# Evolvability of Hsp70 Expression under Artificial Selection for Inducible Thermotolerance in Independent Populations of *Drosophila melanogaster*

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## ABSTRACT

To test whether expression of the inducible heat-shock protein Hsp70 increases under selection for inducible thermotolerance in *Drosophila melanogaster*, we performed artificial selection on replicate sets of *Drosophila* lines founded from two independent populations. Selection entailed pretreatment at 36°C to induce thermotolerance and Hsp70 expression, followed by a more severe heat shock, whose temperature varied between sexes and among generations to achieve 50% mortality. Inducible thermotolerance increased slowly and continuously in selected lines and was 37%–50% greater than in controls after 10–11 generations. Lines founded from the two populations differed in their coevolution of Hsp70 expression. In lines founded from Evolution Canyon, Israel, Hsp70 level initially increased and thereafter was unchanged; replicate lines exhibited two temporal patterns of response to selection. In lines founded from Australia, Hsp70 levels increased throughout selection. In both cases, however, the increase in Hsp70 level averaged only 15%, suggesting that pleiotropy in Hsp70 function constrains evolutionary increase in its expression.

## Introduction

The evolution of gene expression is subject to constraint. In the expression of the genes encoding heat-shock proteins (Hsps), this constraint may arise in part from the multiple roles that the encoded proteins play in the cell. Although best known for their function as molecular chaperones in protein folding, quality control, and deterrence of aggregation (Feder and Hofmann 1999), Hsps also are regulators of growth and apoptosis, intracellular and extracellular messengers, components of major protein secretory and import pathways, and regulators of transcription factor and nuclear receptor activity (DeFranco et al. 1998; Asea et al. 2000; Helmbrecht et al. 2000; Mosser et al. 2000; Fewell et al. 2001; Gabai and Sherman 2002; Ryan and Pfanner 2002; Wallin et al. 2002). Whereas increasing Hsp expression increases overall molecular chaperoning and inducible stress resistance, pathology results if the other functions of Hsps (and the Hsps that regulate them) exceed physiological limits (Dorner et al. 1992; Feder et al. 1992; Dorner and Kaufman 1994; Zatsepina et al. 2001). Indeed, experimental increases in Hsp expression or constitutive expression of normally inducible Hsps disrupt growth and development and can be lethal (Feder et al. 1992; Krebs and Feder 1997*a*, 1998). Thus, these functions may conflict under natural selection for increased Hsp expression (Krebs and Feder 1997*b*; Bettencourt et al. 1999; Sørensen et al. 1999; Lansing et al. 2000).

Several features of Hsps can be viewed as evolutionary solutions to this conflict. First, eukaryotes have evolved protein families of the major Hsps, with family members localizing to specific cell compartments or having distinctive thresholds for their expression and/or degradation (Feder and Hofmann 1999). This pattern may enable evolution of high expression of some family members without disrupting the regulation of others. Second, multiple transcriptional and posttranscriptional mechanisms regulate the heat-shock response, as if ensuring that Hsps never accumulate in abundance except when actually needed (Lindquist 1993).

Despite these putative solutions, however, both comparative (Krebs and Feder 1997*b*; Sørensen et al. 2001; Zatsepina et al. 2001) and laboratory evolution (Bettencourt et al. 1999; Sørensen et al. 1999) studies suggest that evolution at a constant warm temperature (i.e., warm enough to induce low levels of Hsps but never warm enough to realize the protective effects of Hsps against thermal damage) results in decreased Hsp levels, as if the deleterious effects of Hsp levels persist and outweigh any beneficial effects. According to this logic, increased Hsp expression should evolve under environmental regimes in which inducible stress tolerance is crucial. In such circum-

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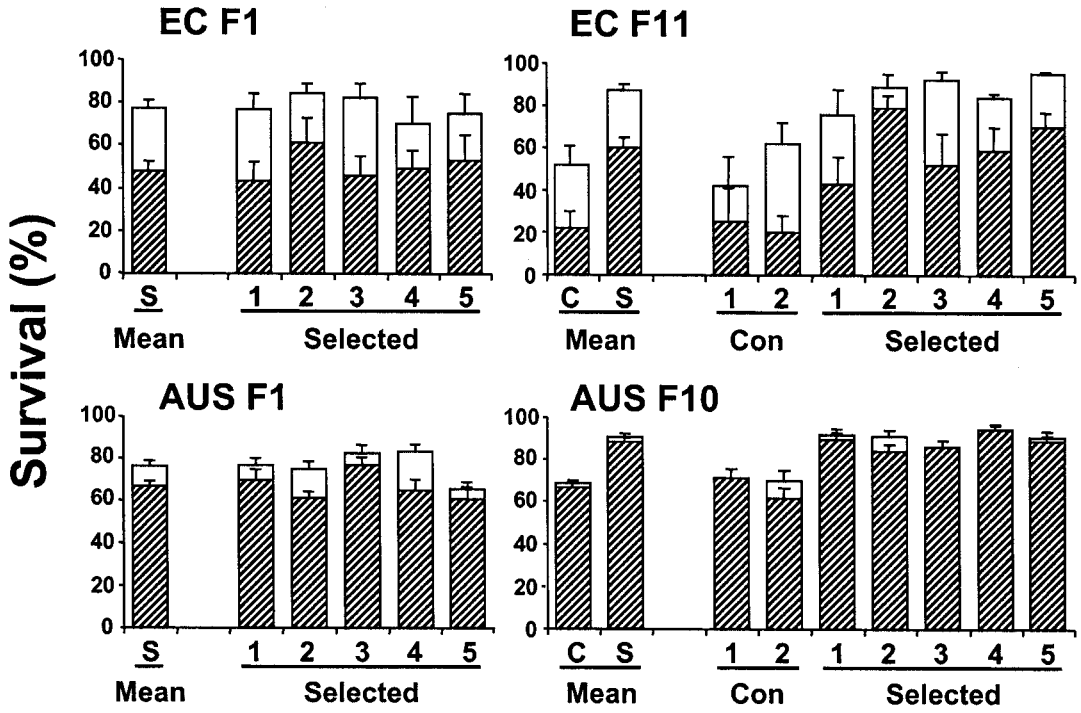


Figure 1. Initial (generation F1) and final (generation F10 or F11) values of inducible thermotolerance for the two experimental populations, EC and AUS. Results are presented separately for each sex for the two control lines (*C* or *Con*) and five selected lines (*S* or *Selected*), each designated by number. Mean survival of a heat shock after pretreatment is plotted ± 1 SE. *Hatched bars*, females; *open bars*, males. For the EC lines, the heat shock was 40.4°C in generation F1 and 40.5°C in generation F11. For females of the AUS lines, the heat shock was 40.5°C in generation F1 and 40.5°C in generation F10. Heat-shock temperatures were 0.1°C higher for AUS males in these generations, which underestimates male-female differences in inducible thermotolerance.

stances, the benefits of increased Hsp expression should outweigh its disadvantages.

Although numerous comparative data are consistent with this latter prediction, increases in Hsp expression through laboratory evolution (Rose 1996) of eukaryotes have thus far not been evident (Bettencourt et al. 1999; Sørensen et al. 1999; Lansing et al. 2000). Most such investigations maintained experimental populations at different temperatures and allowed evolution to proceed but did not explicitly select founders of each generation according to some phenotypic criterion such as inducible thermotolerance. Our contention, which motivated this study, is that direct "artificial selection" (Rose 1996) on inducible thermotolerance would be a stronger test of the underlying biological argument. Accordingly, we have designed replicate laboratory evolution experiments with this criterion in mind. Specifically, we exposed lines of *Drosophila melanogaster* to a selection regime that included, first, an Hsp-inducing thermal pretreatment and, second, a heat shock intended to kill 100% of unpretreated flies and 50% of pretreated flies. Thus, unlike many prior studies of thermal resistance evolution in *Drosophila* (Quintana and Prevosti 1990, 1991; Gilchrist et al. 1997; Gilchrist and Huey 1999), which involved evolution at high temperature or selection on basal thermotolerance (i.e., thermotolerance present without thermal pretreatment), ours involved inducible thermotolerance (i.e., the increased thermotolerance due to pretreatment). Hsps are specifically associated with this latter form of thermotolerance (Feder et al. 1996; Feder and Hofmann 1999). Also, as inducible thermotolerance evolved in our study, we increased the severity of the heat shock to maintain a constant intensity of selection. We ask: Does selection for inducible thermotolerance yield increased Hsp expression as a correlated response? Although the data answer this question positively, they also suggest that the evolution of Hsp expression continues to be highly constrained even under regimes in which its impact on inducible thermotolerance is unambiguously beneficial.

This work used *D. melanogaster* as a model species and Hsp70 as a model Hsp. In *Drosophila*, both basal and inducible thermotolerance evolve in nature and respond to laboratory natural selection (Morrison and Milkman 1978; Quintana and Prevosti 1990, 1991; Krebs and Loeschcke 1996; Loeschcke and Krebs 1996; McColl et al. 1996; Gilchrist et al. 1997; McKechnie et al. 1998; Nevo et al. 1998; Stratman and Markow 1998; Bettencourt et al. 1999; Gilchrist and Huey 1999). Hsp70, a mem-

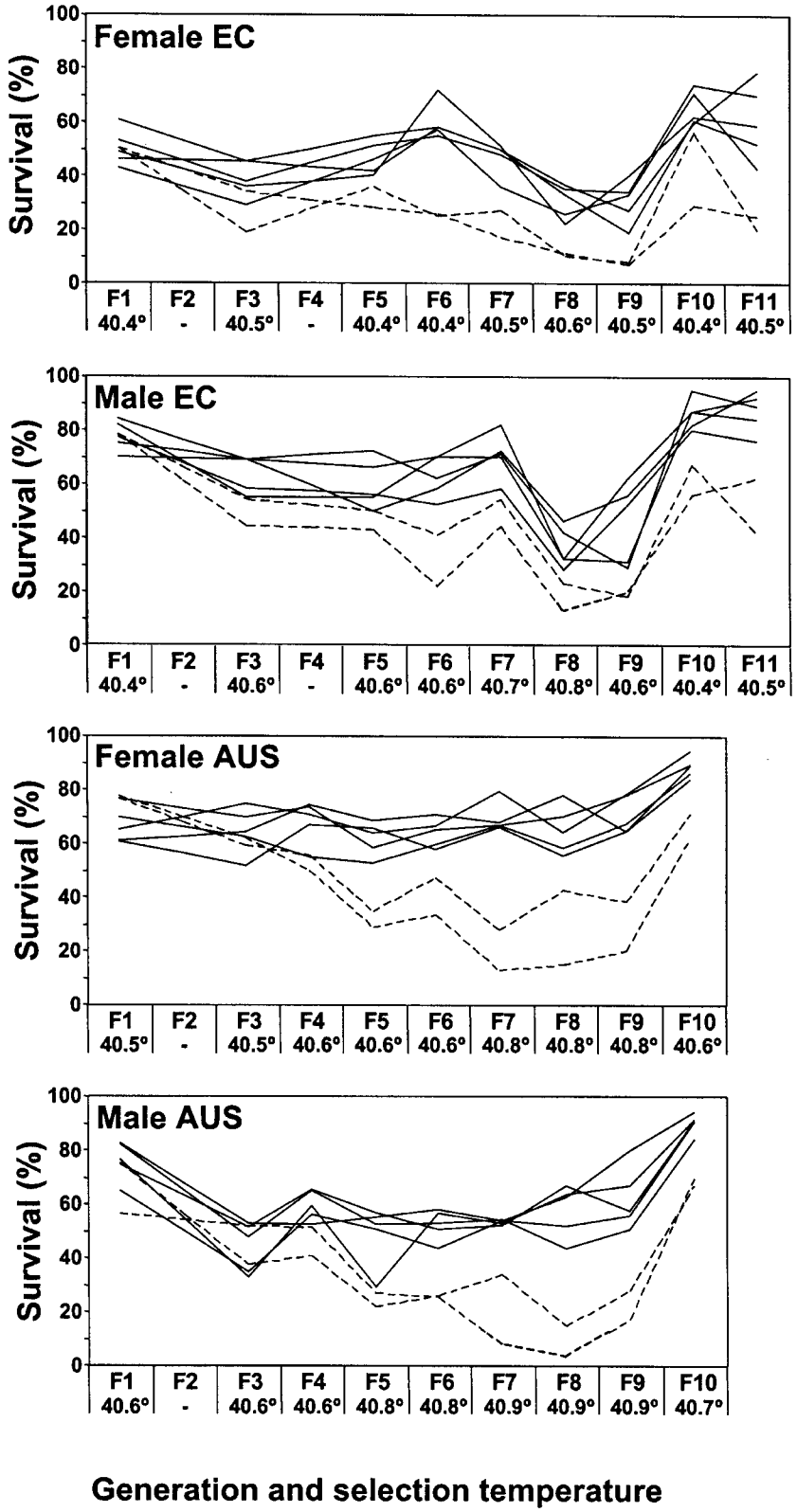


Figure 2. Variation by generation in control replicates (*dashed lines*) and replicates undergoing artificial selection for inducible thermotolerance (*solid lines*). Heat-shock temperatures, which were varied to achieve a constant mortality in the selected lines, are indicated underneath each generation; "−" indicates a generation in which no selection was undertaken. Plotted lines connect mean values; see Table 1 for statistical analysis. Graphs are plotted on the same scale to facilitate comparison.

Table 1: Type III ANOVA of inducible thermotolerance (% survival after pretreatment + heat shock) with generations as repeated measures

| Source | df | Effect MS | Error df | Error MS | *F* | *P* Level |
|---|---|---|---|---|---|---|
| EC females: | | | | | | |
| Treatment (T) | 1 | 1.188 | 17 | .038 | 31.273 | .000 |
| Generation (G) | 7 | .235 | 119 | .030 | 7.770 | .000 |
| Interaction (T × G) | 7 | .076 | 119 | .030 | 2.505 | .019 |
| EC males: | | | | | | |
| Treatment (T) | 1 | .799 | 15 | .032 | 24.742 | .000 |
| Generation (G) | 7 | .230 | 105 | .024 | 9.548 | .000 |
| Interaction (T × G) | 7 | .047 | 105 | .025 | 1.944 | .070 |
| AUS females: | | | | | | |
| Treatment (T) | 1 | 25,107.36 | 19 | 623.932 | 40.240 | .000 |
| Generation (G) | 8 | 2,508.96 | 152 | 455.622 | 5.507 | .000 |
| Interaction (T × G) | 8 | 1,884.82 | 152 | 455.622 | 4.137 | .000 |
| AUS males: | | | | | | |
| Treatment (T) | 1 | 13,557.31 | 20 | 640.299 | 21.173 | .000 |
| Generation (G) | 6 | 3,950.60 | 120 | 720.614 | 5.482 | .000 |
| Interaction (T × G) | 6 | 1,169.92 | 120 | 720.614 | 1.623 | .146 |

Note. Treatment = control or selected. Data for this analysis are plotted in Figure 2.

ber of the DnaK-Hsp70 Hsp superfamily, is the most abundant Hsp in *D. melanogaster* (Feder and Krebs 1998; Feder 1999). Much of the work cited above documents the pleiotropic functions of Hsp70 and its conformity to the beneficial versus deleterious effects concept of Hsp expression evolution. Finally, both experimental and genetic manipulations of Hsp70 expression have established a direct relationship between Hsp70 level and inducible thermotolerance (Feder et al. 1996; Feder and Hofmann 1999).

## Material and Methods

### Drosophila *Strains*

We founded seven replicate lines with 50 flies of each sex from each of two laboratory populations, EC and AUS; two lines from each founder population were randomly assigned as controls, and the remaining five from each founder population underwent selection. EC descended from 25 isofemale lines collected from the midslope station on the north-facing slope of Evolution Canyon (Lower Nahal Oren, Mt. Carmel, Israel) during August and September 1997 (Nevo et al. 1998). Ten females and 10 males of each isofemale line were combined and maintained as a mass culture (>100 adults) with random mating for 50–55 generations. AUS was created by combining 10 synthetic populations, each founded by combining isofemale lines collected from a transect along the east coast of Australia and Tasmania from 16° to 36°S latitude (Bettencourt et al. 2002). Approximately 10 generations with population sizes >100 adults elapsed between the founding of this population and the start of the experiment. Cultures were maintained on standard cornmeal-sugar-yeast-agar medium in half-pint bottles at 25° ± 1°C on a 12L : 12D cycle except when undergoing experimentation.

### *Inducible Thermotolerance and Selection*

Eclosing flies were harvested from cultures previously founded from 100 flies (50 male and 50 female). While still virgin (to allow us to select founders of the next generation), males and females were separated and transferred to fresh glass vials containing 8 mL of medium. Vials usually contained 10–20 animals but occasionally contained as many as 30. One day after eclosion (±1 h), these vials were submerged in thermostated circulating water baths and exposed to 35.9°C for 30 min, 25°C for 30 min (collectively "pretreatment"), and then a heat shock for 30 min. Vial temperatures equilibrated with water temperatures within 30 s. This pretreatment results in near-maximal Hsp70 expression (Zatsepina et al. 2001). Survival was assessed as the proportion of flies that could walk 24 h after heat shock. For each selected replicate, this procedure was repeated for 4–10 d, until enough surviving adults (50 of each sex) accumulated to found the next generation. For each control replicate, eclosing flies were randomly assigned to two groups. One group, used only to determine to measure inducible thermotolerance and Hsp70 levels of controls, underwent the same pretreatment and heat-shock regime as the selected replicates of the same sex and founder population. These were discarded after use. The other group (50 of each sex) underwent sham treatment at 25°C and was used to found the next generation. An alternative control treatment would have been to expose flies to pretreatment and not heat shock. As Loeschcke and colleagues (Sørensen et al. 1999; Lansing et al. 2000) have shown, however,

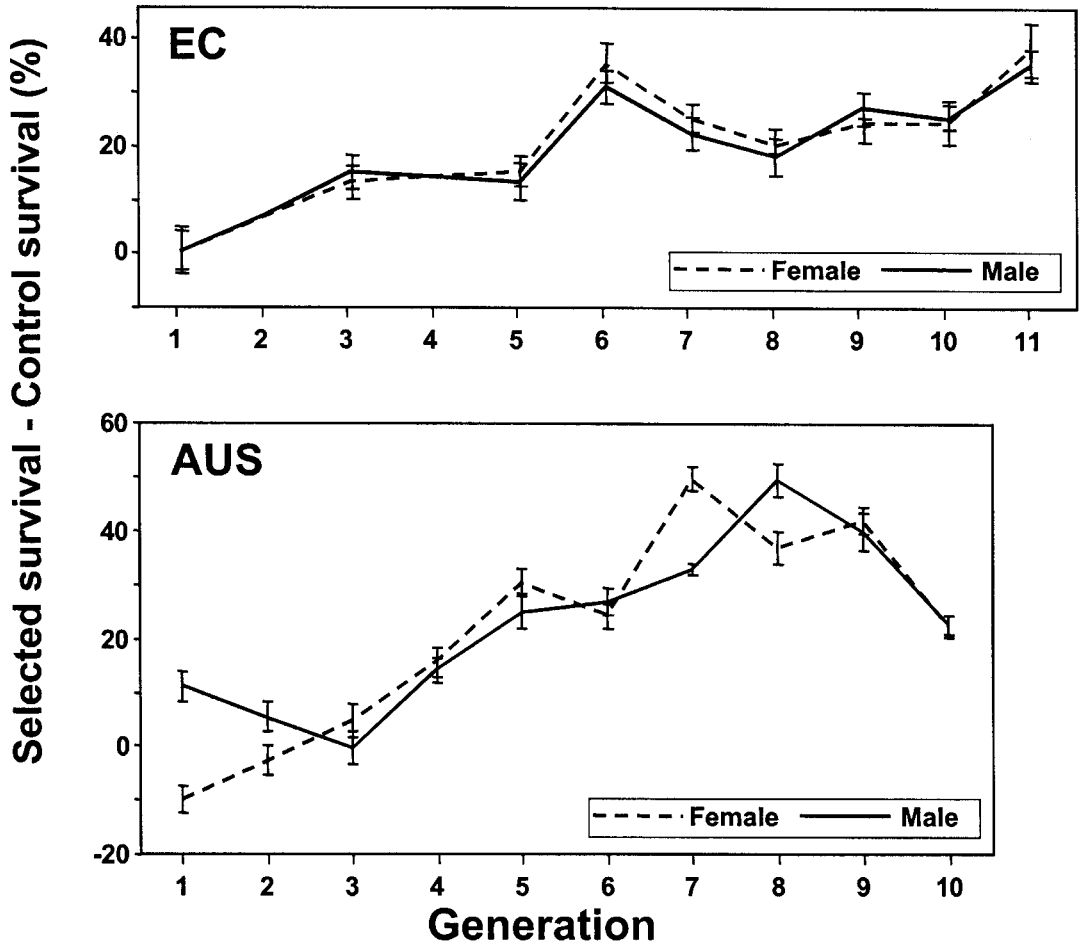


Figure 3. Effect of artificial selection on inducible thermotolerance in each sex of the EC and AUS populations. For each generation, the mean survival of the control lines was subtracted from the values for the selected lines. See Figure 2 for the heat-shock temperatures for each generation. Graphs are plotted on the same scale to facilitate comparison.

this regime selects for decreased Hsp70 level and would thus confound the outcome of selection for inducible thermotolerance. Generations F2 and F4 of the EC lines and generation F2 of the AUS lines were not subjected to heat shock to allow lines to recover. Heat shock without pretreatment resulted in approximately 100% mortality (data not shown).

Heat shock was initially 40.4°–40.6°C, depending on sex and founder population, and was intended to yield 50% mortality. As inducible thermotolerance evolved, the heat-shock temperature was adjusted separately for each sex and founder population to maintain approximately 50% mortality in the selected replicates. Exact mortalities are given below. In the final generation, heat-shock temperature was similar to that in the initial generation.

*Measuring Hsp70 Expression*

Approximately 20 1-d-old virgin males were placed in vials, which were submersed for 30 min in circulating water baths thermostated at 35.9°C. After 30 additional minutes at 25°C, flies were transferred to cryotubes, placed in liquid nitrogen, and stored at −80°C until analysis. For determination of Hsp70, individual flies were lysed in ice-cold 1X Complete Protease Inhibitor (Boehringer-Mannheim) in phosphate-buffered saline, and the Hsp70 content of the lysate was determined by a specific enzyme-linked immunosorbent assay (ELISA; Welte et al. 1993; Feder et al. 1996). Hsp70 concentrations are expressed as percentages of an Hsp70 standard prepared from adult Australian *Drosophila melanogaster* that were treated at 36.5°C for 1 h plus 25°C for 1 h.

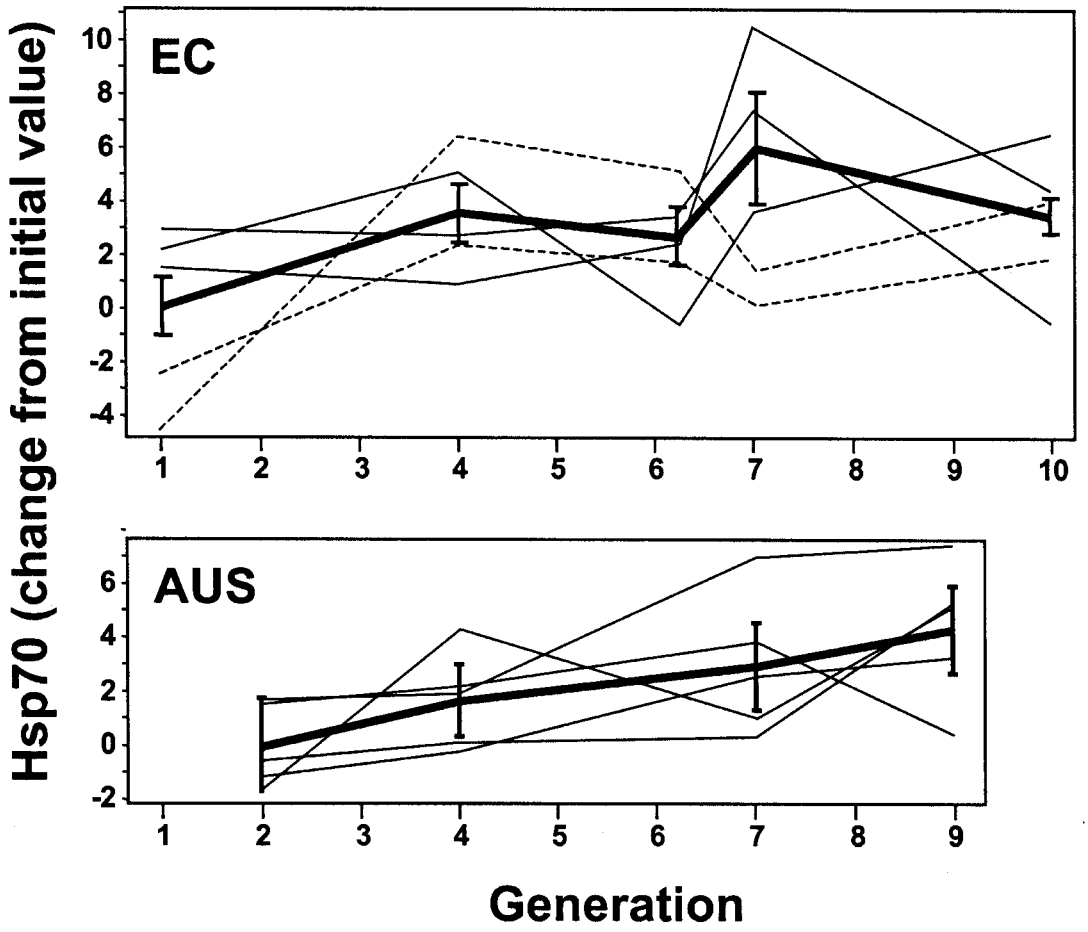


Figure 4. Hsp70 levels of males from the selected lines after a standard heat shock (35.9°C for 30 min and 25°C for 30 min). The *Y*-axis represents change from a standardized ELISA signal, whose initial mean was 24.6 in EC generation 1 and 25.8 in AUS generation 2. The thick lines indicate grand means of all selected lines ± 95% confidence intervals. In the EC lines, two replicates (*dashed lines*) differed from the other replicates (*solid lines*) in initial Hsp70 levels; see text. Tables 2 and 3 present a statistical analysis of these data. Graphs are plotted on the same scale to facilitate comparison.

*Statistics*

ANOVA with repeated measures was used to control for the effect of nonindependence of subsequent generations. Type III sums of squares were computed, and appropriate *F* ratios were used as recommended by Milliken and Johnson (1992, pp. 393–397) for repeated-measures ANOVA with missing/unequal numbers of observations. For determinations of Hsp70 in the EC lines, technical difficulties affected the day-to-day repeatability of assays. Accordingly, values for each day's assay were adjusted according to the deviation of control lines measured on that day from the grand mean of all control values. No such adjustment was necessary for the AUS lines.

**Results**

*Thermotolerance*

At 40.4°C, the initial temperature for the EC replicates, 50% of EC females survived, with lines ranging 43%–51% in survival (Fig. 1). A greater proportion (mean = 78%, range 70%–84%) of EC male flies survived (Fig. 1). A similar pattern was evident in the AUS lines (Fig. 1). Thus, subsequently, males and females of all replicates usually underwent different heat-shock regimes (Fig. 2) and were analyzed separately.

The survival of all lines varied throughout the experiment as the heat-shock temperatures were changed to achieve a constant target mortality in the selected replicates (Fig. 2; Table 1). Selected lines exceeded control lines of the same sex in

Table 2: Type III ANOVA of Hsp70 level (% of standard) with generations as repeated measures

| Source | df | Effect MS | Error df | Error MS | *F* | *P* Level |
|---|---|---|---|---|---|---|
| EC males: | | | | | | |
| Treatment (T) | 1 | 99.047 | 19 | 10.226 | 9.685 | .006 |
| Generation (G) | 4 | 34.891 | 76 | 15.389 | 2.267 | .070 |
| Interaction (T × G) | 4 | 9.124 | 76 | 15.389 | .593 | .669 |
| AUS males: | | | | | | |
| Treatment (T) | 1 | 133.158 | 36 | 78.939 | 1.686 | .202 |
| Generation (G) | 3 | 76.687 | 108 | 8.522 | 8.999 | .000 |
| Interaction (T × G) | 3 | 28.338 | 108 | 8.522 | 3.325 | .022 |

Note. Treatment = control or selected. Data for this analysis are plotted in Figure 4.

inducible thermotolerance ($P < 0.001$ in every case; Table 1). Moreover, the difference between selected and control lines increased with generations of selection and varied similarly in both sexes (Fig. 3). The *P* value of this difference, formally treatment × generation interaction in Table 1, was 0.02 in EC females, 0.07 in EC males, <0.01 in AUS females, and 0.15 in AUS males. Although two probabilities do not exceed the customary threshold for statistical significance, we believe that these reflect the nonlinearity of the changes in survival (Fig. 2) but with an unambiguous evolution of enhanced thermotolerance (Fig. 3).

### *Hsp70 Expression*

Hsp70 level, measured after a standard heat pretreatment, was much greater than in controls maintained at 25°C (data not shown). Artificial selection on inducible thermotolerance affected Hsp70 level but in different ways in the two sets of lines (Fig. 4; Table 2). For the entire data set of the EC lines, treatment (i.e., control vs. selected) significantly ($P = 0.006$) affected Hsp70 level, but generation and treatment × generation did not. Post hoc inspection of the results for the selected lines revealed two patterns. In selected lines 1, 4, and 5, Hsp70 was initially high and did not increase. In selected lines 2 and 3, by contrast, Hsp70 was initially low, but by generation F4 increased to the same level as in selected lines 1, 4, and 5. These patterns are all statistically significant according to an ANOVA (Table 3). This diversity aside, the average proportional change in Hsp70 from initial to final generations in which Hsp70 was determined (F1–F10) was 15%.

Table 3: ANOVA of effect of replicate line and generation on Hsp70 expression in the EC lines

| Source | df | MS | *F* | *P* Level |
|---|---|---|---|---|
| Line | 1 | 253.929 | 18.030 | .0000 |
| Generation | 1 | 96.571 | 6.857 | .0097 |
| Line × generation | 1 | 123.605 | 8.776 | .0035 |
| Residuals | 154 | 14.084 | … | … |

Note. This analysis considered two levels of replicate line (lines 1, 4, and 5 vs. lines 2 and 3) and two levels of generation (1 vs. 4–10) on the basis of Hsp70 levels in generation 1 (see text).

In the AUS selected replicates, average Hsp70 levels increased with successive generations of selection, increasingly diverging from controls (Fig. 4; Table 2). The proportional increase in Hsp70 from initial to final generations in which Hsp70 was determined (F2–F9) was approximately the same as in the EC replicates.

## Discussion

Thermotolerance in *Drosophila* readily responds to laboratory evolution at different temperatures and artificial selection. Most prior work (see "Introduction"), however, has concerned the evolution of basal thermotolerance. As have others (Krebs and Loeschcke 1996; McColl et al. 1996; Bettencourt et al. 1999; Lansing et al. 2000), here we show that inducible thermotolerance likewise responds to laboratory evolution in *Drosophila*. This response was rapid, repeatable, occurred at similar rates (although from different initial levels) in the two sexes, and continued during the 10–11 generations of this study (Figs. 1–3). Although study of both forms of thermotolerance may elucidate the evolvability of thermal physiology, inducible thermotolerance is likely the more relevant to thermal evolution in natural populations of *Drosophila*. In the wild, *Drosophila melanogaster* and similar species most likely encounter lethal thermal stress while confined to necrotic fruit, which serves as oviposition sites, the larval microhabitat, and sometimes pupation sites (Feder 1997). When sunlit, necrotic fruit undergoes a gradual increase in temperature reminiscent of the pretreatment + heat-shock regime of inducible thermotolerance studies (Feder et al. 1997). Indeed, the inducible thermotolerance associated with Hsp70 can be critical for survival of such natural thermal stress (Roberts and Feder 2000).

Inducible thermotolerance also differs from basal thermotolerance in that it stems from mechanisms operating during the pretreatment regime preceding heat shock, including (but not restricted to) the expression of Hsps. In *D. melanogaster*,

Hsp70 itself accounts for a significant component of inducible thermotolerance but neither accounts for all inducible thermotolerance nor is the only Hsp or related protein to increase during pretreatment (Feder and Krebs 1998; Feder 1999). Nonetheless, the mechanistic linkage of Hsp70 and inducible thermotolerance create the expectation that selection on inducible thermotolerance should yield a correlated response (Arnold 1987) in Hsp70 levels unless the coevolution of Hsp70 level is constrained.

In this study, results for two independent sets of replicate lines bear out the expectation that selection for inducible thermotolerance should increase Hsp70 level after pretreatment (Fig. 4). The results also indicate, however, that even this correlated increase in Hsp70, which literally means the difference between life and death, is not without constraint. Average Hsp70 levels increased by no more than approximately 15% during the 10 generations of selection. In the EC replicates, this increase was largely due to two lines with especially low Hsp70 levels increasing to the same average level as in other lines. One of several potential causes for this apparent limitation is exhaustion of genetic variability for Hsp70 expression. This explanation, however, is unlikely. Variation in Hsp70 expression did not decrease in the replicate lines undergoing selection, and indeed, the variation in Hsp70 expression among isofemale lines founded from a natural population (Krebs and Feder 1997*b*) dwarfs that for the synthetic populations of this study. Another potential explanation is that increases in Hsp70 level or some correlated trait that negatively affects fitness (see "Introduction") outweigh their benefit for thermotolerance (Krebs and Feder 1997*a*, 1998; Zatsepina et al. 2001). That these negative impacts are due specifically to Hsp70 and not some correlated trait emerges from studies that experimentally manipulated Hsp70 level against a constant genetic background (Feder et al. 1992; Krebs and Feder 1997*a*, 1998). Thus, numerous prior manipulative studies, several comparisons of natural populations, and laboratory evolution/selection experiments that have now both increased and decreased Hsp70 levels are all consistent with one pattern: evolution in environments with gradually increasing severe heat shocks increases Hsp70 level, while evolution in environments with nonlethal hyperthermia decreases Hsp70 level (Zatsepina et al. 2001).

The specific genetic changes that ensued to vary Hsp70 level are unknown in this study but have involved transposable elements in recent studies of three natural lines with relatively low Hsp70 levels (Michalak et al. 2001; Zatsepina et al. 2001; Lerman et al. 2002). Each of these lines is polymorphic for insertion of a transposable element in the *Hsp70Ba* promoter, whose wild type organization is critical for normal transcription. Indeed, consistent with the aforementioned pattern, morphs lacking a transposon can have higher Hsp70 levels, greater inducible thermotolerance, and lower fitness in the absence of heat shock than morphs with a transposon.

Finally, these data indicate that mechanisms other than an increase in Hsp70 contribute to the laboratory evolution of increased inducible thermotolerance. In the EC lines, inducible thermotolerance increased throughout the latter generations of selection, whereas Hsp70 level did not. Lansing et al. (2000) reported a similar pattern, and Feder and Hofmann (1999) reviewed mechanistic studies that concur with this indication. Notably, trehalose and polyols emerge as candidate deterrents of protein aggregation that are not Hsps (Kimura et al. 1992; Ohtsu et al. 1993, 1998; Salvucci 2000).

In summary, we show that artificial selection for inducible thermotolerance can increase Hsp70, as prior work predicts. The resulting increase in Hsp70 level (approximately 15%) is limited, however. These outcomes are consistent with Hsp70 expression evolving according to a trade-off of beneficial and deleterious phenotypes.

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