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A Comparison of Hsp70 Expression and Thermotolerance in Adults and Larvae of Three Drosophila Species

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A comparison of Hsp70 expression and thermotolerance in adults and larvae of three *Drosophila* species

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Abstract Heat shock proteins (Hsps) and other molecular chaperones perform diverse physiological roles. One is to facilitate, in part, organismal thermotolerance, of which the functional consequences depend on Hsp70 concentration and developmental stage in *Drosophila melanogaster*. To test whether an Hsp70-thermotolerance relationship is a general phenomenon within *Drosophila*, I assayed Hsp70 concentration at a range of temperatures in intact larvae and adults of three species, *D. melanogaster*, *D. simulans*, and *D. mojavensis*, and compared those results to the increase in survival to heat shock that occurs after an Hsp70 inducing pretreatment. Larvae of *D. melanogaster* and *D. simulans* responded similarly to heat; they expressed Hsp70 maximally at 36–37°C, and their tolerance of 1 h heat shocks increased by 1.5–2°C. By contrast, *D. mojavensis*, which tolerates higher temperatures than do *D. melanogaster* and *D. simulans*, expressed Hsp70 only at higher temperatures, although the 36°C pretreatment still increased thermotolerance. Critically, the temperature that maximally induced Hsp70 was a poor inducer of thermotolerance in *D. mojavensis* and may have harmed larvae. Results for *Drosophila* adults, which tolerated heat poorly compared to larvae, likewise suggest that a close link between peak Hsp70 expression and maximal induction of thermotolerance is a feature of *D. melanogaster*, and not of the other species. Neither *D. simulans* nor *D. mojavensis* adults increased tolerance after exposure to the temperatures that maximally induced Hsp70.

INTRODUCTION

Cells and whole organisms become thermotolerant after exposure to non-lethal high temperatures. This response is due at least in part to production of heat shock proteins (Hsps) (Stephanou et al. 1983, Velazquez and Lindquist 1984, Li et al. 1991, Solomon et al. 1991, DiIorio et al. 1996). The *Drosophila* Hsp70, for example, is virtually absent from unstressed cells (Velazquez et al. 1983), but may account for the bulk of protein synthesis minutes after exposure to high temperature (Palter et al. 1986). Thermotolerance of *D. melanogaster* varies with the amount of Hsp70 present prior to exposure to heat stress; where concentrations are low, so too is tolerance, and engineering lines to overproduce Hsp70 can increase thermotolerance in some stages of development (Welte et al. 1993, Feder et al. 1996). Although other aspects of cell physiology also affect responses to temperature change, thermotolerance is much reduced in the absence of Hsps (Riabowol et al. 1988, Solomon et al. 1991, Lee et al. 1993, Jedlicke et al. 1998).

Despite a general role for Hsps in cellular and organismal responses to a variety of stresses (Lindquist 1986), individuals from natural populations may vary in expression of these proteins (Krebs and Feder 1997a). How the evolution of induced Hsp70 expression may correlate with the environment inhabited by different species, however, remains an open question. For one, it is not clear whether individuals from hot environments will express more Hsp70 than will those from cooler localities. Preliminary results suggest that this pattern exists for Chrysmelid beetles, as individuals from low altitudes express more Hsp70 than do those from high altitudes (Dahlhoff and Rank 1998). Because Hsps may impose fitness costs on growth, particularly at non-lethal temperatures (Dorner et al. 1992, Krebs and Feder 1997b),

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environmental correlates with Hsp70 expression are not readily predictable. Potentially, warmer temperatures may select for tighter controls to limit repetitive expression, as suggested from results on *D. melanogaster* maintained for years at 28°C. These flies express less Hsp70 than do comparable lines evolving at 18°C or 25°C (Bettencourt et al. 1999).

To examine evolutionary changes in expression of Hsp70 and thermotolerance, I compared the thermal induction curves between Hsp70 and thermotolerance in three species of *Drosophila*, two common cosmopolitan species, *D. melanogaster* and *D. simulans*, and one distantly related member of the genus, *D. mojavensis*, which inhabits necrotic cactus in the Sonoran desert of the USA and Mexico. One preliminary report suggests that *D. mojavensis* produces Hsp70 mRNA at temperatures above those at which the other two species can synthesize proteins (data of S. Lindquist, in Huey and Bennett 1990). Therefore, I questioned whether differences in these species habitats and their Hsp70 expression patterns correlate with induced tolerance of high temperatures, here defined as the ability to increase tolerance as a response to temperature changes. I also asked whether similar physiological changes occur in both larvae, where growth requirements are inhibited by Hsp70, and in adults, a stage which may benefit from very high Hsp70 expression (Krebs et al. 1998). If Hsp70 expression evolved as a direct response to the thermal environment, similar temperatures should maximally induce both thermotolerance and the expression of Hsp70 in larvae and adults of these three species. I found that only Hsp70 expression of *D. melanogaster* followed these predicted patterns, and for one or both developmental stages, expression peaked at damaging temperatures in *D. simulans* and in *D. mojavensis*.

MATERIALS AND METHODS

Origin of strains

The three species of *Drosophila* used for these studies derived either from a collection in Garwood Orchards, LaPorte, IN, USA, 41° 36.4′ N, 86° 43.2′ W in August 1996 (described in Krebs and Feder 1997a), from which I obtained *D. melanogaster* and *D. simulans*, or from a collection of *D. mojavensis* near San Carlos, Sonora, Mexico in February 1997 (Arizona State strain designation, SOSC0297). Experiments commenced in summer 1998, and, therefore, strains had been reared in the laboratory for 1–2 years. However, laboratory rearing at moderate population sizes, such as four bottles per generation (>200 individuals), has minimal effects on either thermotolerance or Hsp70 expression within this time-frame (R. Krebs, unpublished data).

D. melanogaster and *D. simulans* are well known human commensal species that are readily found almost anywhere fruit may rot, while *D. mojavensis* is a generalist species among those that breed in necrotic tissue of cactus. Its hosts include several columnar cacti, organ pipe and agria, and the fruits of Opuntia in the deserts of SW North America (Heed and Mangan 1986). Consequently, the habitats of these species may simply be characterized as either temperate (*D. melanogaster* and *D. simulans*), where heat stress may be common for larvae in summer (Feder 1996), or hot-arid, where high air temperatures and strong sunlight may expose both larvae and adults to high temperatures for much of the year (Krebs and Bean 1991).

Hsp70 assays

Hsp70 expression was measured from larvae and adults of each species with an Hsp70 specific antibody, 7FB, produced from purified *D. melanogaster* Hsp70 (Velazquez et al. 1983). Western blot analysis indicates that this antibody will react to Hsp70 of *D. simulans* (R. K. unpublished data) and to that of *D. buzzatii* (V. Loeschcke, personal communication). *D. buzzatii* is a close relative of *D. mojavensis*. To support those results, I fixed tissues of heat treated *D. mojavensis* third-instar larvae, and stained them with 7FB and a fluorescent secondary antibody (R. Krebs, unpublished data). Staining of *D. mojavensis* closely matched that of *D. melanogaster* (Krebs and Feder 1997c), which suggests that the antibody recognizes the same protein in these species. The antibody does not react to Hsc70 or to any constitutively produced chaperone in *D. melanogaster*, because non-heat-treated flies fail to stain either in enzyme linked immunosorbent assay, ELISA (Feder et al. 1996), or by immunocytochemistry, except for a few cells (Krebs and Feder 1997c).

Neither do individuals of *D. simulans* of *D. mojavensis* stain at detectable levels in ELISA with the 7FB antibody unless they receive heat treatment (R. Krebs, unpublished data).

For quantitative analysis of Hsp70, I assayed lysates of heat treated flies by ELISA, which has been described elsewhere (Welte et al. 1993, Feder et al. 1996). The ELISA signal is proportional to Hsp70 concentration in the lysates and is expressed as a proportion of the maximum signal obtained for each life stage within these experiments. Adult *Drosophila* normally express only about half the amount per unit protein as do first-instar larvae (Krebs et al. 1998). Control between experimental replicates was obtained by comparing all samples to a pooled lysate of *D. melanogaster* 3rd-instar larvae that were exposed to 36°C for 1 h and then 25°C for 1 h before lysis.

For treatment, I harvested first-instar larvae from the surface of medium 2–8 h after hatching and collected

recently emerged adults after brief anesthesia with CO_2. All flies matured before use; adult *D. melanogaster* and *D. simulans* mature by 3–4 days old, and *D. mojavensis* in 6–8 days (Markow 1982). Different ages among species were required to balance results for Hsp70 expression with those obtained for thermotolerance. Senescence reduces thermotolerance (Stratman and Markow 1999), while immature flies have very high tolerance of stress relative to those that have sexually matured (Krebs et al. 1998). Because the species differ in life history, such that *D. mojavensis* does not reach full sexual maturity until about the time *D. melanogaster* and *D. simulans* begin to senesce, assays focused on ages at which each species exhibited sexual maturity. The collection procedure also gave all flies several days or more to recover from anesthesia before the heat shock, although CO_2 causes little stress (Krebs et al. 1995).

To induce expression of Hsp70, groups of first-instar larvae or pairs of adults were placed within cryotubes with 10 µl phosphate buffered saline (to prevent desiccation). These tubes were submerged in a water bath for 1 h at one of a range of temperatures, placed 1 h at 25°C, and then quick-frozen in liquid N_2.

Thermotolerance assays

Thermotolerance was determined by survival to an extreme stress. Larvae and adult flies for these assays were collected similarly to methods used to estimate expression of Hsp70, except that treatments were performed in glass vials that contained 8 ml of a yeast, cornmeal, molasses and agar *Drosophila* rearing medium. Forty *D. melanogaster* or *D. simulans* larvae were placed in each vial, with 5 vials prepared per temperature treatment. *D. mojavensis*, however, suffers high mortality when larvae compete for pupation sites near the surface of medium; later developing larvae knock loose and drown many individuals that pupate first. Therefore, the analyses presented for this species used 8 vials of 20 larvae at each temperature (although temperature effects were similar whether reared at densities of 20 or 40 per vial). Data on all species were the proportion of emerging adults from each vial.

Each replicate measurement on mature adults involved either 20 males or 20 females either 3–4 days after collection for *D. melanogaster* and *D. simulans*, or 7 days after collection for *D. mojavensis*, as was done for Hsp70 expression. Vials of flies were capped with a moistened stopper above a cotton plug, inverted in a rack with vials evenly spaced, immersed in a water bath for 1 h at the specified temperature or first heated 1 h at 36°C, placed in a 25°C incubator for 1 h, and then immersed in a water bath at the higher temperature. In total, 100 males and 100 females of each species were treated at each temperature. Data were the proportion that could walk 24 h after heat treatment.

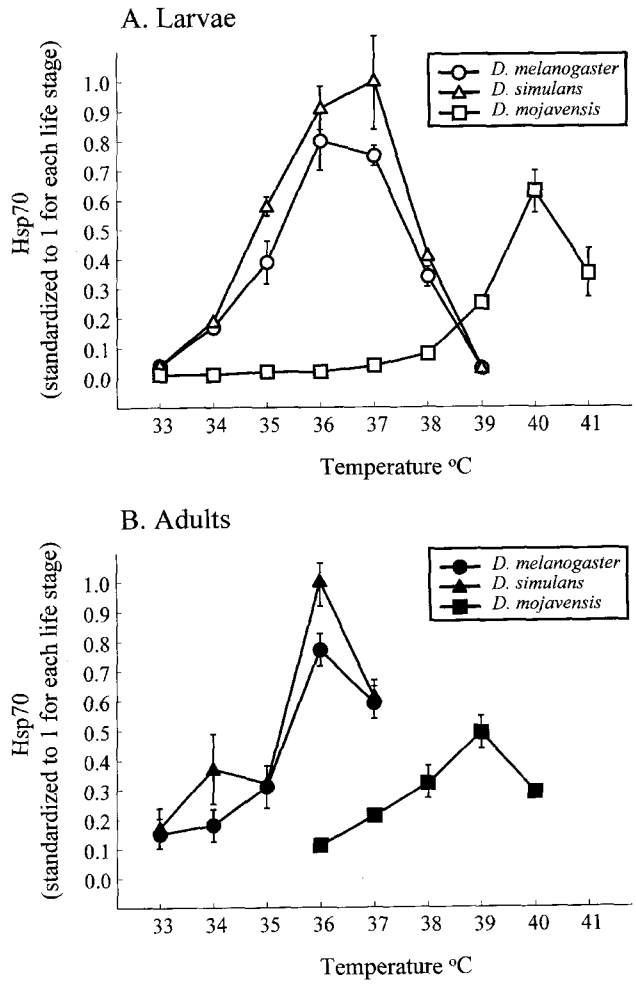

Fig. 1 Expression of Hsp70 in (A) 1st-instar larvae (open symbols) and in (B) adults (filled symbols) of three species of *Drosophila* following a 1 h treatment at the temperature indicated and 1 h at 25°C. Hsp70 concentration for whole tissue samples was standardized to the maximum within larvae and adults across all species to more easily compare the temperature dependence of expression in each species. Expression in adults is about 1/2 that of 1st-instar larvae.

For statistical analysis, frequency data were arcsine-square-root transformed, which increases the variances for means close to 0 or 1, and thereby reduces heterogeneity of variances. For survival data, effects of gender, temperature and the interaction between these two factors were tested in general linear models. Species usually differed for the temperatures where effects became pronounced, making statistical analysis with complete data problematic (i.e., treatment temperatures only partially overlapped among species). Nonetheless, the large magnitude of species differences suggested that exact tests of hypotheses about species variation at each temperature are unnecessary.

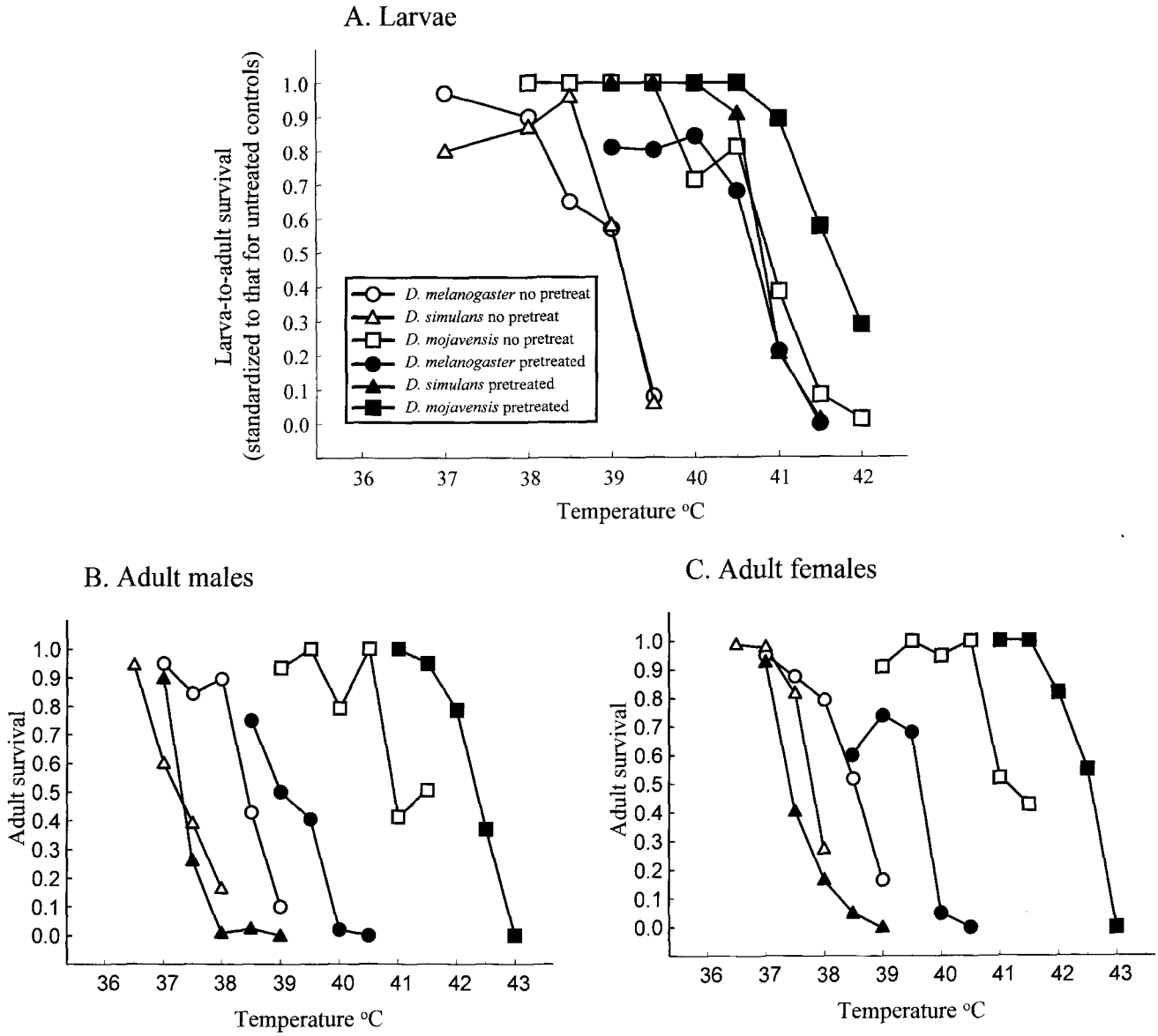

Fig. 2 Thermotolerance of three species of *Drosophila*: (A) first-instar larva-to-adult survival after heat shock and the proportion of adult (B) males or (C) females that could walk 24 h after exposure to a thermal stress. Groups of D. melanogaster (circles), *D. simulans* (triangles) or *D. mojavensis* (squares) either were exposed directly to a high temperature stress for 1 h or they first were pretreated at 36°C for 1 h and then 25°C for 1 h before exposure to the high temperature stress. Filled symbols indicated exposure with the pretreatment

RESULTS

Hsp70 expression

The two related temperate species, *D. melanogaster* and *D. simulans*, expressed Hsp70 in a similar thermal pattern (Fig. 1). Each expressed little Hsp70 below 33°C, and in both species, expression peaked at 36°C in adults and at 37°C in larvae. At higher temperatures, expression declined, although measurements above 37°C in adults of these species were omitted because these temperatures may kill some individuals. In contrast to the fruit-feeding generalists, Hsp70 expression in the cactophilic *D. mojavensis* peaked at a higher temperature, 39°C in adults and 40°C in larvae.

Thermotolerance

Variation in Hsp70 expression among these species enabled a test of the evolutionary relationship between the expression of this one protein and two measures of thermotolerance, basal thermotolerance (survival measured after a direct 1 h exposure to a high temperature) and inducible thermotolerance (the increase in

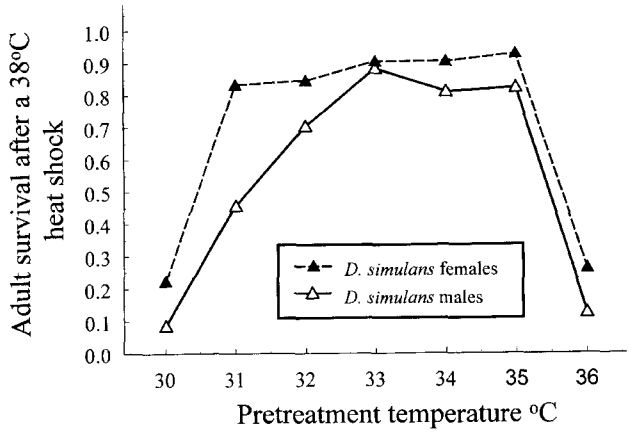

Fig. 3 Survival of *Drosophila simulans* adults to a heat shock after pretreatment at various temperatures. Groups of individuals were first pretreated for 1 h at one of the temperatures indicated, stored at 25°C for 1 h and then exposed to 38°C. Filled symbols indicate results for females, open symbols are those for males.

thermotolerance after pretreatment at a high, but non-lethal temperature). In larvae, survival of *D. melanogaster* and *D. simulans* after a direct exposure to thermal stress was similar, while *D. mojavensis* larvae survived temperatures that killed most larvae of the other two species (Fig. 2A).

Pretreatment at 36°C for 1 h shifted survival curves 1.5 to 2 degrees higher for *D. melanogaster* and *D. simulans* larvae. The increase in thermotolerance of *D. mojavensis* was a more modest one degree. One concern was that the 36°C pretreatment used for all larvae induced little Hsp70 in *D. mojavensis*, but it induced high expression in *D. melanogaster* and *D. simulans*. However, higher pretreatment temperatures failed to improve tolerance of larval *D. mojavensis*, and pretreatment at 39°C, where expression of Hsp70 becomes significant, greatly reduced survival to a 41.5°C heat shock. The proportion of larvae that survived after pretreatment at 36°C was 0.24; at 37°C it was 0.20; at 38°C it was 0.13; and at 39°C, it was 0.04, N=14 vials per group. Mean survival after a 39°C treatment differed significantly from all others ($P<0.05$, Tukey's multiple range test).

Results on adults in these species provided a somewhat different picture of variation among species (Fig. 2B). *D. mojavensis* was again the most thermotolerant, but adults of *D. simulans* survived the stress poorly relative to adults of *D. melanogaster* both with and without a heat pretreatment that maximized Hsp70 expression before the stress exposure. This difference between the closely related species existed in spite of their very similar Hsp70 thermal expression curves. Like larvae of *D. mojavensis*, adults of *D. simulans* do not become thermotolerant from pretreatment conditions that maximize Hsp70; survivorship after a 36°C pretreatment was no better than that without any pretreatment. The adults did become more thermotolerant where pretreatment was performed at lower temperatures: 31–35°C (Fig. 3).

DISCUSSION

Species in the genus *Drosophila* vary both in their expression pattern for Hsp70 and in thermotolerance, but how these traits coevolve is unclear. On one hand, the desert species, *D. mojavensis*, is much more thermotolerant than is either *D. melanogaster* or *D. simulans* and it expresses Hsp70 only at much higher temperatures. On the other hand, the temperatures that induce maximal expression of Hsp70 may not be those which induce thermotolerance best in each species.

Of the three species analyzed, only *D. melanogaster* shows a close relationship between Hsp70 expression and an increase in thermotolerance after exposure to high temperatures in both larvae and adults. Too little Hsp70 expression, induced either by shortening treatments at 36°C, by reducing pretreatment temperature (Krebs and Feder 1998), or by deactivating the heat shock response (Jedlicke et al. 1998), all restrict the level of thermotolerance that develops. Too much Hsp70, however, also can be a problem; *D. melanogaster* lines that carry many extra hsp70 copies tolerate stress less well than do control lines with normal copy number under pretreatment conditions that maximally induce Hsp70 expression (Krebs and Feder 1997b). Use of the transgenic lines verified that Hsp70 variation accounts for at least some of the effects on thermotolerance, and results from them suggest that optimal expression levels may exist in *D. melanogaster* (Krebs and Feder 1998).

In contrast to these results for *D. melanogaster*, here I find that in both *D. simulans* (adult stage) and *D. mojavensis* (larvae and adults), large quantities of Hsp70 in advance of the stress are not required to achieve high thermotolerance. Notable in *D. melanogaster* is that the greatest incremental benefit to thermotolerance appears while expression of Hsp70 remains very low; for example 10 minutes at 36°C greatly enhances survival (Krebs and Feder 1998). While stronger inducing treatments help more, the marginal benefit per additional unit Hsp70 is less. One explanation for how small amounts of Hsp70 present prior to a stress exposure can provide nearly maximal thermotolerance is that when present, Hsp70 may protect its own expression. Drosophila melanogaster continues to make Hsp70 at high temperatures (>38°C) only if some Hsp70 is present, but not when flies are heated directly to this temperature without pretreatment (Krebs and Feder 1997b). Transcription and translation are both temperature sensitive processes (DiDomenico et al. 1982) and molecular chaperones may extend the temperature range at which they occur.

The second phenomenon that requires explanation is why Hsp70 expression follows lethal temperatures much more closely in one species than in others. Temperatures that maximally induce Hsp70 in *D. mojavensis* and in *D. simulans* adults weaken the animal's ability to survive a more extreme stress, which suggests that these treatments cause damage. The relationship between Hsp70 expression and thermotolerance in any species will depend on similarities between the thermal regulation of heat shock proteins and the temperature that causes a lethal lesion. At this time, little is known of how heat kills, although in *D. melanogaster* larvae, gut tissue may be more thermally labile than other tissues and it expresses Hsp70 more slowly (Krebs and Feder 1997c).

To understand how variation in general stress responses relates to variation in Hsp70 induction requires joint investigation of organismal and molecular processes. The present model for the regulation of Hsp70 follows Morimoto et al. (1996) and Satyal et al. (1998): the heat shock transcription factor (HSF) activates expression when trimeric, but is held as a monomer in a competitive complex with Hsp70. When temperatures begin to partially denature proteins, Hsp70 responds to specific exposed non-polar amino acid sequences, binds to these proteins and releases HSF. HSF then trimerizes, binds to the hsp70 promoter, and rapid expression ensues. Species, therefore, should vary in the temperatures that induce Hsp70 where they also differ in the thermostability of cellular proteins. Thermal stability of enzymes often follows environmental gradients in diverse species (Somero 1978). Hsp70 levels also may correlate with changes in ubiquitin in mussels (Hofmann and Somero 1995), a major component of the protein degradation pathway, which suggests a link between Hsp70 expression and protein damage. However, by this model, the breakdown of non-essential proteins may be as likely to induce Hsp70 as would damage to critical elements, and, therefore, species may differ for the relationship between Hsp expression and mortality due to variation in the thermolability of a small number of proteins.

The complexity of Hsp70 regulation extends to differences between thermal expression curves in larvae and in adults. Quantifiable expression of Hsp70, as determined by ELISA, first appears at 37–38°C in *D. mojavensis* larvae and at 1–2°C lower temperatures in their adults (Fig. 1). This developmental difference is consistent for all three species and it therefore, may be a general stage-specific phenomenon in the genus. Because protein expression across development differs considerably (Bate and Martinez Arias 1993), as does the thermal environment (Feder 1996), lower Hsp70 induction temperatures in larvae and in adults also are consistent with the presence of elements that vary in thermolability. The data necessary to directly test this explanation for thermotolerance variation will, however, be difficult to obtain. Perhaps a comparison of protein concentration between heat-treated and untreated individuals or heat-treated larvae and adults on 2-D gels might provide some insight on variation in protein sensitivity to thermal stress, but new synthesis would likely obscure differences in protein loss.

An explanation for the evolutionary relationship between Hsp70 expression and thermotolerance depends on how Hsp70 protects the organism, and how great its role is relative to other cellular components. The relationship between expression and survival among species suggests that cells make Hsp70 as a response to damage, and that this protein does not prevent damage, i.e. its function is to repair cells and to return their function to normal. Support follows from comparative staining of heat shocked larval tissues with vital dyes; similar damage occurs with and without pretreatment (Krebs and Feder 1997c), Hsp70 aids tissue recovery (Krebs and Feder 1998), and, likewise, Hsp70 may speed the rate of recovery of alcohol dehydrogenase activity after heat denaturation (Feder and Krebs 1998). The important point is that no studies indicate that Hsp70 in *Drosophila* stops proteins from becoming denatured, although this function is proposed for other Hsps, like the 104 kD chaperone of yeast (Parsell et al. 1993).

Thus, differences between species suggest that variation in Hsp70 expression lies not simply in regulatory variation in the heat shock response, but in the physiological nature of the cell environment. Caution is, therefore, recommended in the pursuit to understand co-variation between thermotolerance and the expression of chaperones, because variation may be unrelated to the normal control elements of the heat shock response. Furthermore, these results raise a conceptual question as to how the current view of heat shock proteins would differ had the relationship between the thermal expression curve and the inducibility of thermotolerance in *D. melanogaster* have more closely resembled relationships in *D. simulans*, or particularly, those in *D. mojavensis*. Results for neither of these species show close correspondence between these traits. Would the often ill-cited presumption that many Hsps protect cells from damage persist, or would research have focused more on mechanisms of cellular damage and repair? The comparative evidence from *Drosophila* clearly implicates this latter role as primary for Hsp70, and suggests that variation in cell damage may underlie species differences in the regulation of the heat shock response.

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