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EFFECT OF ENGINEERING *Hsp70* COPY NUMBER ON Hsp70 EXPRESSION AND TOLERANCE OF ECOLOGICALLY RELEVANT HEAT SHOCK IN LARVAE AND PUPAE OF *DROSOPHILA MELANOGASTER*

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Summary

To determine how the accumulation of the major *Drosophila melanogaster* heat-shock protein, Hsp70, affects inducible thermotolerance in larvae and pupae, we have compared two sister strains generated by site-specific homologous recombination. One strain carried 12 extra copies of the *Hsp70* gene at a single insertion site (extra-copy strain) and the other carried remnants of the transgene construct but lacked the extra copies of *Hsp70* (excision strain). Hsp70 levels in whole-body lysates of larvae and pupae were measured by ELISA with an Hsp70-specific antibody. In both extra-copy and excision strains, Hsp70 was undetectable prior to heat shock. Hsp70 concentrations were higher in the extra-copy strain than in the excision strain at most time points during and after heat shock. Pretreatment (i.e. exposure to 36 °C before heat shock) significantly improved thermotolerance, and this improvement was greater and more rapid in larvae and pupae of the extra-copy strain than in those of the excision strain. The experimental conditions resemble thermal regimes actually experienced by *Drosophila* in the field. Thus, these findings represent the best evidence to date that the amount of a heat-shock protein affects the fitness of a complex animal in the wild.

Key words: *Drosophila melanogaster*, heat-shock protein, Hsp70, thermotolerance.

Introduction

Heat and many other stresses elicit expression of a suite of highly conserved proteins termed stress or heat-shock proteins (Hsps). While the cellular role of Hsps and the regulation of their expression is becoming understood at ever finer levels of resolution (Morimoto *et al.* 1994; Feder *et al.* 1995), their physiological significance at the level of the whole organism remains largely enigmatic, particularly in complex eukaryotes. One reason for this enigmatic status is that the thermal phenotype of higher eukaryotes comprises hundreds if not thousands of traits – a background that often confounds the assessment of the role of a particular Hsp or any other single trait (Feder, 1996). Modern molecular techniques, however, offer an unparalleled opportunity to manipulate a single trait against a constant genetic background and thereby to assess the trait's significance (Welte *et al.* 1993; Feder, 1996). We have exploited such a manipulation to examine the consequences of varying Hsp70 expression for tolerance of ecologically relevant heat shock in *Drosophila melanogaster*.

Hsp70 family proteins, including some constitutively expressed cognates, play diverse cellular roles in the folding of nascent polypeptides, the intracellular distribution of peptides to organellar compartments such as mitochondria, the degradation of proteins and their removal from the cell, and the renaturation or disposal of proteins damaged by heat and other stresses (Morimoto *et al*. 1994). In *D. melanogaster*, one heat-inducible member of this family has been amplified in the genome (Ish-Horowicz *et al.* 1979) and it dominates Hsp expression in this species (Lindquist and Craig, 1988). At least 10 nearly identical genes in the diploid genome encode this protein, Hsp70, which is strongly induced by high temperatures and hypoxia but is virtually absent from the cell at other times (Velazquez and Lindquist, 1984; Palter *et al.* 1986; Lindquist, 1993). Genetic and biochemical manipulations of Hsp70 levels have clearly established the importance of this protein for thermotolerance of cells in culture (Riabowol *et al.* 1988; Li *et al.* 1991; Solomon *et al.* 1991; Feder *et al.* 1992). To examine whether parallel consequences ensue in entire complex eukaryotes, Welte *et al.* (1993) combined P-element-mediated germ-line transformation and site-specific homologous recombination to generate *Drosophila* strains bearing 12 extra copies of *Hsp70* and an 'excision' strain lacking the extra copies. Because both

the 'extra-copy' and the excision strain derive from a single integration of the original transgene construct, the excision strain serves as a control for positional mutagenesis, i.e. inadvertent interruption of a background gene. Comparison of these strains uncovers differences that are attributable to differences in *Hsp70* copy number because the two strains otherwise have the same genetic background (Welte *et al.* 1993). Welte *et al.* (1993) used two independently derived pairs of transformant strains to demonstrate that Hsp70 levels are rate-limiting for thermotolerance in embryos and that extra copies of *Hsp70* are sufficient to protect embryonic development against heat stress.

The present study examines the significance of *Hsp70* copy number for more advanced developmental stages, wandering third-instar larvae and pupae. It extends the work of Welte *et al.* (1993), moreover, by using experimental treatments that resemble the actual temperature regimes that *Drosophila* experience in the field and by examining developmental stages that more clearly undergo natural thermal stress. In nature, *Drosophila* larvae infest necrotic (i.e. fallen and rotting) fruit. Solar heating of necrotic fruit can expose larvae dwelling within to severe if not lethal thermal stress. Temperatures above 35 °C are not uncommon for infested fruit at temperate latitudes, and temperatures may exceed 45 °C (Feder, 1996). *Drosophila* larvae are quite sensitive to these temperatures; many physiological functions relevant to fitness fail with chronic exposure to temperatures above 35 °C, and death ensues rapidly at 39–41 °C (David *et al.* 1983; Ashburner, 1989; Feder *et al.* 1996). One naturally occurring response to these temperatures is expression of Hsp70 (Feder *et al.* 1996). To examine the consequences of Hsp70 expression for whole larvae and pupae, we exploited the extra-copy and excision strains constructed by Welte *et al.* (1993) to generate controlled variation in Hsp70 expression. We then analyzed the consequences of this variation for tolerance of naturally encountered temperatures. The results demonstrate that variation in copy number of this single gene, *Hsp70*, affects Hsp70 concentrations in whole larvae and pupae, which in turn affects their tolerance of natural thermal stress and, potentially, their fitness.

Materials and methods

We examined one pair of extra-copy and excision strains, whose construction and analysis were described by Welte *et al.* (1993), designated 59+ and L-59 respectively. These are third chromosome integrants that are distinguished as adults by the presence or absence of the eye color marker associated with the transgene. Wandering-phase third-instar larvae were collected without anesthesia from the walls of glass bottles in which stocks were maintained on yeasted molasses–agar medium. To gather other stages, eggs were collected by placing Petri dishes filled with yeasted molasses–agar medium inside population cages of each strain. Eggs were then allowed to develop, and larvae were collected at the following times after laying: first instar, 36 h; second instar, 60 h; 1-day-old third instar, 84 h. Filter paper cones were placed onto the medium as pupation neared. Larvae crawled onto these cones to pupate. Cones were replaced daily, with each cone containing only pupae that had formed within the previous 24 h. Unless indicated, larvae and pupae were maintained at 25 °C on a 14 h:10 h L:D photoperiod.

To assess variation in Hsp70 concentration, larvae and pupae were exposed to a variety of experimental thermal regimes, immediately frozen in liquid N_2, and stored at –80 °C until analysis. Typically, a single sample consisted of two third-instar larvae or pupae, 10–15 second-instar larvae or 20–35 first-instar larvae. Wandering-phase third-instar larvae and pupae were placed within a 35 mm plastic culture dish on paper toweling moistened with phosphate-buffered saline (PBS); the dishes were covered, sealed with Parafilm, and placed in water baths thermostatted at the experimental temperatures or in an incubator at 25 °C for recovery. Samples of younger larvae were transferred to a 1.5 ml microfuge tube containing PBS. Each tube was centrifuged briefly, the PBS was drawn off, and the entire tube was maintained in a thermostatted water bath for heat shock and an incubator at 25 °C for recovery. Larvae or pupae in each sample were lysed without pre-thawing *via* intermittent sonication in ice-cold 2 mmol l^{-1} *p*-methylsulfonyl fluoride (PMSF) in PBS. Lysates were centrifuged at 14 000 ***g*** for 30 min at 4 °C, and then stored on ice. The protein content of the supernatant was determined (BCA Assay, Pierce Biochemicals) for 10 µl samples in triplicate in a 96-well microplate format, according to the manufacturer's instructions, and was standardized against bovine serum albumin diluted in PBS.

Hsp70 standards were prepared in bulk from *Drosophila* Schneider 2 cells grown to confluence in 25 cm^2 tissue culture flasks (Solomon *et al.* 1991). The cells were loosened by gentle pipetting of the medium, and the entire flask was placed in a water bath at 36.5 °C for 1 h and then incubated at 25 °C for 1 h. The contents of several flasks were then quickly pooled, rinsed with ice-cold PBS, centrifuged briefly, and the supernatant drawn off. The cells were lysed, the lysate centrifuged, and the protein content of the supernatant determined as described above. Samples of supernatant were stored at –80 °C.

Hsp70 concentrations were measured by enzyme-linked immunosorbent assay (ELISA) (Welte *et al.* 1993). Supernatants of whole-animal lysates prepared the same day were diluted to 20 µg ml^{-1} protein in ice-cold coating buffer and used to coat micro-well plates (Falcon 3915 ProBind). Plates were left overnight at 4 °C to allow proteins to adsorb. After extensive rinsing, bound Hsp70 was detected with the *Drosophila* Hsp70-specific antibody 7FB (Velazquez *et al.* 1980, 1983; Velazquez and Lindquist, 1984) (1:5000) coupled to alkaline phosphatase *via* secondary and tertiary antibodies [1:1000 rabbit anti-rat IgG (Cappel Organon Teknika, Durham, NC) and 1:1000 alkaline-phosphatase-conjugated goat anti-rabbit IgG (Sigma, St Louis, MO), respectively]. Plates were incubated at 37 °C with the phosphatase substrate *p*-nitrophenyl phosphate (1 mg ml^{-1}), prepared according to the

manufacturer's instructions (Sigma), and the colored reaction product was measured at 405 nm in a micro-plate reader. For at least one replicate of each sample or standard, the primary antibody was omitted to allow correction for non-specific signal. The corrected signal has been shown to be a linear function of the quantity of the Hsp70 in the lysate over a very broad range (Welte *et al*. 1993). Each assay included multiple replicates of the Hsp70 standard; all results are expressed as a percentage of the signal of this standard.

In preliminary investigations with wandering third-instar larvae, the Hsp70 concentration was 2.9±0.3 % (mean ± S.E.M.) of standard after 2 h of heat shock at 32 °C, 15.1±2.7 % at 34 °C, 70.8±5.5 % at 36 °C and 53.0±6.5 % at 38 °C (pooled values for extra-copy and excision larvae, N=8–14). We therefore chose 36 °C as a standard pretreatment temperature to assess the relationship between Hsp70 accumulation and thermotolerance. Hereafter, 'controls' refer to *Drosophila* that were maintained at 25 °C with neither pretreatment nor heat shock.

To measure acute thermotolerance of wandering-phase third-instar larvae, groups of 8–10 larvae were transferred to 35 mm plastic culture dishes in which the bottom was covered with a paper towel moistened in PBS. Each dish was covered and sealed with Parafilm. This entire assembly was submerged approximately 3 cm beneath the surface of a thermostatted water bath. Larvae were either transferred directly from 25 °C to 39 °C (heat shock) or given various pretreatments preceding the heat shock. Pretreatments were 60 min at 36 °C followed by specified periods at 25 °C. In each case, survival time at 39 °C was recorded as follows: when the beam of a fiber-optic illuminator was directed through the culture dish, larvae inevitably sought to evade the beam as long as they were able to move. Larvae at 39 °C were periodically probed with the light beam. Cessation of the photoaversive behavior was equated with death, as larvae that did not move in response to the beam were moribund and never recovered thereafter. Occasionally, larvae pupated during determinations; such individuals were excluded from the analysis.

To measure developmental thermotolerance of pupae, larvae that had pupated during the preceding 24 h were transferred to plastic culture dishes and heat-shocked with and without pretreatment as described above. For pupae, however, the pretreatment was either 36 °C for 2 h or 36 °C for 1 h followed by 25 °C for 1 h, and the heat shock was 41 °C for 1 h. After heat shock, pupae were scored once or twice daily for emergence of adult flies until eclosion ceased. Samples of control pupae were treated similarly but were not exposed to the 41 °C heat shock.

Results

No Hsp70 was detected in larvae or pupae that had not undergone pretreatment or heat shock.

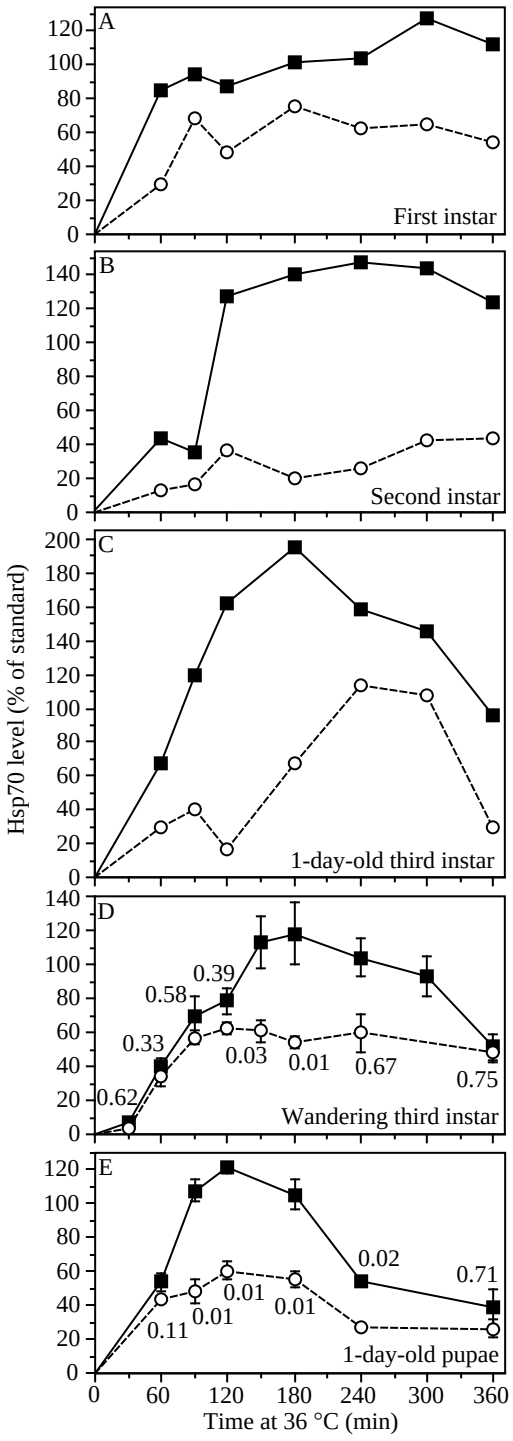

Fig. 1. Hsp70 accumulation during exposure to 36 °C in *Drosophila melanogaster* at several developmental stages. (A) First instar; (B) second instar; (C) first day of third instar; (D) wandering-phase third-instar; (E) 1 day after the start of pupation. Hsp70 levels were measured by ELISA in whole-body lysates using a monoclonal antibody specific for Hsp70. Results are expressed relative to the signal obtained with a standard derived from heat-shocked *Drosophila* tissue culture cells (see Materials and methods). Each point in A–C represents a single pooled lysate of multiple (N=2–35) larvae. D and E plot means ± one standard error; N=5–13. Solid line and filled squares, extra-copy strain; broken line and open circles, excision strain. The number by each pair of mean values is the P value resulting from a Mann–Whitney U-test comparing the Hsp70 levels of extra-copy and excision larvae.

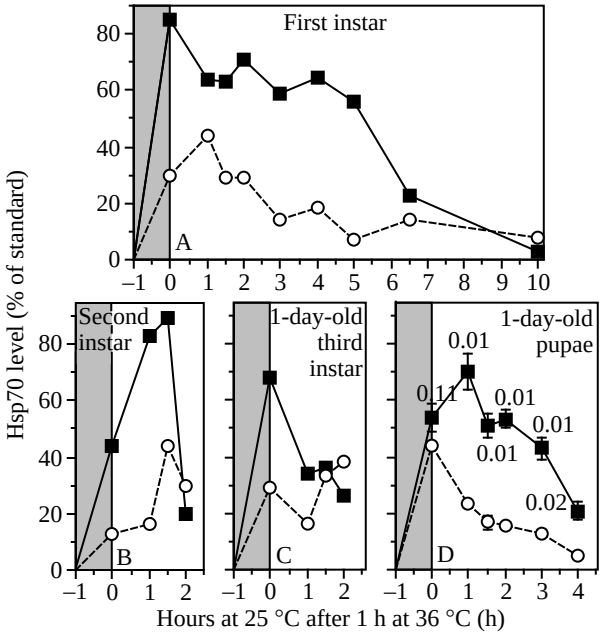

Fig. 2. Hsp70 accumulation in *Drosophila melanogaster* at several developmental stages during and after recovery from a 1 h heat shock at 36 °C. (A) First instar; (B) second instar; (C) first day of third instar; (D) 1 day after the start of pupation. Data are plotted as in Fig. 1. Shaded areas represent the time of exposure to 36 °C. Note that comparable data for wandering-phase third-instar larvae are given in Fig. 4B.

In wandering third-instar larvae, Hsp70 concentrations were much higher after a 2 h exposure to 36 °C than after a 2 h exposure to 32 °C, 34 °C or 38 °C (see Materials and methods). Accordingly, subsequent analyses used 36 °C to explore how length of heat shock, developmental stage and genotype affected Hsp70 accumulation (Fig. 1): Hsp70 levels increased with duration of exposure to 36 °C, and then either stabilized or decreased with additional exposure. The various developmental stages differed in the kinetics and magnitude of Hsp70 accumulation. In virtually every case, extra-copy larvae and pupae exhibited higher levels of Hsp70 than excision larvae and pupae, with the difference ranging from 10 to 700 %.

During recovery at 25 °C after 36 °C pretreatment, Hsp70 levels in larvae required at least 2 h to return to control levels, and sometimes much more (Figs 2–4). After heat shocks of 1 and 1.5 h, Hsp70 concentrations still had not returned to initial levels after 7 h of recovery at 25 °C in wandering-phase third-instar larvae (see Figs 3, 4B). Moreover, Hsp70 levels often (but not always) continued to increase after cessation of heat shock. For example, in second-instar larvae (Fig. 2B) and in wandering-phase third-instar larvae of the extra-copy strain (see Fig. 4B), Hsp70 concentrations more than doubled during the first hour at 25 °C after a 1 h heat shock. With few exceptions, Hsp70 levels were again significantly greater in extra-copy larvae than in excision larvae at comparable times and developmental stages.

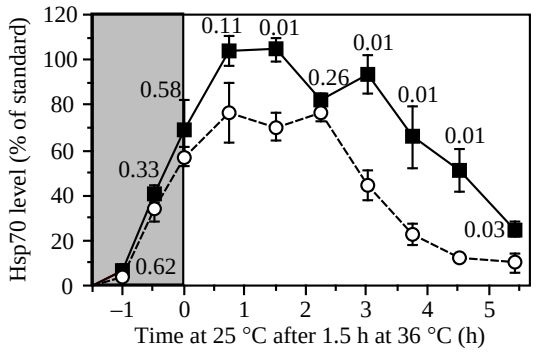

Fig. 3. Hsp70 levels during and after recovery from a 1.5 h heat shock at 36 °C in lysates of wandering-phase third-instar larvae. Data are plotted as in Fig. 1. Shaded areas represent time of exposure to 36 °C.

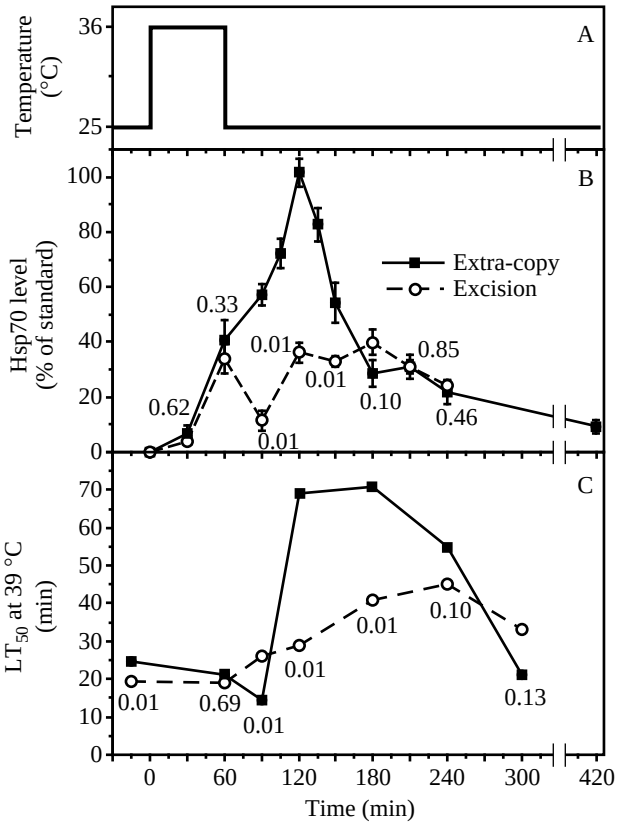

Fig. 4. The relationship between Hsp70 levels and thermotolerance in wandering-phase third-instar larvae of the extra-copy and excision strains. (A) Temperature during the experiment. (B) Hsp70 concentrations. Data are plotted as in Fig. 1D. (C) Thermotolerance. At each time point, larvae were transferred to 39 °C and the LT_{50} was determined. The number underneath each pair of values is the *P* value resulting from a log rank test comparing the survival times of extra-copy and excision larvae. Each point represents 13–18 larvae.

1-day-old pupae resembled larvae in Hsp70 accumulation during and after exposure to 36 °C (Figs 1E, 2D, 5C,E). No increase in Hsp70 was detectable in pupae either during or after a 1 h exposure to 41 °C without pretreatment (data not shown). If pupae were exposed to 36 °C before transfer to 41 °C,

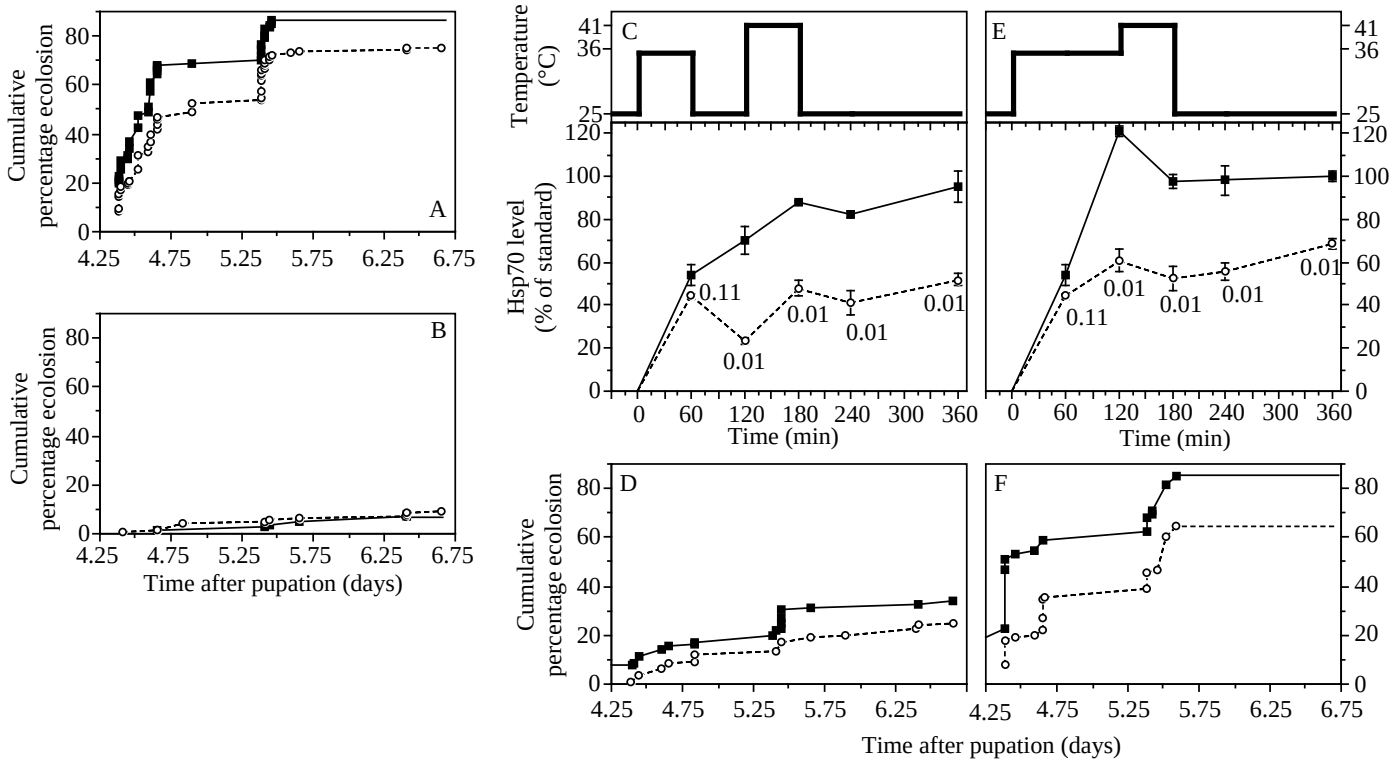

Fig. 5. Relationships among pretreatment regimes, Hsp70 accumulation and thermotolerance of pupae from the extra-copy and excision strains. Temperatures, Hsp70 levels and strains are plotted as in Figs 2–5. Cumulative percentage eclosion is the percentage of pupae in a sample from which adults had emerged by the indicated time point. (A) Eclosion in pupae maintained at 25 °C. (B) Eclosion in pupae administered a 1 h heat shock at 41 °C after 1 day of pupation. No Hsp70 was detectable in the pupae represented in A and B. C and E show temperatures and Hsp70 levels of pupae during exposures to two different pretreatment regimes and a 1 h heat shock at 41 °C after 1 day of pupation. Each point represents the mean of 4–6 samples. For further details, see Fig. 1. D and F show eclosion success of pupae that underwent the temperature regimes depicted in C and E, respectively. Initial sample sizes in percentage eclosion studies (extra-copy, excision): A, 105, 120; B, 180, 195; C, 105, 105; D, 75, 90.

however, Hsp70 levels were elevated above control levels during the 41 °C heat shock and remained elevated for at least several hours thereafter. Extra-copy pupae had significantly higher levels of Hsp70 after 36 °C pretreatment than did excision pupae, and this difference persisted during and after a 1 h exposure to 41 °C. Hsp70 accumulated to a higher level during and after a 2 h heat shock at 36 °C than after a 1 h heat shock at 36 °C followed by 1 h at 25 °C.

Pretreatment at 36 °C markedly increased thermotolerance in wandering third-instar larvae and in 1-day-old pupae of both strains (Figs 4, 5). At 39 °C, control larvae had an LT_{50} of 20 min; the LT_{50} more than doubled after a 1 h pretreatment at 36 °C. Approximately 75 % of pupae maintained at 25 °C successfully eclosed as adults. This proportion decreased to 8 % in pupae exposed to 41 °C for 1 h without pretreatment. Pretreatment for 1 h at 36 °C followed by 1 h at 25 °C increased eclosion success after a 41 °C heat shock to 30 %; pretreatment for 2 h at 36 °C restored eclosion success to the same level as in pupae maintained at 25 °C.

While these changes in thermotolerance are consistent with the presumed cytoprotective effects of Hsps, they neither exclude other cytoprotective mechanisms nor establish which of the many Hsps may be mediating the changes. The differences between the extra-copy and excision strains, however, strongly implicate Hsp70 (Figs 4, 5): in the wandering third-instar larvae, extra-copy larvae exhibited a greater and a more rapid increase in both Hsp70 accumulation and thermotolerance than did excision larvae (Fig. 4). The improvement in thermotolerance, moreover, corresponds with the peak in Hsp70 accumulation in the extra-copy strain. Not until 3 h after heat shock did excision larvae attain the same thermotolerance as extra-copy larvae, by which time thermotolerance had declined from its maximum in the extra-copy line. In pupae, strain differences in tolerance are equally striking. For both pretreatment regimes, the protective effect of pretreatment was greater in the extra-copy pupae than in the excision pupae. A 2 h pretreatment at 36 °C was sufficient to protect pupae of the extra-copy strain against the effects of exposure to 41 °C (Fig. 5).

Discussion

Methodology

The *Hsp70* family of *Drosophila* comprises several genes, which differ in the kinetics and magnitude of their inducibility and encode proteins that play diverse cellular roles (see

Introduction). Frequently, however, investigators use techniques that cannot distinguish among these genes or their products either qualitatively or quantitatively, yielding equivocal results and making interpretation difficult (Feder, 1996). The present study exploits a specific antibody and an ELISA to quantify Hsp70 (*sensu strictu*) accumulation. While this technique resolves some of the problems of earlier work, it is not without pitfalls. We have analyzed whole-body lysates of larvae and pupae, and have standardized our results on the basis of the Hsp70-specific signal per unit of soluble protein. Thus, non-specific differences in total soluble protein (e.g. gut contents, cells that are dead or unresponsive to heat shock) have the potential to bias results. Accordingly, while comparisons of similar developmental stages are likely to be robust, comparisons among stages may be particularly prone to such bias.

ELISA of whole-body lysates, moreover, cannot resolve whether Hsp70 is uniformly distributed throughout larvae and pupae or whether it is highly concentrated in certain cell types or tissues and absent in others. The few studies employing Hsp70-specific probes show that diverse cell types of larvae express Hsp70 during and after stress in wild-type *Drosophila* (Velazquez and Lindquist, 1984; Palter *et al.* 1986). Malpighian tubules, by contrast, show no evidence of detectable Hsp70 after heat shock (Singh and Lakhotia, 1995).

Results

Expression and cellular concentration of Hsp70 are tightly regulated at multiple levels (Lindquist, 1993). Accordingly, although the construction of the experimental strains added 12 extra copies of *Hsp70* to the genome, increased concentrations of Hsp70 during heat shock or recovery is not a foregone outcome. Welte *et al.* (1993), however, demonstrated that 6 h embryos of the extra-copy strain achieved nearly twice the Hsp70 concentration of excision embryos of similar age. Moreover, Welte *et al.* (1993) demonstrated specific enrichment in mRNA transcribed from the transgenic allele, as opposed to a general increase in *Hsp70* transcription. The present study shows that the differences between extra-copy and excision strains in Hsp70 concentration extend to all pre-adult developmental stages, except pre-blastoderm embryos. A similar pattern is evident in adults (M. Tatar and J. Curtsinger, personal communication). At some stages (e.g. Fig. 2), the strains differ by as much as sixfold in Hsp70 levels. Strains also differ in the kinetics of change in Hsp70 concentration. In first-, second- and early third-instar larvae at 36 °C, for example, extra-copy larvae achieve peak Hsp70 concentrations more rapidly than do excision larvae of the same stage. Similarly, Hsp70 levels remain elevated after heat shock for longer in first- (Fig. 2A) and early third-instar (Fig. 2C) larvae and in pupae (Fig. 2D) than in excision organisms of the same stage.

In general, these findings validate the comparison of extra-copy and excision larvae and pupae to elucidate the importance of Hsp70 for thermotolerance and emphasize the importance of specifying the time course of variation in Hsp levels before investigating their physiological significance. The differing kinetics of Hsp70 accumulation specify optimal time points for comparing the extra-copy and excision strains. In wandering third-instar larvae, for example, extra-copy and excision strains do not differ significantly in Hsp70 levels after 60 min at 36 °C, and do not differ maximally until after an additional 60 min of recovery at 25 °C (Fig. 4B). During continual exposure to 36 °C, such larvae do not differ maximally in Hsp70 accumulation until 2.5–3 h has elapsed (Fig. 1D). With even more prolonged exposure to 36 °C, Hsp70 levels decline and eventually become similar in the two strains. Times of maximal difference in Hsp70 accumulation, moreover, vary among developmental stages.

Drosophila undergo profound changes in thermotolerance during embryogenesis (Welte *et al.* 1993). In wild-type strains maintained at 25 °C, basal thermotolerance increases progressively during the first 12 h of embryogenesis and is constant thereafter. The effect of *Hsp70* copy number on inducible thermotolerance is most dramatic in 6 h embryos, in which Hsp70 accumulation was rate-limiting for inducible thermotolerance. In 12 h embryos, in which basal thermotolerance has completed its initial increase and a heat shock response is well developed, extra-copy strains show no advantage over excision strains in the induction of thermotolerance (Welte *et al.* 1993). Thus, according to these findings of Welte *et al.* (1993), the dramatic benefits of increasing *Hsp70* expression might be limited to a particularly sensitive stage of embryogenesis. To the contrary, the present study demonstrates that extra copies of *Hsp70* result in increased inducible thermotolerance in wandering third-instar larvae and in 1-day-old pupae (Figs 4, 5).

A second way in which the present study extends the work of Welte *et al.* (1993) is in its ecological relevance. In *Drosophila*, oviposition peaks late in the photophase (David *et al.* 1983). Although rates of embryonic development vary broadly with temperature and other conditions, a large fraction of *Drosophila* embryos in the field will probably have developed beyond the critical period for Hsp70-dependent thermotolerance before they experience significant thermal stress. Larvae and pupae, by contrast, clearly experience thermal stress in the field during developmental stages at which Hsp70 expression plays a critical role in tolerance. Larvae inhabit necrotic fruit, which is prone to overheating if it is sunlit, and can experience temperatures that are both harmful in their own right and sufficient to induce Hsp70 expression in the field (see Introduction). Pupae, which are immobile, are at similar risk if attached to a warm fruit or otherwise exposed. Although necrotic fruits do not naturally undergo the stepwise temperature transitions we have imposed in the laboratory (Feder, 1996), they clearly attain the temperatures of our laboratory pretreatment, stress and recovery (25–41 °C). Thus, unless wild *Drosophila* differ dramatically from our laboratory strains, both the generalized inducible thermotolerance demonstrated in wandering third-instar larvae and 1-day-old pupae and the enhanced inducible thermotolerance in the extra-copy strain ought to have significant consequences for the

fitness of *Drosophila* in their natural setting. Moreover, *Drosophila* in the wild vary significantly in their capacity for Hsp70 expression, and this variation is correlated with their thermotolerance (R. A. Krebs and M. E. Feder, unpublished data). Many studies have previously demonstrated a correlation between the heat-shock response and natural thermal stress in a manner that is consistent with adaptive significance (e.g. Lindquist and Craig, 1988; Hofmann and Somero, 1995), but this work is the first to demonstrate such a direct link; it sets the stage for experimentation with extra-copy and excision strains in the field.

Hsp70 of *Drosophila* is unusual in several respects. First, *Drosophila* lack any detectable expression of Hsp100 family proteins (Sanchez and Lindquist, 1990; Feder and Lindquist, 1992), which occur in many other organisms and play an important role in inducible thermotolerance (Parsell and Lindquist, 1993). Second, *D. melanogaster* has naturally undergone extensive duplication of the *Hsp70* gene, with at least 10 copies per diploid genome in modern strains (Ish-Horowicz *et al*. 1979). In yeast, overexpression of Hsp70 can partially compensate for the loss of thermotolerance caused by deletion of the major Hsp100 gene involved in thermotolerance, *HSP104* (Sanchez *et al*. 1993). The amplification and overexpression of *Hsp70* in *Drosophila* are clearly resonant with the findings of the present study. It may be that *Drosophila* has amplified its *Hsp70* genes in part to compensate for the loss of Hsp100 expression, and that *Drosophila* depends more heavily upon Hsp70 for thermotolerance than most other organisms.

However, although Hsp70 is beneficial in tolerating heat stress and extra copies of *Hsp70* extend these benefits, the present *Hsp70* copy number may represent an upper limit to the evolutionary amplification of this gene. Indeed, the wild-type *Hsp70* copy number and levels of Hsp70 expression may have evolved as a compromise between the beneficial and deleterious effects of Hsp70. Expression of Hsp70 is strongly repressed in the absence of stress (Velazquez *et al*. 1983; Lindquist, 1993); if not, growth and cell division suffer as a result in *Drosophila* cells in culture (Feder *et al.* 1992). Furthermore, newly hatched larvae of the extra-copy strain die at greater rates than excision larvae when their Hsp70 levels are increased chronically by repeated mild heat shock (R. A. Krebs and M. E. Feder, in preparation). Possibly, in such circumstances, Hsp70 may bind target proteins inappropriately, doing more harm than good. In any event, the elucidation of the costs and benefits of Hsp70 expression remains a challenge for future research.

In a broader context, the present study is significant because it represents the first use of genetic engineering as a tool in animal ecological and evolutionary physiology. The use of site-specific homologous recombination yields sister strains of *Drosophila* differing solely in copy number of a single selected gene, with the excision strain serving as a matched control for insertional mutagenesis. In so doing, it directly addresses the '*ceteris paribus*' problem of comparing complex phenotypes unambiguously (Lewontin, 1978; Feder, 1996). Although the mechanisms whereby variation in Hsp70 accumulation results in variation in thermotolerance still require physiological analysis for their resolution, the ability of genetic engineering techniques to create controlled variation at the most fundamental level will facilitate such ecophysiological analysis enormously (Feder and Block, 1991; Welte *et al.* 1993).

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