1999

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Robert A. Krebs
Cleveland State University, r.krebs@csuohio.edu

Brian R. Bettencourt
University of Chicago

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This is a pre-copyedited, author-produced PDF of an article accepted for publication in American Zoologist following peer review. The version of record Krebs RA and Bettencourt BR. 1999. Evolution of thermotolerance and variation in the heat shock protein, Hsp70. American Zoologist 39(6):910-9. is available online at: http://icb.oxfordjournals.org/content/39/6/910.abstract.

Evolution of Thermotolerance and Variation in the Heat Shock Protein, Hsp70[1]

ROBERT A. KREBS*,[2] AND BRIAN R. BETTENCOURT†

*Department of BGES, Cleveland State Univ., 2399 Euclid Ave, Cleveland Ohio 44115
†Department of Organismal Biology and Anatomy, The University of Chicago, 1027 East 57th St., Chicago, Illinois 60637

SYNOPSIS. **Low to moderate levels of stress induce a class of molecular chaperones called heat shock proteins (Hsps), which protect cells, tissues and whole organisms from more severe stress. In higher Eukaryotes, Hsp70 is one of the principle heat-induced chaperones. This response is general, and how much Hsp70 an animal produces correlates with the level of stress to which it is exposed. Nonetheless, definitively linking high Hsp70 expression as an adaptation to stress tolerance is problematic, because organisms and cells respond to stress in many ways. By molecular manipulation of Hsp70 in one animal group, *Drosophila*, differences in *hsp70* copy number are shown to directly influence heat-induced expression of Hsp70 and tolerance of heat. However, too high an expression level of Hsp70 can harm individuals during periods of rapid growth. This strong physiological relationship between Hsp70 concentration and thermotolerance, along with Hsp70's remarkable degree of interspecific coding sequence conservation, suggest that *hsp70* regulatory elements may evolve as an adaptation in diverse species to their thermal environments. To examine this possibility, correlative studies within species and research on phylogenetic covariation between these traits is reviewed with a focus on *Drosophila* species. However, the techniques and results discussed should broadly apply to other animal groups where evolutionary approaches can be used to test whether genetic variation in both thermotolerance and Hsp expression within and among species select locally on either *hsp70* sequence and/or expression.**

INTRODUCTION

Expression of Hsp70 and related Hsps is nearly universal, and these proteins are conserved across all organismal kingdoms (Lindquist, 1986). These protein families, therefore, must have appeared early in cell evolution, and they have diverged into three functional groups: some are constitutively expressed and respond little (if at all) to heat; some possess developmental functions and are also up-regulated after stress; and a smaller group, which has little or no function in organisms in the absence of stress, is massively upregulated after stress. Prominent members of this latter group are some of the 70kD class of Hsps, which are true "heat-shock" proteins. Their function has long been presumed to be protection of individuals from thermal stress (Pelham, 1986).

That heat shock proteins underlie an important part of inducible thermotolerance, and responses to other forms of stress in many organisms is now incontrovertible (Feder, 1999). Cells that lack Hsps certainly will tolerate less stress than will those which express normal levels (Solomon *et al.*, 1991). Phylogenetic analyses link the magnitude of inducible thermotolerance and of expression of Hsps. They suggest, as well, that the temperature initiating expression may vary with average environmental temperature in lizards (Ulmasov *et al.*, 1992) and in ants (Gehring and Wehner, 1995). However, these and similar data from fish and insects (Carretero *et al.*, 1991; Koban *et al.*, 1991; Fader *et al.*, 1994; diIorio *et al.*, 1996) are largely correlative and indicate only that similar conditions induce both Hsp expression and thermotolerance (Hoffmann and Parsons, 1997). Nat-

[1] From the Symposium *Organismal, Ecological and Evolutionary Significance of Heat Shock Proteins and the Heat Shock Response* presented at the Annual Meeting of the Society for Comparative and Integrative Biology, 6–10 January 1999, at Denver, Colorado.

[2] E-mail: r.krebs@popmail.csuohio.edu

urally, because Hsps are induced by a variety of stresses (Lindquist, 1986), their evolution must involve more than climate considerations. Nonetheless, the consistent relationship between Hsp expression and climate in diverse species favor a real Hsp/thermotolerance relationship.

To evaluate what is currently known about the evolution of Hsp70 and its role in thermotolerance we review research, primarily from *Drosophila,* that has used two complementary approaches. First we examine effects of manipulating the phenotype or genotype and then searching for effects on heat tolerance. Such studies can provide a functional link between gene expression and physiology. Second, we examine comparative studies that monitored covariation between Hsp70 expression and thermotolerance. Such experiments can link environmental variation and change in physiology over evolutionary time.

A candidate species on which both manipulative and population analysis are feasible is *Drosophila melanogaster,* which is also a member of a genus that is well studied phylogenetically. Therefore, we examine molecular and comparative evidence on the role of Hsp70 in thermotolerance in this species and in several related Drosophilids, and ask whether expression of this protein has evolved as an adaptation to thermal stress. In *Drosophila melanogaster,* the primary inducible heat-shock protein is Hsp70. At high temperatures (>31°C), the physiology of this species changes; individuals produce Hsp70, and by consequence, they tolerate higher temperatures than were possible before this change (Parsell and Lindquist, 1994; Feder *et al.,* 1996). These conditions are well above those in which the fly normally thrives (Feder and Krebs, 1998). Structurally, the *hsp70* loci of *D. melanogaster* are complex. Five active gene copies encode *hsp70,* and these genes are distributed between two loci (Ish-Horowicz *et al.,* 1979). Two duplication events produced this structure from an ancestral arrangement of two copies in the genus (Leigh-Brown and Ish-Horowicz, 1981). Spofford (1972) proposed that gene duplication is a simple means to change gene expression and regulation, but whether *hsp70* copy number, Hsp70 expression, and environmental stress covary among species is not known.

The success in tracking change within the genus *Drosophila* followed a series of experiments that began with a molecular manipulation of *D. melanogaster* that enabled a link to understand differences among other species. As will become apparent, these latter analyses are still in their infancy, but they are largely the target of independent ongoing research projects by the authors.

Manipulating HSP70 Expression

Two manipulations of the *Drosophila* genome demonstrate the specific importance of Hsp70 to thermotolerance. Jedlicka *et al.* (1997) produced lines with a temperature-sensitive mutation in the heat shock transcription factor, HSF. These flies possess normal *hsp70* genes, but their expression is not induced by heat. Consequently, flies from these lines express very little Hsp70, and they have little inducible thermotolerance (Jedlicka *et al.,* 1997). Welte *et al.* (1993) created strains that contain 12 additional gene copies per diploid genome. These lines enabled several tests of how thermotolerance changes relative to the concentration of Hsp70, including consequences of super-maximal expression levels.

A strong inducible response to heat in *Drosophila* requires Hsp70 (Solomon *et al.,* 1991; Li and Duncan, 1995; Feder, 1996). No expression virtually eliminates inducible thermotolerance, low levels of expression improve thermotolerance greatly, and while very high expression increases thermotolerance more, too much can reduce thermotolerance (Krebs and Feder, 1998*a*).

The relationship between Hsp70 induction and the change in thermotolerance is therefore complex, and it varies across development (Fig. 1). Pupae and young adults are much more tolerant of heat than are other life-stage, but Hsp70 expression cannot explain this difference. Both pupae (Feder and Krebs, 1997) and adults produce less Hsp70 than do young larvae (Krebs *et al.,* 1998). Benefits of overexpression likewise vary, as supranormal Hsp70 levels increase

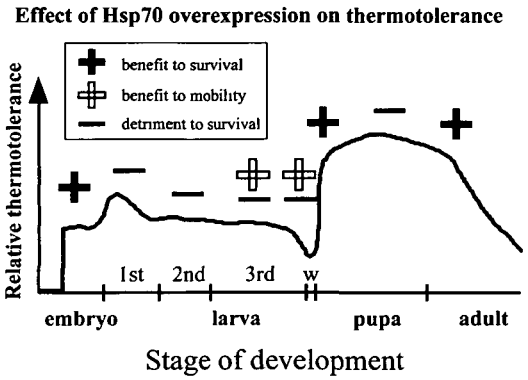

FIG. 1. Thermotolerance and the effect of overexpressing Hsp70 in *D. melanogaster* change during development. Benefits from super-normal Hsp70 concentrations result either as increased survival after stress or as an extended period of activity before the animal becomes dormant from the stress. Negative effects are recorded only by increased mortality. The data summarize various experiments on two pairs of transgenic lines, one from each pair possess 12 extra-*hsp70* gene copies, and one had these additional copies excised. Individuals with the extra copies produce more Hsp70 as a response to heat than do either the excision or any wild-type strains. Data originate as follows: embryos (Welte *et al.*, 1993), wandering larvae and pupae (Feder *et al.*, 1996), growing larvae (Krebs and Feder, 1997*a*); pupae (Feder and Krebs, 1998); and adults (Krebs *et al.*, 1998).

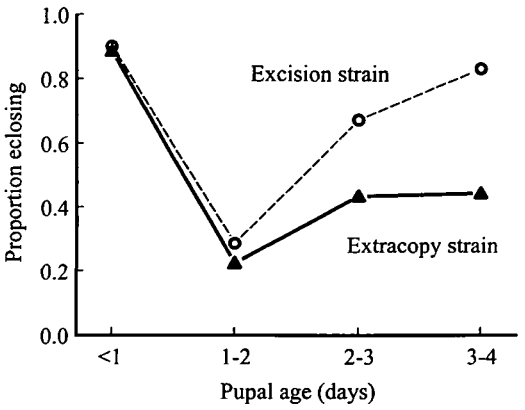

FIG. 2. Thermotolerance of pupae after heat shock is affected by Hsp70 level. Survival of pupae to adult was recorded for excision and extra-copy individuals treated 1 h at 36°C (pretreatment) followed by 1 hr at 25°C (recovery) and then 1 h at 41°C (severe heat shock) between 1 and 4 full days after pupation. Non-darkened pupae (those <6 hr old) were not collected. In young pupae (0.25–1 day old), breakdown of the larval tissues predominates and extra-Hsp70 either has little effect or benefits pupae (Feder *et al.*, 1996).

embryonic survival after heat stress (Welte *et al.*, 1993), lengthen the duration larvae remain mobile during heat stress, and increase survival of both very young pupae (Feder *et al.*, 1996) and young adults (Krebs *et al.*, 1998). None of these gains, however, is large. At the same time, potential costs are prevalent, and one is large: fewer larvae that overexpress Hsp70 survive to adulthood after a heat shock than do larvae that express normal amounts of Hsp70 (Krebs and Feder, 1997*a*). Likewise, pupae that overexpress Hsp70 survive to adulthood less often (Fig. 2, unpublished data of R.K.).

The developmental schedule of costs versus benefits of Hsp70 overexpression follows a pattern dictated by growth: benefits occur in stages where growth is minimal, and costs increase where growth is rapid (Fig. 1). Embryos benefit only briefly, within a short time frame after which cells rapidly sequester Hsp70 into granules (Welte *et al.*, 1993), while larvae and pupae fair poorly. Fully grown larvae, young pupae and adults, which show little division or enlargement of cells, gain most in survival. Fitness costs do appear from overexpression in adult gametes, as female fecundity may decline (Tatar, 1999). Similarly, expression of Hsp70 in cell lines at low temperature, which is made possible by inserting a heterologous promoter/*hsp70* construct, also stops growth (Feder *et al.*, 1992). Thus, developmentally, a trade-off exists wherein higher expression may benefit an individual at some stages of development, but harm it if exposed to heat during periods of rapid growth.

Hsp70 expression can both benefit and harm individuals even in the same developmental stage. A very short pretreatment that induces little Hsp70 will increase survival relative to individuals given no pretreatment. Under these stringent conditions, individuals that possess greater *hsp70*-copy number possess an advantage, whereas strong inducing treatments favor wild-type copy number over extra-copy lines (Fig. 3). This difference probably occurs because flies express Hsp70 under near-lethal stress conditions only when some Hsp70 is present before they encounter extreme stress

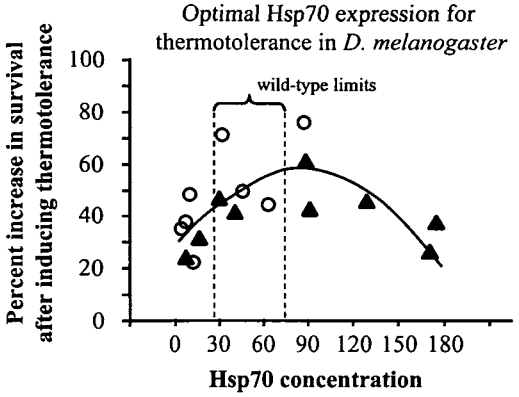

FIG. 3. Inducible thermotolerance of *D. melanogaster* larvae varies with the amount of Hsp70 present prior to heat-shock. Too little or too much Hsp70 (presented relative to a standard) changed how much survival to a heat shock increased within lines compared to survival where no pretreatment was given. Concentrations of Hsp70 were varied in either the extra-copy larvae (filled triangles) or an respective excision control strains (open circles) by manipulating pretreatment duration and temperature (fig. modified from Krebs and Feder, 1998*a*). The stippled area represents the range of maximal expression levels for wild type strains (Krebs and Feder, 1997*b*).

(Krebs and Feder, 1997*a*). In nature, temperatures probably increase slowly relative to the laboratory treatments applied, whether in orchard fruit (Feder, 1996) or in necrotic cactus (Krebs and Loeschcke, 1994), which gives *Drosophila* larvae time to respond.

Natural selection may constrain expression of Hsp70 to a level below that optimal for thermotolerance. Otherwise, costs to growth may exceed benefits to survival. Indeed, most wild-type *D. melanogaster* strains produce sub-optimal Hsp70 levels under conditions set to maximize expression (Fig. 3). The actual level maintained in populations should depend on two factors, the frequency of severe stress relative to mild inducing stress levels, the stage that most likely encounters stress, and the evolutionary independence within the species to respond specifically at particular life stages. These questions may only be answered by studies of natural populations.

HSP70 IN NATURAL POPULATIONS

While manipulation of the genome can delineate a functional relationship between Hsps and thermotolerance, these manipulations tell only the contemporary consequences of change and not how a process actually evolved. Many factors affect protein expression in natural populations. Another problem with transgenic lines, from an evolutionary perspective, is that they often possess similar genetic backgrounds, and therefore they vary little except for the manipulated trait. Consequently, these techniques may overstate the importance of Hsps to thermotolerance because they focus only on how variation in one trait affects phenotypic variation. The lines provide no information on how much variation that Hsp70, or any specific protein, may explain in a "natural" thermotolerant phenotype.

Classical genetic approaches may estimate the number of genes or mechanisms that underlie natural variation in thermotolerance, and therefore, they complement manipulative genetic studies (Feder and Krebs, 1997). One procedure to quantify variation is to produce isofemale lines in the laboratory. These are genetically distinct lines that each derive from a single once-mated female. When reared at low density and high population size, these lines possess a relatedness among individuals similar to that between full-siblings (Parsons, 1980). Where genetic variation occurs in a population, individuals of the same line are more alike for thermotolerance than are individuals from different lines in *Drosophila* (Hosgood and Parsons, 1968; Parsons, 1973) and in other insects (White *et al.*, 1970; Niklasson and Parker, 1994). In genetically variable lines, Hsp70 expression correlated with inducible thermotolerance (Krebs and Feder, 1997*b*). Furthermore, Hsp70 expression covaried across development, *i.e.*, lines that produced high amounts of Hsp70 did so at all developmental stages tested (newly hatched larvae, wandering 3rd-instar larvae, and young adults, Krebs *et al.*, 1998). Therefore, trade-offs across development can be critical. Selection on Hsp70 expression at one stage may oppose changes at another.

In addition to developmental differences in the tolerance of both stress and the expression of Hsp70, trade-offs may also affect selection on Hsp70 within one life

stage. Larvae from lines that naturally express more Hsp70 tolerate heat better, but they succumb more often in the absence of stress (Krebs and Feder, 1997*b*). This result suggests that a trait can be beneficial in a stressful environment but detrimental or costly under benign conditions. Similarly, the transgenic lines differed for survival in the absence of stress: extra *hsp70* copies reduced larva-to-adult survival at 25°C, particularly when larvae received an Hsp70-inducing treatment without a subsequent severe heat shock (Krebs and Feder, 1997*a*; 1998*b*). Those individuals pay a cost from too much Hsp70, but gain nothing.

Evidence from Variation among *Drosophila* Populations and Species

Study of variation in Hsp70 expression among *Drosophila* populations and species lags behind physiological studies, but divergence among groups may reveal how genetic or evolutionary changes may underlie variation in temperature tolerance (Levins, 1969; Huey and Bennett, 1990). Bettencourt *et al.* (1999) demonstrated that environmental trade-offs can change Hsp70 expression after long-term rearing at different constant temperatures. They compared Hsp70 expression in five *D. melanogaster* lines that Sandro Cavicchi (University of Bologna, Italy) raised at 18, 25 and 28°C for more than 20 years. All lines originated from a single stock, and they evolved over several hundred generations. These lines, which vary in thermotolerance (Cavicchi *et al.*, 1995), also vary in Hsp70 expression (Fig. 4): the 28°C lines produce less Hsp70 and induce heat tolerance less well than do either the 18 or 25°C lines. Results of Bettencourt *et al.* (1999) are best explained by selection against Hsp70 expression at 28°C. This temperature will not denature proteins, but if some tissues express even low levels of Hsp70, selection might reduce the sensitivity of the induction response. The 18°C line may be more sensitive to induction at low temperatures (Fig. 4). Heat shock protein induction typically is 5–10°C above conditions typical for normal growth (Lindquist, 1986), even for species adapted to extremes of cold (Vayda and Yuan, 1994). Notably, these long-term laboratory lines

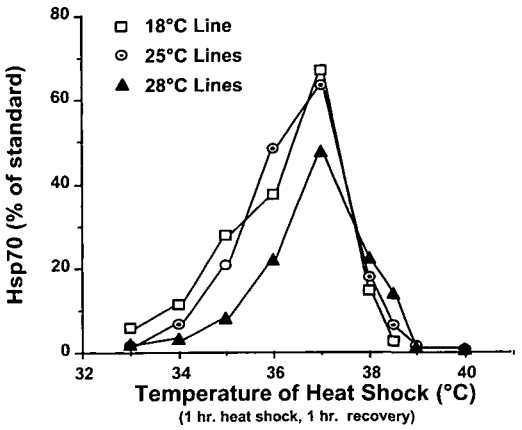

FIG. 4. Expression of Hsp70 among lines of *D. melanogaster* that evolved independently at either 18, 25 or 28°C for over 20 years (modified from Bettencourt *et al.*, 1999). results at 25 and 28°C are those pooled for two independent lines. All temperature treatments were for 1 hr, followed by 1 hr at 25°C. Larvae from the 28°C lines expressed significantly less Hsp70 than did those from other lines except at temperatures above 37°C, where induction may be due to a mix of factors after cell damage/protein denaturation begins to occur.

all possess the same number of *hsp70* copies, which means that other factors must vary among them to produce the observed variation in Hsp70 expression. Thermotolerance in these lines varies due to effects at multiple chromosomes (Cavicchi *et al.*, 1995).

In contrast to laboratory evolution experiments, tests for correlation between environmental variation and induced responses to stress suggest only rough relationships between stress level and tolerance (*e.g.*, Coyne *et al.*, 1983; Krebs and Loeschcke, 1995; 1999). Therefore, applying adaptive hypotheses is tenuous, particularly because the thermal microenvironment at each collection site varies unpredictably, and information on *Drosophila* ecology and behavior in nature is limited (Feder, 1996).

An alternative approach to assess variation in thermotolerance is to compare related species from ecologically diverse areas. Hoffmann and Parsons (1997) suggest that natural selection on stress tolerance should reduce the number of genetic variants that affect a trait. Stress susceptible species therefore should show more quantitative variation for tolerance while selected spe-

cies should vary at few loci. Although limited in resolution, genetic crosses among populations that vary in thermotolerance provide data to test this prediction. Krebs *et al.* (1996) performed such crosses in *D. melanogaster* and in *D. buzzatii* (the latter is a species that breeds in necrotic prickly-pear cactus of dry grasslands and survives exposure to conditions that rapidly kill the hardiest *D. melanogaster*). They found differences in the genetic structure of stress tolerance between the two species. Most population differences in *D. buzzatii* could be explained by as few as two loci, while crosses among *D. melanogaster* populations indicated that complex gene interactions may underlie survival to thermal stress, a result supported in separate experiments by Cavicchi *et al.* (1995). Thus, for *D. melanogaster,* in which transgenic techniques definitively identified an important role for Hsp70 on thermotolerance, many other traits also influence this trait. For now, the relationship between Hsp70 expression and thermotolerance of *D. buzzatii* is not known.

Progress on the importance of Hsps in other species will come soon. For *Drosophila,* recent results suggest that the antibody used to quantify Hsp70 in *D. melanogaster* will recognize this protein in diverse lineages. Tissues of heat-treated larvae of *D. mojavensis* (closely related to *D. buzzatii*) stain for Hsp70 similarly to those of *D. melanogaster,* which suggests similar expression of Hsp70 in these species that diverge at the base of the genus *Drosophila.* However, the thermal patterns of Hsp70 expression vary between larvae of *D. melanogaster* and its sibling species, *D. simulans,* which are similar, and the desert adapted *D. mojavensis.* Expression in both temperate species peak at 36–37°C, but *D. mojavensis* requires much higher temperatures before induction begins (Fig. 5A), and expression peaks several degrees higher, at 40°C. Unfortunately, potential differences in binding affinities limit conclusions that can be drawn from quantitative variation between species, although comparison of induction temperatures and those for maximum expression are not hindered.

These phylogenetic changes in Hsp70

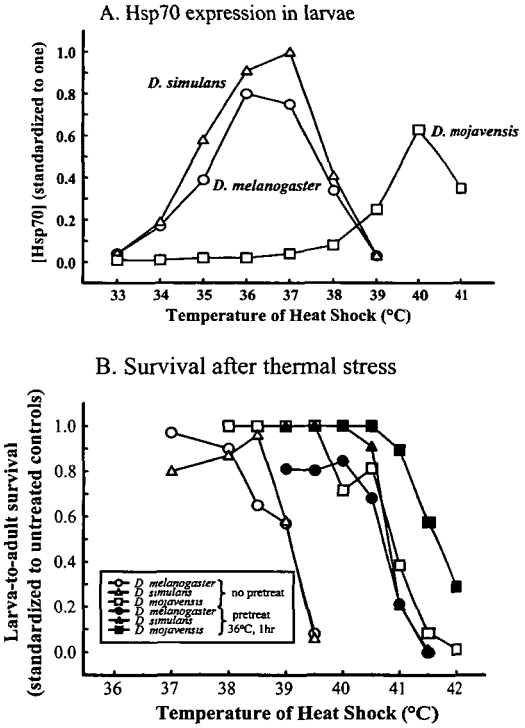

FIG. 5. (A) Hsp70 expression differs among larvae from one wild-type stock each of *D. melanogaster, D. simulans* and *D. mojavensis* after a 1 hr exposure to the temperature indicated followed by 1 hr at 25°C. Concentrations were standardized to that of the maximum for the highest expressing species, which was *D. simulans* at 37°C. (B) Survival of these same species likewise differed when exposed to varied levels of thermal stress. Larvae either were directly exposed to the stress for 1 hr (open symbols) or first were conditioned by pretreatment for 1 hr at 36°C followed by 1 hr at 25°C (closed symbols) before they were exposed to the potentially lethal stress. Thermotolerance was recorded as survival to adult, which was standardized to control (untreated) larvae of each species.

expression correlate with variation in thermotolerance (Lindquist unpubl. in Huey and Bennett, 1990). Without pretreatment, a 1 hr 38.5°C treatment killed a significant proportion of both *D. melanogaster* and *D. simulans,* but similar mortality of *D. mojavensis* required 40°C (Fig. 5B). Pretreatment shifted the survival curves of each species higher, but similar differences occurred for each. If Hsp70 expression is an adaptation to heat stress, we predicted that Hsp70 expression would correlate across temperatures with the strength of induction—the increase in thermotolerance after

pretreatment at a specific temperature. This change did not occur. Induction of thermotolerance began at temperatures well below those that induce Hsp70 expression in *D. mojavensis* (Results presented in Fig. 5B are for larvae pretreated at 36°C), and higher pretreatment temperatures reduce thermotolerance (Krebs, 1999). Similarly, pretreatments either at 36°C for less than 1 h or treatments at slightly lower temperatures improve survival of *D. melanogaster* as well as do the 1 hr 36°C treatment, which induces the most Hsp70 (Krebs and Feder, 1998*a*). Therefore, higher thermotolerance in *D. mojavensis* likely evolved from changes in aspects of physiology other than a change in the regulation of Hsp70.

Furthermore, the optimal temperature for Hsp70 induction and the best temperature to increase thermotolerance, a relationship which appears close in *D. melanogaster,* may not hold for many species. The relationship between Hsp70-inducing conditions and those that induce tolerance are similarly complex in fish (diIorio *et al.,* 1996; Iwama *et al.,* 1998). Therefore, an understanding of how various environmental factors induce Hsps awaits more detailed molecular study of *hsp* regulatory elements.

Phylogenetic Diversity in *HSP70*

One means to elucidate evolutionary change in *hsp70* is to examine its molecular evolution in a systematically well known group, such as the genus *Drosophila.* Do genes from residents of particular environments show consistent changes, or has the conserved nature across phyla limited variation within the genus too much to facilitate new adaptive responses? Does variation, for example in *hsp70* copy number, reflect an increase in Hsp70 expression?

As yet, these questions remain largely unanswered. However, two general points apply: First, despite their age and ubiquity, *hsp70* genes are surprisingly evolutionarily malleable in *D. melanogaster* and its relatives. Second, while *hsp70* copy number evolution is a demonstrable and intuitively appealing mechanism underlying Hsp70 expression variation, it is certainly not the only one.

The primitive condition for Diptera is a

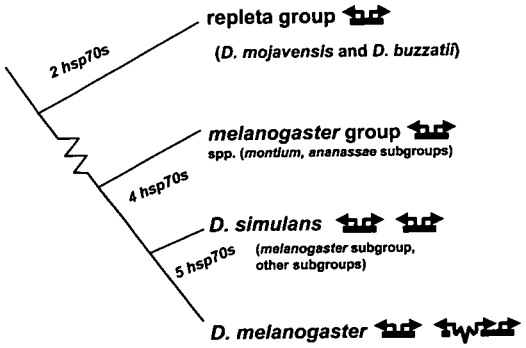

FIG. 6. Phylogenetic relatedness and putative *hsp70* copy numbers for the 3 species compared for Hsp70 expression and thermotolerance in Figure 7. Copy number is known for *D. melanogaster* and *D. simulans,* but not for *D. mojavensis* or any of its relatives, although the ancestral number of *hsp70* copies in the genus is two.

characteristic inverted pair of *hsp70* genes. In the *melanogaster* species group, *hsp70* genes evolved by amplification and rearrangement (Fig. 6). While some *melanogaster* group species possess only two gene copies (Bettencourt and Feder, 1998), the *melanogaster* subgroup species have at least four *hsp70* genes. Within this subgroup, *Drosophila melanogaster* possesses a unique arrangement of five *hsp70* genes in which interlocus variability is low, and gene conversion is apparently rampant (Leigh Brown and Ish-Horowicz, 1981). Thus, the present structure of the *hsp70* loci in the *melanogaster* lineage likely evolved by chromosomal duplication, tandem gene duplication, and conversion.

The ecology of the *melanogaster* subgroup species reflects this trend towards increased *hsp70* copy number, higher Hsp70 expression and variation in thermotolerance. The species with cosmopolitan distributions, *D. melanogaster* and *D. simulans,* express more Hsp70 and are more thermotolerant than are other members. *Drosophila erecta,* a phylogenetically basal species, has a very limited montane distribution and low thermotolerance, while *Drosophila yakuba,* which has a wide but not cosmopolitan distribution, shows intermediate thermotolerance (unpublished data of D. A. Benson, personal communication). Thus, changes in Hsp70 expression and thermo-

tolerance may facilitate adaptation to the greater thermal variability encountered by more wide-ranging species.

Although comparison on Hsp70 expression and thermotolerance in the melanogaster group suggests an adaptive cause, the generality of the result is not known. Drosophilids are an ancient and speciose group (>3,000 species, Ashburner, 1989), and *hsp70* genes can evolve rapidly. Therefore, genes may duplicate independently in different lineages, and thus, phylogenetic breadth should be considered carefully when testing adaptive hypotheses for the *hsp70* genes. Copy number of *hsp70* in other species, like *D. mojavensis* (Fig. 6), cannot be inferred from the basal number in the genus. Evolutionary change in Hsp70 expression also may occur for reasons other than variation in copy number; *D. simulans* has only 4 *hsp70* genes, yet this species actually expresses more Hsp70 than does *D. melanogaster,* which has 5-copies (Fig. 5A). Selection may target *cis* and *trans* modifiers of Hsp70 expression, which may include promoter elements, coding sequence, and both 5′ and 3′ untranslated regions (Petersen and Lindquist, 1989, Fernandes *et al.,* 1994, Hess and Duncan 1996) or modification of HSF or HSBP1 (Saytal *et al.,* 1998). In a notable example, early workers found two distinct types of the 87A7 *hsp70*-containing locus (bearing or lacking a large insertion in the shared *hsp70Aa-Ab* regulatory region) in the Oregon R strain of *D. melanogaster* (Goldschmidt-Clermont *et al.,* 1980). These variant alleles exist in other natural lines, lines vary at several other sites, and they respond to thermal selection (B. B., unpublished data).

Summary

Hsp70 expression enhances thermotolerance, but the benefits of too much Hsp70 restrict growth. Therefore cells must limit expression at periods when damage may result. Trade-offs like this may select to maintain genetic variability in natural populations, and mean expression therefore may depend on environmental variation. As a consequence, expression may change rapidly. However, Hsp70 does not appear to facilitate shifts to warmer climates, and its expression is not necessarily higher in species from these habitats. Instead, it appears to mitigate damage following short-term exposures to stress. Therefore, the species that may evolve greater expression of Hsp70 are those that live in the greatest range of environments. Expression in each species may differ for many reasons, or be similar for different reasons; in the end, we predict that variation in Hsp70 expression will follow more microclimatic differences rather than either large-scale phylogenetic or climatic patterns. Testing this prediction will require direct analysis of sequence variation of *hsp70* and modifier loci for many species.

Acknowledgments

We particularly thank Martin Feder, who remains an adviser to us both, Noemi Cartaño, John Kim, Tony Nguyen (Univ. of Chicago), Cathy Bender and Antoinette Hereford (Cleveland State) for their technical assistance in various studies reported here. BRB was supported by a HHMI Predoctoral Fellowship.

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