains that differ in sites of transgene integration, effects potentially attributable to either the length of the insertion or to minor differences in genetic background may be separated from those specific to the presence or absence of the transgenic sequence.

Previous work has documented several advantages of extra *hsp70* copies in terms of enhanced inducible thermotolerance in both cells in culture (Solomon et al 1991) and in whole *Drosophila* (Welte et al 1993; Feder et al 1996). The present study focuses on instances in which extra *hsp70* copies compromise survival and growth, both after mild and severe heat shock and in the absence of thermal stress.

METHODS

Origin of strains

We examined two independently derived pairs of extra-copy and excision strains, with the sites of transgene integration mapping to chromosomes II and III, respectively. Chromosome numbers (II or III) are henceforth used to designate particular pairs of strains. Welte et al (1993) described the construction of these strains via unequal homologous recombination. Each extra-copy strain is homologous for a transgene construct including three *hsp70* genes and an eye-color marker, w^{hs}, all flanked by yeast recombination targets and P-elements. Each corresponding excision strain shares the same chromosomal site of transgene integration and flanking elements, but lacks the *hsp70* transgenes and eye-color marker.

Most experiments examined the chromosome III pair of extra-copy and excision strains. In theory, because each excision strain shares the site of transgene integration with its sister extra-copy strain, and possesses a similar genetic background, differences between each excision and extra-copy pair should be primarily attributable to *hsp70* copy number. In rare cases, a difference in the length of DNA between the P-element sequences can itself result in a phenotype due to unusual genetic circumstances (Stearns and Kaiser 1996); however, the likelihood that two independent chromosomal interruptions would yield the same phenotype in relation to the size of the transgene insert is negligibly small (Welte et al 1993). For this reason, we repeated our studies with the chromosome II pair of extra-copy and excision strains in selected instances.

Larval handling

Newly hatched larvae were collected directly from the surface of the egg-laying substrate (a yeast–cornmeal –molasses–agar medium sprinkled with live yeast) using dissecting needles. Third-instar larvae were first separated from the medium in 3 mol/l NaCl, and diluted until the medium sank, with larvae remaining on the surface (Ashburner 1989). These larvae were poured to filter paper and transferred as necessary with a moistened paint brush.

Choosing treatments

Experimental temperatures for the functional analysis of extra *hsp70* copies were determined by seeding sets of 40 1st-instar excision III larvae to vials containing medium, and these vials were submerged in high-temperature water baths within 2 h of larval transfer. Figure 1 depicts temperature treatments. Controls (25°C) were run with each set of high-temperature treatments to account for possible variation in collection and handling techniques, and after the stress exposure, all vials were placed at 25°C. From these determinations (Fig. 1), we chose 36°C as the pretreatment temperature; i.e. a temperature known to induce heat shock protein expression (Feder et al 1996), but not in itself kill larvae. We chose 38.5°C as a heat shock temperature for inflicting substantial mortality, unless otherwise specified. Typical experimental designs involved exposure to this heat shock for 1 h with or without prior pretreatment, which was 1 h at 36°C and 1 h at 25°C.

Hsp70 expression

To document Hsp70 levels in 1st-instar larvae, 40–60 recently hatched (0- to 2-h-old) larvae were collected onto small petri dishes (3 cm diameter) of yeast–cornmeal –molasses–agar medium. These dishes were wrapped in Parafilm® and exposed to a designated temperature

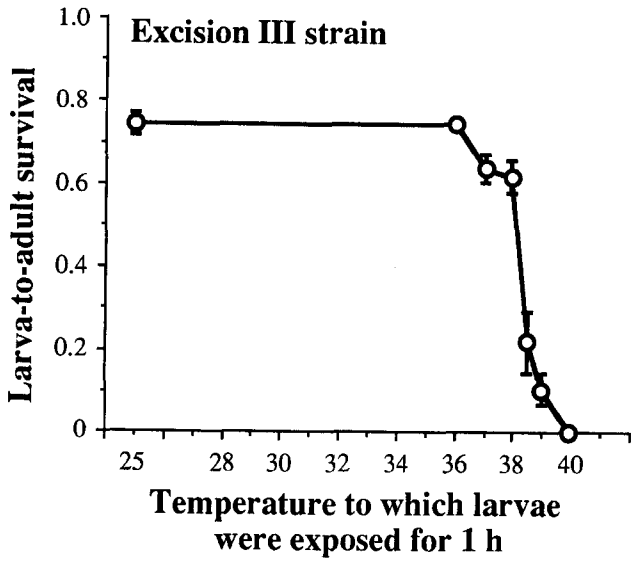

Fig. 1 First-instar larva-to-adult survival for individuals of the excision III strain following direct exposure without pretreatment at various temperatures. After treatment, larvae developed at 25°C. Symbols are means ± 1 SE.

treatment in a thermostatted water bath. Treatments varied in the presence/absence of pretreatment, heat shock temperature (38.5°C, 39.5°C), and time at 25°C following heat shock or pretreatment. After treatment, 35–40 larvae were rapidly transferred to a 1.5 ml cryotube, which was immersed in liquid nitrogen. Third-instar larvae for each strain were similarly handled, except that only four of these larger individuals were necessary per replicate. Any larvae that died during treatment were discarded.

Methods for measurement of Hsp70, modified from Welte et al (1993), are presented in Feder et al (1996) and Krebs and Feder (1997). In summary, larvae were lysed via sonication, centrifuged, and protein content of the supernatant was determined (BCA Assay, Pierce Biochemicals). Hsp70 concentrations in whole animal lysates were estimated by an enzyme-linked immunosorbent assay (ELISA) in 96 well microplates using the primary antibody, 7.FB, which is specific for the heat inducible Hsp70 family member of *D. melanogaster*. Although the method cannot discriminate between a decline of Hsp70 levels due to degredation and sequestration of Hsp70 in a complex that masks its antigenic determinant, different methods of removal should similarly affect cell physiology. Each datum was the mean signal from 4 wells with the primary antibody present minus the mean of 3 wells where primary was omitted to allow correction for non-specific signal. The corrected signal has been shown to be a linear function of the quantity of Hsp70 in the lysate over a very broad range (Welte et al 1993). Concentrations were expressed as a percentage of the signal by an Hsp70 standard, which was prepared in bulk from *Drosophila* Schneider 2 cells heated to 36.5°C for 1 h and subsequently placed 1 h at 25°C. Each assay included two replicates of this Hsp70 standard.

Larval survival

We examined survival of larvae to multiple developmental time-points, and used several assays of survival as appropriate for each. To examine 1st or 3rd-instar larva-to-adult survival, 40 excision or extra-copy larvae of the desired age were seeded to vials that contained 7 ml of medium. At least 10 vials for each strain and age were exposed to each of the same temperature regimes used to characterize Hsp70 levels: pretreatment only (36°C for 1 h), heat shock only (38.5°C for 1 h), pretreatment and heat shock (36°C 1h, 25°C 1 h and then 38.5°C for 1 h), with controls left at 25°C. During treatment, vials were spaced to facilitate uniform heating within the water baths. Vials were then placed at 25°C, and adult flies counted as they emerged.

To determine age-specific mortality, 40 1st-instar larvae were seeded to each of 20 replicate petri dishes per strain (prepared as for Hsp70 samples) within each of five blocks of these replicates. After completing all temperature treatments, the Parafilm® was removed. On each of the following 5 days, surviving larvae of each strain were counted in one dish per treatment, and then discarded. Where necessary, the census was aided by slowly heating a dish from the bottom, which induced larvae to crawl to the surface. No larvae could be found thereafter. As a dish of 40 larvae provided only one count, each datum was independent.

In separate sets of replicates, larval survival was scored 0–1 h after stress for 1st-instar larvae. Larvae were judged to be alive if they responded to gentle prodding. Similar treatment of control dishes at constant 25°C established rates of death due to handling and the proportion of larvae recoverable. The number of live and dead larvae were scored, from which the proportion escaping detection was obtained. At 36°C, some larvae responded by exiting the petri dish or moving to the lid, and they would have been scored as dead in analyses where only those individuals still alive were counted. At 38.5°C, larvae quickly became immobile, and all those that died from the heat stress were scored. Therefore, in the age-specific mortality experiment, larval survival in the pretreatment only and pretreatment plus heat shock groups was adjusted for the proportion of the 40 larvae whose fate could not be determined.

Larvae at 3rd-instar were similarly handled, and with fewer developmental time points before pupation, larvae

were collected together for thermotolerance measurements immediately after stress and for those at 24 h and 48 h after stress. By 48 h, many control larvae pupate, and pupae are difficult to score as alive or dead. However, as rapid growth was required to pupate within the 48 h time period for 3rd-instar larvae or 120 h for 1st-instar larvae, and very few heat-shocked larvae grew fast enough to pupate in this time, pupae were scored as alive.

Larval growth after heat stress

Larval growth was estimated by measuring total larval protein in pooled sets of 1st-instar larvae of the chromosome III lines. One or 3 days following treatment in petri dishes, as above, a sample of surviving larvae was collected from each petri dish and was placed in 0.1 mol/l NaOH in an eppendorf tube: 200 µl for fifteen 24-h-old larvae or 400 µl for each of 2 tubes of five 72-h-old larvae. Larvae were subsequently frozen at -80°C overnight to fractionate cells, thawed, heated for 2 h at 80°C, and left to stand overnight. Freezing and heating were repeated for the larger larvae. Total protein content was measured by the BCA reaction against a BSA standard dissolved in 0.1 mol/l NaOH. Pilot experiments with both 1st and 3rd instar larvae indicated that dissolved protein is a linear function of larval numbers, and that 15 1st-instar and five 3rd-instar larvae contain protein quantities within the linear range of the protein assay. Therefore, by assuming that larger larvae contain more total protein, variation in size could be estimated reliably.

Effect of multiple pretreatment

First and 3rd-instar larvae of the excision III and extra-copy III strains were pretreated 0, 1, 2, or 3 times in yeast–cornmeal–molasses–agar medium. Larvae were handled in four experimental groups, which were composed of 40 larvae in each of 10 vials: (0) no treatment; (1) exposed to 36°C for 1 h at 2–4 h after hatching; (2) as for group 1, with another 36°C treatment 24 h after hatching; and (3) as for group 2, with a third treatment another 24 h later. Pretreatment of 3rd-instar larvae began immediately after collection for groups 1, 2 and 3, and followed at subsequent 18 h interval(s). The shorter time interval was necessary to complete all treatments before any larvae began to pupate. For pretreatments, vials were submerged in water baths, as above, with larvae developing to adulthood at 25°C. Because the medium, as well as larvae, receive the heat treatment, a separate experiment verified that repeated pretreatment of the medium itself had no effect on larvae. First-instar larva-to-adult survival was not significantly different in medium exposed to 36°C for 1 h 0, 1, 2 or 3 times prior to addition of larvae (data not shown).

The persistence of induced thermotolerance was examined in 3rd-instar larvae of the chromosome III lines. Forty 3rd-instar excision or extra-copy larvae were seeded to each of 10 vials per strain, half of which were pretreated shortly after collection. After 24 h at 25°C, all larvae were exposed to 38.5°C for 1 h, and returned to 25°C. For a separate set of 3rd-instar larvae, Hsp70 concentration was determined (as above). Larvae (72-h-old) either were frozen (at –80°C) immediately after exposure to 36°C for 1 h or were exposed to 36°C for 1 h and then held at 25°C for 24 h before freezing.

Statistics

For each experiment, significance of effects between strains and the various treatments was determined by fixed-effects factorial analyses of variance in Statview (Statview 1992). Effects of pretreatment and heat shock were tested by coding treatment groups of larvae as pretreated or not, and as heat shocked or not, which enabled a test for the interaction between the effects of pretreatment and heat shock. Although survival after heat shock was performed in three temporally separate sets of experiments for 1st-instar larvae and in two sets for 3rd-instar larvae, due to constraints on experimental size, results were combined for analyses across development. As linearity of effects across development cannot be assumed, and all data were collected as a single measurement for groups of 40 larvae per petri dish or vial, each of the developmental time points was coded independently. Because this analysis provides no information as to when differences first occur or diminish, separate analyses also were made at each developmental time point.

As done for survival-to-stress, analysis of variance with ELISA data on Hsp70 concentration considered each time point of recovery at 25°C after treatment as independent. Because differences among treatment groups were large relative to the low variance among replicates at each treatment, these results are described as a process over time although statistical probabilities for differences have been included. Treatment effects on larval growth, estimated as total protein, required separate analysis for larvae 24 h after stress and those 72 h after stress because variances were much larger for the larger larvae. Effects of multiple pretreatment on the main effects, strain and pretreatment number, were tested by ANOVA and by regression of pretreatment frequency on larva-to-adult survival in each strain. The large sample sizes required in many of these experiments required replicates to be prepared in blocks that were separated in time of preparation. Statistically, these block effects could be factored into ANOVA, but we preferred to take the more conservative approach of considering any time-of-handling variation as part of the error variation. As for all

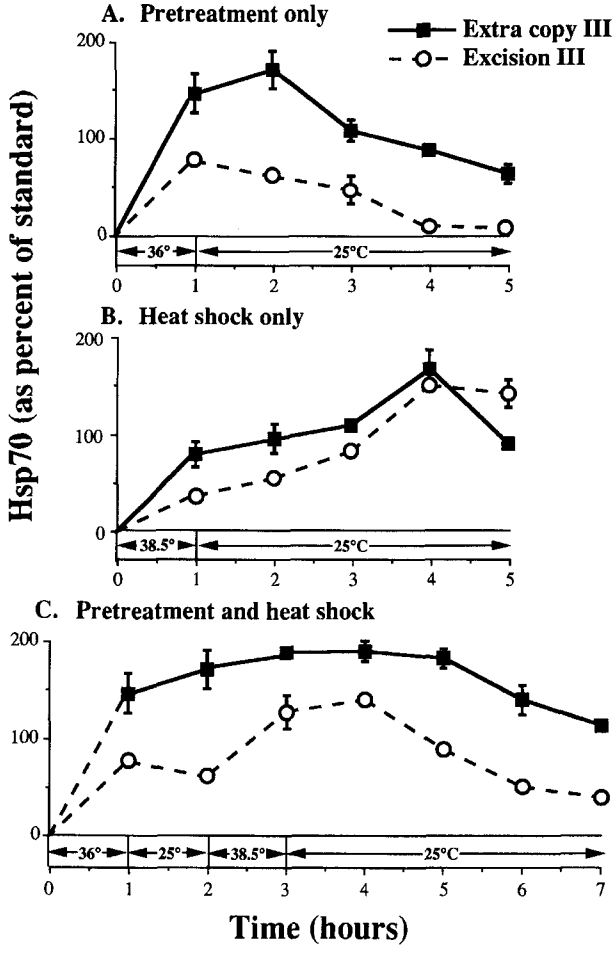

Fig. 2 Hsp70 levels (measured relative to a standard) in 1st-instar larvae of the extra-copy III and excision III lines. Temperature treatments and durations are imbedded in the X axis. Initial concentrations represent that prior to stress, at which time they are below detectable levels. Symbols are means ± 1 SE.

survival analyses, each set of larvae used to score total protein or Hsp70 concentration was used only once; thus each datum was independent. Survival data were arcsine-square root transformed for use in ANOVA, although actual means are presented in each figure. Protein data were not found to deviate from normality.

RESULTS

Hsp70 expression

Hsp70 was not detectable by ELISA in larvae maintained at 25°C. As previously described for diverse development stages from 6-hr embryos through adults (Welte et al 1993; Feder 1996; Feder et al 1996a; M. Tatar and J. Curtsinger, personal communication), transformation with 12 extra copies of *hsp70* almost always increased Hsp70 levels during and/or after exposure to a mild or severe heat shock (Figs 2–4). This effect was evident in 1st-instar and early

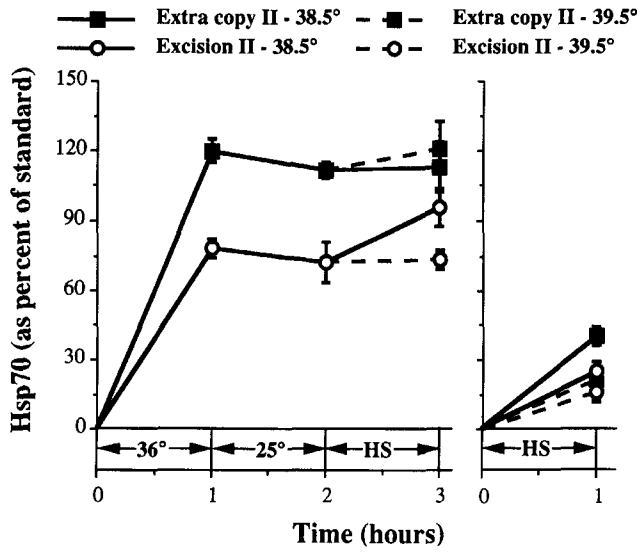

Fig. 3 Hsp70 levels (measured relative to a standard) in 1st-instar larvae of the extra-copy II and excision II lines. Heat shocks were of 38.5°C (solid lines) or 39.5°C (dashed lines). Symbols are means ± 1 SE.

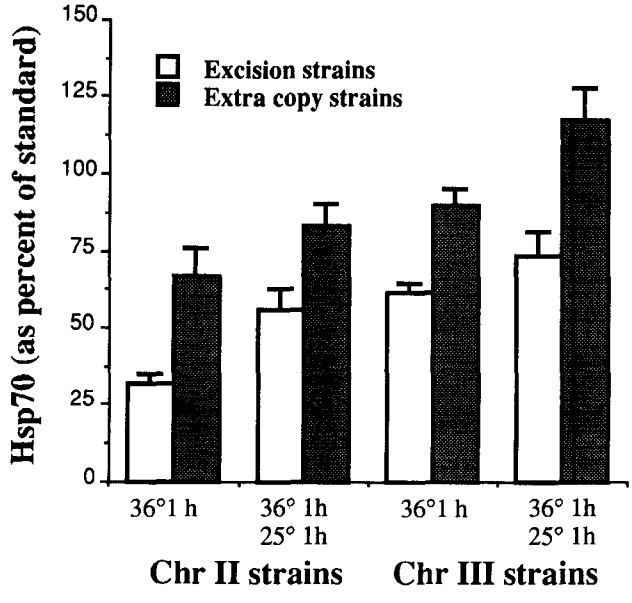

Fig. 4 Hsp70 levels (measured relative to a standard) in 3rd-instar larvae of both the chromosome II and chromosome III extra-copy and excision control lines. Values are means ± 1 SE.

3rd-instar larvae of both chromosome II and chromosome III transformants (Figs 2–4). The only exception was after 4 h recovery from a 1 h exposure to 38.5°C in the chromosome III lines, in which Hsp70 levels in the excision strain exceeded those in the extra copy strain (Fig. 2B).

Pretreatment enhanced Hsp70 levels during and after ensuing heat shock (Figs 2–3). For example, in the

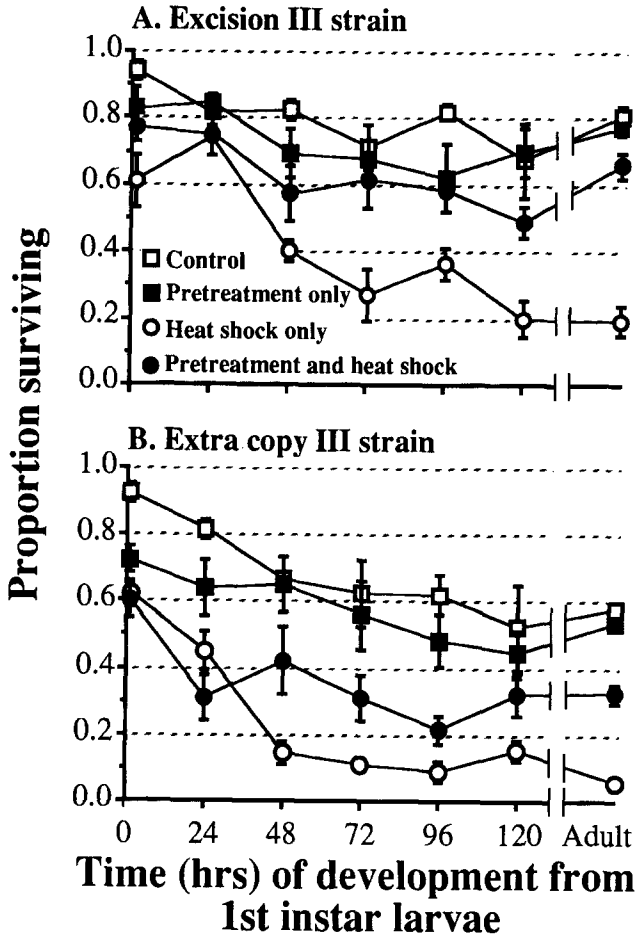

Fig. 5 Survival of individuals across development following exposure of recently hatched 1st-instar chromosome III larvae of the (A) excision or (B) extra-copy strains to one of four treatment conditions: pretreatment only 1 h at 36°C; heat shock only 1 h at 38.5°C; pretreatment and heat shock 1 h at 36°C, followed by 1 h at 25°C, and then 1 h at 38.5°C; or control-constant 25°C, which was the environment in which individuals developed except when undergoing treatment. Points represent independent samples and are connected only for clarity. Larvae were measured once. Symbols are means ± 1 SE.

chromosome III extra-copy line, Hsp70 levels in 1st instar larvae 1 h after a 38.5°C heat shock were 95% of standard in larvae without pretreatment and 185% of standard in pretreated larvae (Figs 2B,C).

Survival of 1st-instars, chromosome III

In newly hatched larvae at constant 25°C (i.e. the normal culture temperature), survival was nearly identical in the extra-copy III and excision III strains 1 h after sham transfer (Fig. 5). However, more of these larvae survived to adulthood in the excision strain than in the extra-copy strain ($P < 0.05$).

Pretreatment alone did not significantly affect survival to adulthood in either the extra-copy or excision strains ($P > 0.1$ in both strains), although differences from controls were greater in the extra-copy strain. Pretreatment did, however, alter temporal patterns of survival. In the extra-copy line, more pretreated larvae died within the first 24 h after hatching than did control larvae (1 h after pretreatment, $P < 0.01$; 24 h later, $P < 0.05$). In the excision line, more larvae also died 1 h after pretreatment than did controls, but the difference was not significant.

Heat shock alone greatly reduced survival, and this decline was greater in the extra-copy larvae than in the excision larvae ($P < 0.01$) at all developmental time points in the two strains.

Pretreatment significantly mitigated the effect of heat shock in both extra-copy and excision larvae ($P < 0.001$), although increased survival with pretreatment was not expressed until 48 h after heat shock. However, even though extra-copy larvae had higher Hsp70 concentrations than excision larvae before, during, and after heat shock, pretreated excision larvae survived heat shock significantly better than did extra-copy larvae throughout development (Fig. 5).

Survival of 1st-instars, chromosome II

For this set of strains, survival was assessed at only two times, immediately after heat shock and/or pretreatment, and at adulthood. At the earlier time, the following patterns were evident: As in the chromosome III lines, survival immediately after sham transfer was identical in the excision and extra-copy lines, averaging >98%. Heat shock alone markedly reduced survival from controls ($P < 0.001$), with the decrease significantly larger in the excision strain (to 84.7±1.4%) than in the extra-copy strain (to 91.3 ± 1.6%, $P < 0.01$). Although their effects were statistically significant ($P < 0.05$), both pretreatment alone and pretreatment plus heat shock only slightly diminished survivorship (to about 96%). The extra-copy and excision strains did not differ in these treatment effects.

By adulthood, however, other patterns emerged, which again indicated more Hsp70 may not always be better for withstanding thermal stress. Survival of both strains was significantly reduced by heat shock without pretreatment ($P < 0.001$), with the excision line (49.5 ± 2.2%) surviving stress significantly better than the extra-copy line (40.3 ± 1.7%, $P < 0.01$). No differences among the other treatments, control, pretreatment only, and heat shock plus pretreatment, were significant, nor were interaction effects significant. Mean survival ranged between 65% and 68% in both strains for these treatments. Due to this higher thermotolerance of the chromosome II than of the chromosome III strains, these experiments were repeated at a heat shock temperature of 39.5°C to compare thermotolerance differences between strains after pretreatment. No larvae survived long enough to pupate

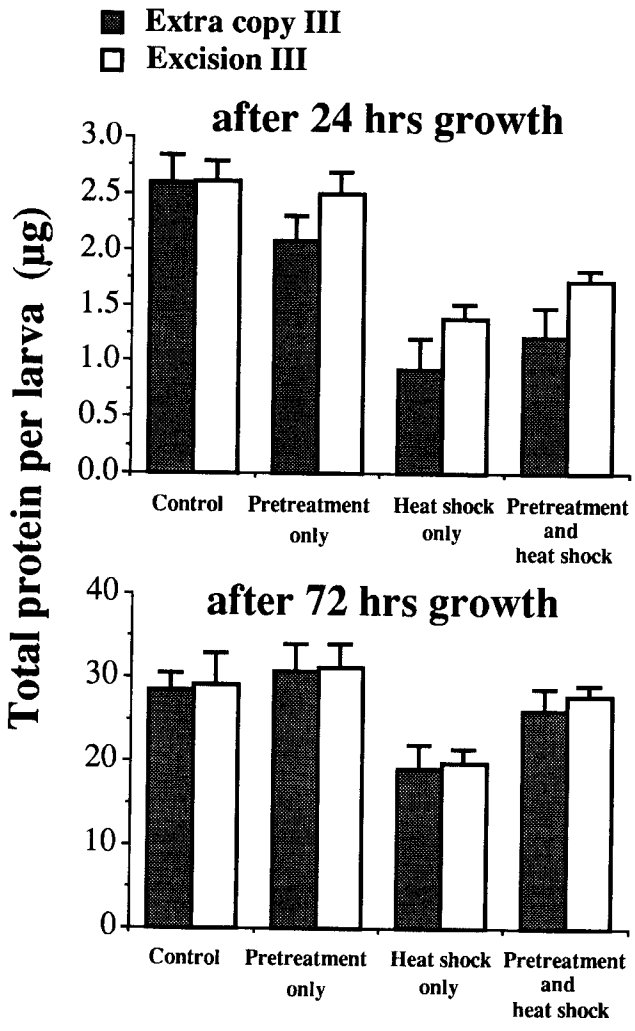

Fig. 6 Larval mass, as total soluble protein, after 24 and 72 h of development. Recently hatched 1st-instar larvae of the excision III and extra-copy III strains were exposed to one of four treatment conditions: pretreatment only 1 h at 36°C; heat shock only 1 h at 38.5°C; pretreatment and heat shock 1 h at 36°C, and 1 h at 25°C, and then 1 h at 38.5°C; or were maintained at constant 25°C, which was the environment in which larvae developed except when undergoing treatment. Values are means ± 1 SE.

after this higher stress temperature unless they received pretreatment. With pretreatment, excision larvae survived to adulthood in significantly higher proportions (82.6% of controls) than did extra-copy larvae (34.0% of controls, $P < 0.001$).

Larval growth after heat stress

To ascertain whether the temperature treatments affect growth as well as survivorship, the authors measured total protein of surviving chromosome III larvae 24 h and 72 h after a 38.5°C heat shock administered 2–4 h after hatching (Fig. 6). Heat shock significantly reduced total protein measured at both times relative to that of controls (at 24 h and at 72 h, $P < 0.001$). At 24 h, larvae that had undergone pretreatment alone contained slightly less protein than did control larvae, but contained significantly more protein than larvae that underwent heat shock without pretreatment (pretreatment × heat shock interaction, $P < 0.05$). At 72 h, larvae that had undergone heat shock without pretreatment still contained less protein than controls, but larvae that underwent pretreatment before heat shock or those receiving pretreatment alone were indistinguishable from controls in total protein. Except in controls, extra-copy larvae contained less protein than excision larvae at 24 h after heat shock and/or pretreatment ($P < 0.05$); this strain difference was no longer evident at 72 h.

Larval survival, 3rd-instars

Survival of 3rd-instar chromosome III larvae after stress (38.5°C) resembled that of 1st-instars, although overall tolerance of 3rd-instars was lower. Without pretreatment, fewer than 1% of individuals survived to adulthood after heat shock (Fig. 7). Larvae heat-shocked without pretreatment grew little (by visual estimation), and almost all died without pupating. Similarly, less than 10% of chromosome II larvae survived to adulthood after this heat shock (38.5°C) as 3rd-instars (Fig. 8). For survival immediately after the stress challenge, extra-copy III larvae tolerated heat shock without pretreatment better than did excision III larvae (Fig. 7). Subsequent survival in extra-copy III declined more rapidly than in excision III ($P < 0.05$), which was similar to results for 1st-instar larvae; larvae of the excision strain survived heat shock at higher proportions relative to larvae of the extra-copy strain ($P < 0.1$). A comparable result occurred for chromosome II larvae; higher survival after heat shock for excision than extra-copy larvae was evident both with and without pretreatment ($P < 0.001$).

Effects of multiple pretreatment

The foregoing results suggest that the higher levels of Hsp70 occurring from extra copies of the *hsp70* gene are at least neutral, and probably harmful, and led us to hypothesize that repeated overexpression of Hsp70 should increase mortality still further. To test this hypothesis, we exposed 1st-instar larvae of both chromosome III strains to 0, 1, 2, or 3 pretreatments (Fig. 9). Survival in the extra-copy strain decreased with number of pretreatments by an average of 12% each pretreatment (regression equation: proportion surviving = 0.617–0.121 pretreatment frequency, $r^2 = 0.56$). Pretreatment number had no effect on survival in the excision strain, in which survival was significantly higher than in the extra-copy strain ($P < 0.001$).

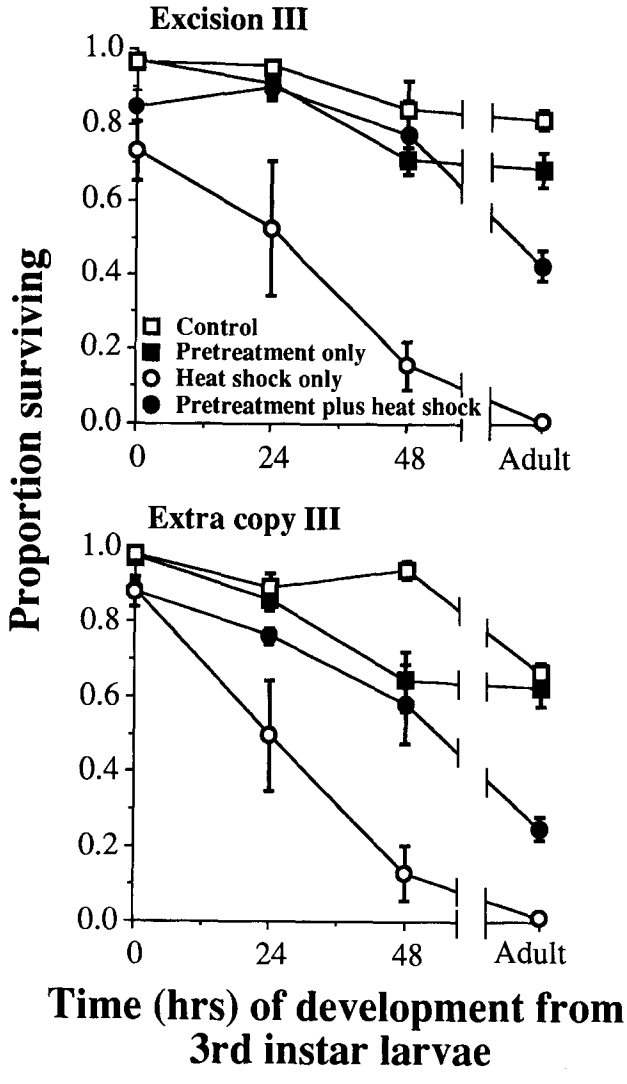

Fig. 7 Survival of 3rd-instar larvae of the excision III and extra-copy III strains. Larvae either received pretreatment only 1 h at 36°C; heat shock only 1 h at 38.5°C; pretreatment and heat shock 1 h at 36°C, and 1 h at 25°C, and then 1 h at 38.5°C; or were maintained at constant 25°C, which was the environment in which individuals developed except when undergoing treatment. Points represent independent samples and are connected only for clarity. Larvae were not measured more than once. Symbols are means ± 1 SE.

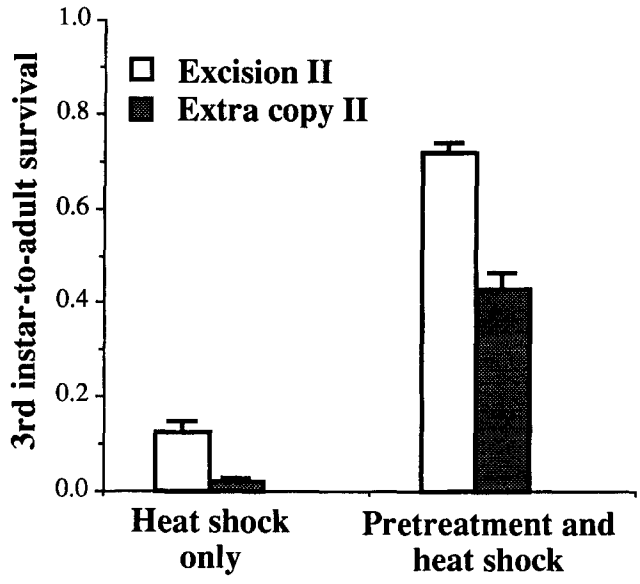

Fig. 8 Larva-to-adult survival of 3rd-instar chromosome III larvae of the excision and extra-copy strains following exposure directly to 38.5°C or with a 1 h 36°C pretreatment and 1 h at 25°C before the stress exposure. Values are means ± 1 SE.

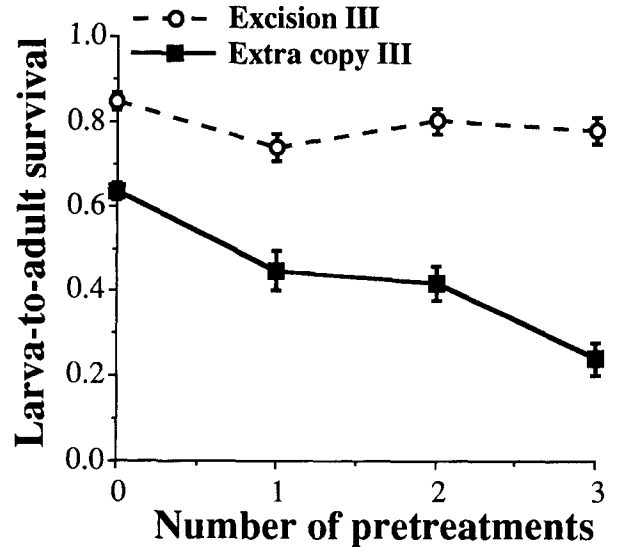

Fig. 9 Larva-to-adult survival of recently hatched 1st-instar larvae of the chromosome III excision and extra-copy strains following exposure to one of four treatments. Conditions were pretreatment once (2–4 h after hatching), twice (after hatching and 24 h later), or 3 times (as for 'twice' and another 24 h later). The untreated control was maintained at constant 25°C, which was the environment in which larvae developed except when undergoing treatment. Symbols are means ± 1 SE.

We repeated this experiment on 3rd-instar larvae of both chromosome II and chromosome III strains (Fig. 10). Survival decreased with number of pretreatments in all 3rd-instar larvae ($P < 0.001$), was higher in excision than extra-copy lines across all treatments ($P < 0.001$), and was higher in chromosome II larvae than in chromosome III larvae ($P < 0.001$). However, no interaction effects were significant, indicating that number of pretreatments similarly reduced survival of 3rd-instar larvae in all strains.

Finally, to test long-term benefits of pretreatment, 3rd-instar larvae of both chromosome III strains were administered a heat shock at 96 h after hatching, either preceded or not by a single pretreatment 24 h earlier (Fig. 11). Larvae of both strains had similar and low (averaging < 5%) survival if not pretreated. Pretreatment 24 h

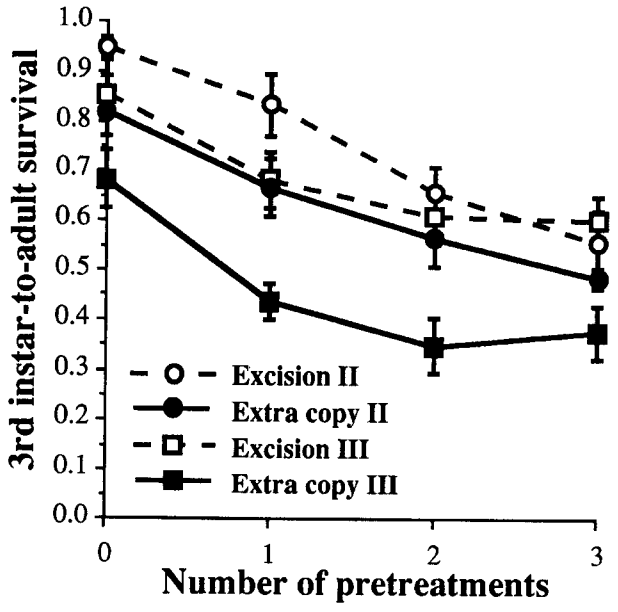

Fig. 10 Larva-to-adult survival of 3rd-instar larvae from the chromosome II and chromosome III excision and extra-copy strains following exposure to one of four treatments. Conditions were pretreatment once (2–4 h after collection), twice (after collection and 18 h later), or 3 times (as for 'twice' and another 18 h later). The untreated control was maintained at constant 25°C, which was the environment in which larvae developed except when undergoing treatment. Values are means ± 1 SE.

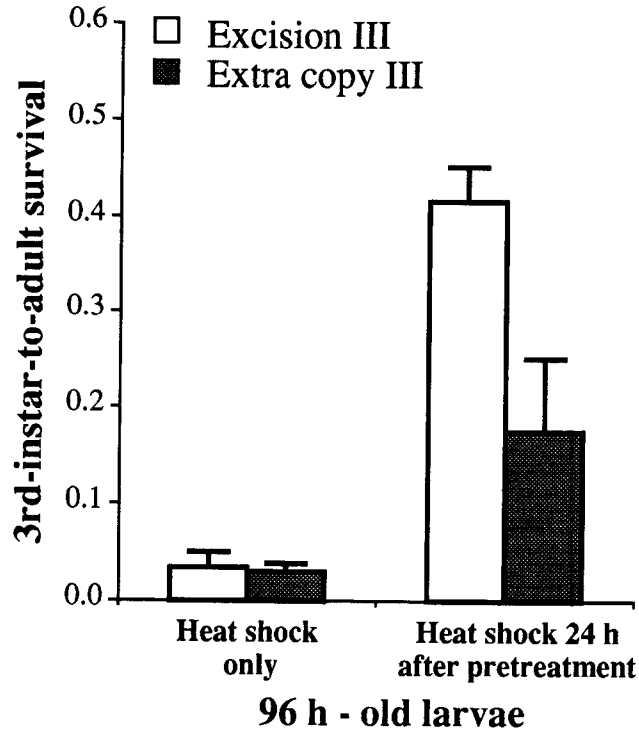

Fig. 11 Larva-to-adult survival of 96-h-old 3rd-instar larvae from the chromosome III excision and extra-copy strains following heat shock. Larvae either were pretreated once, at 72-h-old, before heat shock or were exposed directly to 38.5°C for 1 h without pretreatment. Values are means ± 1 SE.

beforehand significantly improved survival of larvae ($P < 0.001$), and more so in the excision strain than in the extra-copy strain ($P < 0.01$). At the time of the heat shock, Hsp70 resulting from the pretreatment was no longer detectable (data not shown).

DISCUSSION

The experimental regime, in which larvae undergo a mild-heat pretreatment before encountering a severe heat shock, reflects conditions that *Drosophila* experience in nature. Fruit inhabited by larvae routinely exceeds 36°C and may exceed 45°C in nature, and these natural heat shocks induce expression of Hsp70 in wild larvae (Feder et al 1997) similar to that in the excision strains. As has long been known, pretreatment induces Hsps, especially Hsp70 in *Drosophila* (Lindquist 1993), and mitigates some harmful effects of heat shock on growth and survival (Feder et al 1996). Pretreatment also protects the expression of Hsp70 during and after heat shock (Figs 2,3). These beneficial effects presumably derive from the function of Hsps as molecular chaperones, and, as studies of thermal stress in nature suggest (Feder 1996; Feder et al 1997), are likely to enhance the evolutionary fitness of *Drosophila* in nature. As revealed by comparisons of extra-copy and excision strains, transformation with extra copies of a single gene, *hsp70*, is sufficient to enhance inducible thermotolerance.

Accompanying these beneficial effects of extra *hsp70* copies, however, are pronounced deleterious effects. First-instar larvae that survive heat shock thereafter die at higher rates in extra-copy strains than in excision strains, and this difference in mortality is greater if larvae are given a Hsp-inducing pretreatment before heat shock. Furthermore, in 1st-instar larvae of the chromosome III extra-copy strain, survival decreased in direct proportion to the number of pretreatments, while pretreatment number did not affect survival in its corresponding excision line. Both in 1st-instar (Fig. 5) and 3rd-instar (Fig. 7) chromosome III larvae, survival of the extra-copy line, but not the excision line, becomes lower than that of control larvae 24 h after heat shock. Growth delays at 24 h after heat shock are likewise greater in extra-copy larvae than in excision larvae (Fig. 6), and strain differences disappear by 72 h after heat shock.

The cellular mechanisms underlying these deleterious effects are unclear and deserve further study. As discussed in the Introduction, candidate mechanisms include inappropriate diversion of limiting cellular resources (energy, substrate, protein synthetic

apparatus) into the heat shock response, suppression of background protein synthesis, and toxicity of Hsps; these mechanisms are not mutually exclusive. Hsp70 associates with a diversity of intracellular structures during and after heat shock, each of which could be the site of a toxic effect if Hsp70 is superabundant. These include several structures related to cell division, including the nucleus in general (Velazquez et al 1984; Palter et al 1986), chromosomes themselves (Velazquez et al 1980; Laran et al 1990; Kutejova et al 1993) and the centrosome (Perret et al 1995), where physiological levels of Hsps may mitigate heat-induced damage (Pelham 1984; Mamon et al 1990, 1992; Vidair et al 1993). Interestingly, in cultured *Drosophila* cells that were engineered to express Hsp70 at normal temperatures, cell proliferation initially slows, and the resumption of cell proliferation is specifically associated with the sequestration of cytoplasmic Hsp70 into intracellular granules (Feder et al 1992). In experiments with whole larvae, the bulk of the increased mortality associated with extra *hsp70* copies occurred not during or immediately after heat shock, but in the ensuing days; i.e. after Hsp70 was no longer detectable. Extra copies of *hsp70* were also associated with a short-lived depression of growth after heat shock. These effects single out growth, and tissues and life-cycle stages in which growth and chromosome replication of polytene tissue are manifest, as particularly susceptible to effects of Hsp70 overexpression. Mitotic and S phases of cell growth are heat sensitive (Westra and Dewey 1971), and indeed, our ongoing work suggests that the larval gut, in which continual cell growth in size is essential for normal digestive function, is among the most heat-sensitive of all tissues.

Curiously, extra-copy strains exhibit greater mortality than excision strains even when maintained at 25°C, a temperature thought not to induce Hsps in *Drosophila* larval tissues and cells in culture, and at which many assays, including our ELISA, have failed to detect Hsp70. A potential explanation for this phenomenon is Hsp70 induction in a small number of critical cells and/or cell types. In fact, Hsp70-specific immunofluorescence is characteristically detectable in several cells in the brain of larvae maintained at 25°C (R. Krebs, unpublished data).

Drosophila melanogaster is distinctive in having undergone extensive duplication of its major inducible Hsp70 family member. At the same time, *Drosophila* deploys an array of regulatory mechanisms that guard against excessive Hsp70 expression (Yost et al 1990; Solomon et al 1991) or of stress related damage. Only when these autoregulatory mechanisms are overwhelmed may negative consequences ensue. The lack of any negative effects in young, multiply-pretreated excision III larvae, and the lower larval-to-adult survival of extra-copy than excision lines under some low stress and all higher stress treatments is consistent with this interpretation. The inability to increase larva-to-adult survival after stress by increasing *hsp70* copy number and consequently, Hsp70 expression, suggests that natural Hsp70 expression levels approach or are at the maximum for an effective function in stress defense. Rat cells expressing human Hsp70 receive no additional benefit, or cost, from expression levels twice that necessary for providing maximal thermotolerance (Li and Nussenzweig 1996) and Norris et al (1995), who measured Hsp concentrations after slowly heating fish, *Poeciliopsis gracilis,* to 41°C, interpreted their results as supporting higher thermotolerance for fish expressing intermediate levels of Hsp70.

Thus, the present study suggests why this gene duplication has not been even more extensive. This balance between beneficial and negative effects suggests an evolutionary trade-off, or antagonistic pleiotropy. A trade-off occurs when a trait that increases fitness is linked to a trait that decreases fitness (Stearns and Kaiser 1996). Here the linked aspects of Hsp70 expression could be surviving until stress abates and recovery and growth after stress abates. Alternatively or additionally, benefits at one developmental stage could trade-off with costs at another point in development. Most evolutionary biologists have investigated trade-offs by characterizing genetic or phenotypic correlations among traits in extant populations and species or in populations undergoing experimental selection (Hoffmann and Parsons 1991; Leroi et al 1994; Promislow 1995; Krebs and Loeschcke 1996). These approaches, while valuable, may be unable to attribute trade-offs to specific genes or to discover how specific gene products have both beneficial and negative effects. Molecular manipulation, such as that of Welte et al (1993), target specific gene products that have been well characterized in advance. Therefore, they are particularly promising for elucidating the way in which a gene product can both positively and negatively affect life-history traits (e.g., growth and survival), and for complementing analyses of genetic and phenotypic correlations (Krebs and Feder 1997). In the present study, Hsp70 concentration emerges as a trait likely subject to strong stabilizing selection in nature.

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REFERENCES

Ashburner M (1989) *Drosophila*: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Coleman JS, Heckathorn SA and Hallberg RL (1995) Heat-shock proteins and thermotolerance: linking ecological and molecular perspectives. Trends Ecol. Evol. **10**, 305–306.

DiDomenico BJ, Bugaisky GE and Lindquist S (1982) Heat shock and recovery are mediated by different translational mechanisms. Proc. Natl. Acad. Sci. USA **79**, 6181–6185.

Dorner AJ, Krane MG, and Kaufman RJ (1988) Reduction of endogenous GRP78 levels improves secretion of a heterologous protein in CHO cells. Mol. Cell Biol. **8**, 4063–4070.

Dorner AJ, Wasley LC and Kaufman RJ (1992) Overexpression of GRP78 mitigates stress induction of glucose regulated proteins and blocks secretion of selective proteins in Chinese hamster ovary cells. EMBO **11**, 1563–1571.

Dura JM (1981) Stage dependent synthesis of heat shock induced proteins in early embryos of *Drosophila melanogaster*. Mol. Gen. Genet. **184**, 381–385.

Feder JH, Rossi JM, Solomon J, Solomon N and Lindquist S (1992) The consequences of expressing Hsp70 in *Drosophila* cells at normal temperatures. Genes Dev. **6**, 1402–1413.

Feder ME (1996) Ecological and evolutionary physiology of stress proteins and the stress response: the *Drosophila melanogaster* model. In: Phenotypic and Evolutionary Adaptation to Temperature, eds IA Johnston and AF Bennett, Cambridge University Press, Cambridge, 79–102.

Feder ME, Cartaño NV, Milos L, Krebs RA and Lindquist SL (1996) Effect of engineering Hsp70 copy number on Hsp70 expression and tolerance of ecologically relevant heat shock in larvae and pupae of *Drosophila melanogaster*. J. Exp. Biol. **199**, 1837–1844.

Feder ME, Blair N and Figueras H (1997) Natural thermal stress and heat-shock protein expression in *Drosophila* larvae and pupae. Funct. Ecol. (in press).

Hartl FU (1996) Molecular chaperones in cellular protein folding. Nature **381**, 571–580.

Heckathorn, SA, Poeller GJ, Coleman JS and Hallberg, RL (1996a) Nitrogen availability alters patterns of accumulation of heat stress-induced proteins in plants. Oecologia **105**, 413–418.

Heckathorn, SA, Poeller GJ, Coleman JS and Hallberg, RL (1996b) Nitrogen availability and vegetative development influence the response of ribulose 1, 5–bisphophate carboxylase/oxygenase, phosphoenolpyruvate carboxylase, and heat-shock protein content to heat stress in *Zea mays* L. Int. J. Plant Sci. **157**, 533–546.

Heikkila JJ, Kloc M, Bury J, Schultz GA and Browder LW (1985) Acquisition of the heat-shock response and thermotolerance during early development of *Xenopus laevis*. Dev. Biol. **107**, 483–489.

Hoffmann AA and Parsons PA (1991) Evolutionary Genetics and Environmental Stress. Oxford University Press, NY.

Ish-Horowicz D, Pinchin SM, Schedl P, Artavanis TS and Mirault ME (1979) Genetic and molecular analysis of the 87A7 and 87C1 heat-inducible loci of *D. melanogaster*. Cell **18**, 1351–1358.

Krebs RA and Feder ME (1997) Natural variation in the expression of the heat-shock protein Hsp70 in a population of *Drosophila melanogaster*, and its correlation with tolerance of ecologically relevant thermal stress. Evolution **51**, 173–179.

Krebs RA and Loeschcke V (1994) Costs and benefits of activation of the heat-shock response in *Drosophila melanogaster*. Funct. Ecol. **8**, 730–737.

Krebs RA and Loeschcke V (1996) Acclimation and selection for increased resistance to thermal stress in *Drosophila buzzatii*. Genetics **142**, 471–479.

Krebs RA, La Torre V, Cavicchi S and Loeschcke V (1996) Heat-shock resistance in *Drosophila* populations: analysis of variation in reciprocal cross progeny. Hereditas **124**, 47–55.

Kutejova E, Durcova G, Surovkova E and Kuzela S (1993) Role of heat-shock proteins in the recovery of mitotic chromosome damage induced by high temperature in *Drosophila melanogaster*. Genetika **29**, 604–612.

Laran E, Requena JM, Jimenez RA, Lopez MC and Alonso C (1990) The heat shock protein hsp70 binds in vivo to subregions 2–48BC and 3–58D of the polytene chromosomes of *Drosophila hydei*. Chromosoma **99**, 315–320.

Leroi AM, Chen WR and Rose MR (1994) Long-term laboratory evolution of a genetic life-history trade-off in *Drosophila melanogaster*. 2. Stability of genetic correlations. Evolution **48**, 1258–1268.

Li GC and Nussenzweig A (1996) Thermotolerance and heat shock proteins: Possible involvement of Ku autoantigen in regulating Hsp70 expression. In: Stress-inducible Cellular Responses, eds RI Feige, I Yahara and B Polla, Birkauser Verlag, Basel, 425–450.

Lindquist S (1993) Autoregulation of the heat-shock response. In: Translational Regulation of Gene Expression 2, ed J Ilan, Plenum Press, NY, 279–320.

Mamon LA, LV Barabanova and NN Kostromina (1992) Frequency of non-disjunction and loss of sex chromosomes in oogenesis in the *Drosophila melanogaster* mutant I(1) ts 403 with a defect in the heat-shock protein system in anoxia and at high temperature. Genetika **28**, 64–71.

Mamon LA, EL Mazur, IV Churkina and LV Barabanova (1990) The effect of high temperature on the frequency of nondisjunction and loss of sex chromosomes in females of *Drosophila melanogaster* strain I(1)ts403 with a defect in the heat shock protein system. Genetika **26**, 554–556.

McKenzie JA (1993) Measuring fitness and intergenic interactions: the evolution of resistance to diazinon in *Lucilia cuprina*. Genetica **90**, 227–237.

Morimoto RI, Tissiéres A and Georgopoulos C, eds (1994) The Biology of Heat Shock Proteins and Molecular Chaperones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Muller WU, Li GC and Goldstein LS (1985) Heat does not induce synthesis of heat shock proteins or thermotolerance in the earliest stage of mouse embryo development. Int. J. Hyperthermia **1**, 97–102.

Norris CE, diIorio PJ, Schultz RJ and Hightower LE (1995) Variation in heat shock proteins within tropical and desert species of Poeciliid fishes. Mol. Biol. Evol. **12**, 1048–1062.

Palter KB, Watanabe M, Stinson L, Mahowald AP and Craig EA (1986) Expression and localization of *Drosophila melanogaster* hsp70 cognate proteins. Mol. Cell Biol. **6**, 1187–1203.

Pelham HR (1984) Hsp70 accelerates the recovery of nucleolar morphology after heat shock. EMBO J **3**, 3095–3100.

Perret E, Moudjou M, Geraud ML, Derancourt J, Soyer-Gobillard MO and Bornens M (1995) Identification of an HSP70–related protein associated with the centrosome from dinoflagellates to human cells. J. Cell Sci. **108**, 711–725.

Promislow DEL (1995) New perspectives on comparative tests of antagonistic pleiotropy using *Drosophila*. Evolution **49**, 394–397.

Sanchez Y and Lindquist SL (1990) HSP104 required for induced thermotolerance. Science **248**, 1112–1115.

Sanchez Y, Taulien J, Borkovich KA and Lindquist S (1992) Hsp104 is required for tolerance to many forms of stress. EMBO **11**, 2357–2364.

Solomon JM, Rossi JM, Golic K, McGarry T and Lindquist S (1991) Changes in Hsp70 alter thermotolerance and heat-shock regulation in *Drosophila*. New Biologist **3**, 1106–1120.

Statview (1992) Abacus Concepts, Inc., Berkeley, CA.

Stearns SC and Kaiser M (1996) Effects on fitness components of P-element inserts in *Drosphila melanogaster*: analysis of tradeoffs. Evolution **50**, 795–806.

Velazquez JM and Lindquist S (1984) hsp70: nuclear concentration during environmental stress and cytoplasmic storage during recovery. Cell **36**, 655–662.

Velazquez JM, DiDomenico BJ and Lindquist S (1980) Intracellular localization of heat shock proteins in *Drosophila*. Cell **20**, 679–689.

Vidair CA, Doxsey SJ and Dewey WC (1993) Heat shock alters centrosome organization leading to mitotic dysfunction and cell death. J. Cell Physiol. **154**, 443–455.

Welte MA, Tetrault JM, Dellavalle RP and Lindquist SL (1993) A new method for manipulating transgenes: engineering heat tolerance in a complex, multicellular organism. Current Biol. **3**, 842–853.

Westra A and Dewey WC (1971) Variation in sensitivity to heat shock during the cell-cycle of Chinese hamster cells *in vitro*. Int. J. Rad. Biol. **19**, 467–477.

Yost HJ, Petersen RB and Lindquist S (1990). Posttranscriptional regulation of heat shock protein synthesis in *Drosophila*. In: Stress Proteins in Biology and Medicine, eds RI Morimoto, A Tissiéres and C Georgopoulos, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 379–409.