The Journal of Experimental Biology 209, 3964-3973
Published by The Company of Biologists 2006
doi:10.1242/jeb.02463

Selection on knockdown performance in *Drosophila melanogaster* impacts thermotolerance and heat-shock response differently in females and males

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Accepted 31 July 2006

Summary

We studied adaptive thermotolerance in replicate populations of *Drosophila melanogaster* artificially selected for high and low knockdown temperature (T_{KD}), the upper temperature at which flies can no longer remain upright or locomote effectively. Responses to selection have generated High T_{KD} populations capable of maintaining locomotor function at ~40°C, and Low T_{KD} populations with T_{KD} of ~35°C. We examined inducible knockdown thermotolerance, as well as inducible thermal survivorship, following a pretreatment heat-shock (known to induce heat-shock proteins) for males and females from the T_{KD} selected lines. Both selection for knockdown and sex influenced inducible knockdown thermotolerance, whereas inducible thermal survivorship was influenced only by sex, and not by selection. Overall, our findings suggest that the relationships between basal and inducible thermotolerance are contingent upon the methods used to gauge thermotolerance, as well as the sex of the flies. Finally, we compared temporal profiles of the combined expression of two major heat-shock proteins, HSC70 and HSP70, during heat stress among the females and males from the selected T_{KD} lines. The temporal profiles of the proteins differed between High and Low T_{KD} females, suggesting divergence of the heat-shock response. We discuss a possible mechanism that may lead to the heat-shock protein patterns observed in the selected females.

Key words: *Drosophila melanogaster*, laboratory selection, knockdown, thermotolerance, heat-shock response, HSC70, HSP70.

Introduction

Most organisms in nature are exposed chronically to fluctuating environmental conditions, and as a consequence, have evolved stress response networks that activate when ambient conditions approach stressful levels. One means by which virtually all organisms combat stress is through the heat-shock response (Lindquist and Craig, 1988; Morimoto et al., 1994; Feder and Hofmann, 1999). Heat-shock proteins (HSPs) are principle constituents of this response and typically function as molecular chaperones, which assist in maintaining cellular protein structure. During thermal stress, members of the HSP70 family mitigate cellular damage in *Drosophila* (Georgopoulis and Welch, 1993; Parsell and Lindquist, 1993; Hartl, 1996; Sørensen et al., 2003; Gong and Golic, 2006). HSC70 and HSP70 are the most abundantly expressed members of this family, and both have been implicated in tolerance to hyperthermia. HSC70 is expressed constitutively and is linked to basal thermotolerance, whereas the expression of HSP70 is heat-induced and is linked to acquired (inducible) thermotolerance (Lindquist, 1980; Velaquez, 1983; Palter et al., 1986; Leung et al., 1996; Feder and Hofmann, 1999; Sung and Guy, 2003). The role of HSP68, the third principle HSP70 family member, in thermotolerance is not well-established (McColl et al., 1996).

Drosophilids are a model system used to study the contribution of the heat-shock response to thermotolerance. Drosophilids are thermoconformers, that is, their small body size demands that body temperature conform to ambient temperatures. In *Drosophila*, exposure to stressful temperature generally induces synthesis of HSP70 (e.g. Lindquist, 1981; Welte et al., 1993; Krebs and Loeschcke, 1994; Feder et al., 1996; Sørensen et al., 2003). HSP70 induction comes with costs and has been shown to adversely impact development and reduce reproductive capacity. In *D. melanogaster,* HSP70 induction is implicated in reduced fecundity (Krebs and Loeschcke, 1994), and HSP70 overexpression is associated with increased larval mortality (Krebs and Feder, 1997b), retarded growth (Feder et al., 1992), and reduced egg hatching (Silbermann and Tatar, 2000).

While HSP70 expression in *D. melanogaster* incurs high costs, induced thermotolerance due to HSP70 accumulation is well-established (e.g. Welte et al., 1993; Feder et al., 1996; Feder and Krebs, 1998; Krebs and Feder, 1997a). Juvenile drosophilids are confined to their feeding environment and,

therefore, may be unable to seek refuge from thermal stress. In contrast, adults can fly and may rely on behavior to escape. Despite a capacity for escaping heat stress *via* flight, temperatures in nature can increase quite suddenly (Dahlgaard and Loeschcke, 1997), and adults may find themselves in transit during stress-inducing temperature transitions. In these situations, the maintenance of flight allows adults to seek a thermal refuge. Induction of HSP70 appears to protect adult *D. melanogaster* against locomotor dysfunction during thermal stress (Roberts et al., 2003; Klose and Robertson, 2004), suggesting that HSPs have ecologically relevance for adults, as well as juveniles (see also Michalak et al., 2001; Sørensen et al., 2003; Gong and Golic, 2006).

Relatively few studies have examined the impact of HSPs on locomotor function in insects, but one of the few is particularly germane to our current work (Newman et al., 2004). In the study, the effect of HSPs on locomotion was compared between a desert and a temperate species of *Drosophila*. Thermosensitivity of locomotor behavior in larvae of the desert species (*D. arizonae*) was reduced, relative to that of the temperate species (*D. melanogaster*). The upper temperature limit for locomotor function in *D. arizonae* exceeded that of *D. melanogaster* by 6°C. Conversely, exposure to 40°C was associated with improved synaptic function at neuromuscular junctions only in *D. melanogaster*, not in *D. arizonae*. The high basal thermotolerance in *D. arizonae* was correlated with high levels of constitutively expressed HSC70, whereas the low inducible thermotolerance of this species was correlated with the absence of inducible HSP70 at 40°C. In general, desert species of *Drosophila*, as well as species' strains from hot climates, appear to have high constitutive levels of HSC70 and relatively high temperature set-points for HSP70 induction (Sørensen et al., 2001; Zatsepina et al., 2001). This profile of high constitutive levels coupled with high temperature induction of HSPs has also been observed in desert lizards (Ulmasov et al., 1992; Zatsepina et al., 2000).

In this study, we used artificial selection to generate replicate populations of *Drosophila melanogaster* capable of locomotor function at high temperatures (~40°C). The adults from these populations were selected for high knockdown temperature (T_{KD}), the upper temperature at which flies are unable to locomote effectively or remain upright (Gilchrist and Huey, 1999). The High T_{KD} flies display enhanced thermotolerance, relative to the Low T_{KD} populations selected for low knockdown temperature. In nature, populations of *Drosophila melanogaster* exhibit clinal variation in knockdown time and temperature along the eastern coast of Australia, with flies from low latitudes having a significantly higher knockdown than those from high latitudes (Hoffmann et al., 2002) (G.W.G. and R. B. Huey, unpublished). Adaptive changes in thermotolerance may be mediated by modifications within the heat-shock response, which has been demonstrated in a variety of natural and experimental *Drosophila* populations (McColl et al., 1996; Krebs and Feder, 1997a; Dahlgaard et al., 1998; Feder and Krebs, 1998; Lerman and Feder, 2001; Michalak et al., 2001; Sørensen et al., 2001; Zatsepina et al., 2001; Bettencourt et al., 2002).

We propose that the knockdown lines provide a useful model of adaptation to thermal stress, and that locomotor performance at high temperature in the High T_{KD} lines is supported, in part, by modifications within the heat-shock response. Furthermore, selection for high or low knockdown temperature has altered basal T_{KD} thermotolerance in our selected lines, and we argue that inducible thermotolerance may be influenced as well. More specifically, we propose that the HSP profile of the High T_{KD} flies may resemble that of high-temperature adapted *Drosophila*. We predict that females and males from the High T_{KD} lines, when compared to the same sex from the Low T_{KD} lines, will have: (i) higher constitutive levels of HSC70, and (ii) a temperature set-point for HSP70 induction that is higher than 36°C, a temperature that induces HSP70 synthesis in adults from temperate populations (Zatsepina et al., 2001). Assuming that the temperature set-point for HSP70 induction has shifted upward in the High T_{KD} lines and is >36°C, we predict that, following a 36°C pre-treatment, (iii) locomotor capacity during thermal stress will be improved in Low T_{KD} lines (and Control lines) only, and (iv) inducible thermotolerance, indicated by enhanced thermal survival, will be evident in Low T_{KD} lines (and Control lines) and absent in High T_{KD} lines.

Materials and methods

Establishment of artificially selected fly lines

The founding population of *Drosophila melanogaster* used in this study was established in 1991 by combining ~1000 isofemale lines collected by L. Harshmann and M. Turelli in Escalon, California, USA. In April 1992, a subset of this population (~1000 females) was used to generate a large number of offspring, which were transferred to population cages, each containing 2000–3000 adults (for details, see Gilchrist and Huey, 1999). The flies were maintained in discrete 2-week generations at 25°C with a 12 h:12 h L:D photoperiod. In 1993–1994, six High Knockdown lines ('High T_{KD}'), three Low knockdown lines ('Low T_{KD}'), and six Control lines were established. The lines underwent artificial selection (as described below) for 46 generations, ending in September 1997, at which time they were maintained in discrete 3-week generations without selection. In June 2004, selection on these original lines was resumed and continued for 7 generations.

In January 2005, we combined flies from the original lines within each selection group and use these flies to generate new High T_{KD}, Low T_{KD} and Control lines (Fig. 1). To establish each of the four new High T_{KD} lines ($HN_{1–4}$), 25 males and 25 females from each of the six original High T_{KD} lines were haphazardly chosen and combined. The same procedure was repeated using flies from the six original Control lines to establish four new Control lines ($CN_{1–4}$). Each of the four new Low T_{KD} lines ($LN_{1–4}$) was generated by combining 50 males and 50 females haphazardly chosen from each of the three

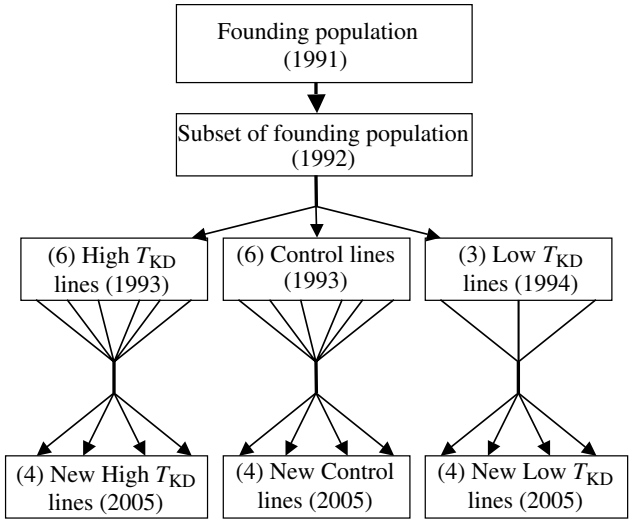

Fig. 1. A diagrammatic depiction of the selection history of the original and newly established High $T_{KD,}$ Low $T_{KD,}$ and Control lines. Details of the selection history are discussed in Materials and methods.

original Low T_{KD} lines. We generated these new selection lines in order to: (1) reduce inbreeding by crossing the original lines within each treatment group, and (2) create equal numbers of lines within the HN, LN and CN treatment groups. All experiments in this study were done using the new selection lines that had undergone selection for up to 9 generations.

Measuring knockdown temperature

The knockdown protocol is detailed elsewhere (Huey et al., 1992; Gilchrist and Huey, 1999). Approximately 1000 flies from each line are poured into the inner tube of a water-jacketed Weber column (Weber, 1988). The water jacket is heated by a Haake DC-10 immersion heater (Haake-Buchler Inc., Paramus, NJ, USA) in a 26 liter water bath and contains a copper tube through which air is heated to the same temperature and forced through the column. The flies are poured into the inner tube when the air core temperature is 30°C. Mesh baffles within the inner tube provide places for the flies to cling during the experiment. The water temperature is ramped from 30°C up to 50°C over ~50 min; the average rate of change is ~0.4°C min^{-1}. T_{KD} is that temperature at which a fly can no longer locomote or cling to the baffles or column walls; they fall out of the column. As they fall out, the flies are fractionated at 0.5°C intervals between 32°C to 46°C. Recovery is nearly instantaneous (Gilchrist and Huey, 1999). The knockdown flies are then separated by sex, counted, and the distribution of knockdown temperatures recorded separately for males and females from each line.

Selection and maintenance protocols of fly lines

To maintain each HN line, ~30% of the flies with the highest T_{KD} are selected and retained for breeding. For each LN line, ~30% of the flies whose T_{KD} generally ranges from 35.5–37°C are selected and retained for breeding. After each Control line is run through the KD column, ~30% of all the flies are haphazardly chosen for retention and breeding. Following a round of selection, the selected flies from each line (~300 individuals with approximately equal number of males and females) are divided into two groups of ~150 flies, each of which is placed into a bottle containing 30 ml cornmeal–molasses–yeast–agar–tegosept medium sprinkled with yeast. The flies are maintained at 25°C for 5–6 days following knockdown to ensure mating between selected males and females (Gilchrist and Huey, 1999). They are then transferred to fresh food sprinkled with yeast and allowed to oviposit for ~24 h. The eggs are transferred to fresh food vials at a density of ~50 eggs/vial. Twenty-eight vials of eggs are collected from each line. Flies develop and mature at 25°C (12 h:12 h L:D) for 13 days, at which time the adults are transferred to fresh food bottles sprinkled with yeast in preparation for the next knockdown experiment, which is run on days 14 and 15 following egg collection. (The selection and maintenance protocols have remained consistent for the original and the new knockdown lines.)

Induction of HSP70 and knockdown temperature

Flies from each selection line were removed from selection for two generations prior to egg collection to minimize cross-generational effects. Twenty vials of eggs (~50 eggs/vial) were collected from each CN, LN and HN line. At 14 days following egg collection, flies from four food vials were transferred into a single food bottle; this was repeated until we had collected five food bottles from each line, each containing ~200 flies. Immediately prior to the pretreatment (36°C for 1 h, followed by 25°C for 1 h), flies in each bottle were transferred to an empty food bottle containing a piece of moistened filter paper (~1 cm^2). Immediately following pretreatment, the flies were run through the knockdown column, and the distribution for knockdown temperatures was recorded (as described above). The distribution of T_{KD} of flies not subjected to the pretreatment was also recorded within the same generation for all CN, LN and HN lines.

Basal and induced thermal survival

To minimize cross-generational effects, all flies were removed from selection for one generation prior to egg collection. Eggs (~50 eggs/vial; 28 vials/line) were collected from all CN, LN and HN lines. Each fly line was treated according to the following protocol. Thirteen days following egg collection, adults were CO_2-anesthetized and combined. Two groups of ~180 flies were generated haphazardly, and each group was transferred to a fresh food bottle. Three days later, the flies in both bottles were CO_2-anesthetized, combined, separated according to sex, and distributed haphazardly into two groups of food vials: five vials with ~15 males in each (Group 1) and five additional food vials with ~15 males in each (Group 2). The same collection scheme was repeated for the females. All flies were allowed to recover from CO_2-anesthesia for ~36 h. Groups 1 and 2 were treated according to the following protocols.

(1) Pretreatment+heat stress

The flies from each food vial in Group 1 were transferred to a 12 mm×75 mm glass culture tube containing 1 ml of 2% agar. Culture tubes were capped (pin holes were made in caps), placed into a 36°C water bath for 1 h, and then held at 25°C for 1 h. Immediately following this pretreatment, the flies were heat-shocked at 38.5°C for 1 h, held at 25°C for 24 h, and then scored for survivorship.

(2) Heat-stress only

Flies in Group 2 were treated exactly as described above for Group 1, except the pretreatment (i.e. 36°C for 1 h, and then 25°C for 1 h) was omitted. Proportional survivorship of flies in each culture tube was determined by dividing the number of flies alive in a tube at 24 h by the number of flies initially placed in the tube.

The heat-shock response: heat-stress treatment and sample preparation

Two major HSP70 family members, HSC70 and HSP70, were assayed separately in males and females following exposure to 36°C at 0 min, 10 min, 20 min, 30 min or 60 min. Samples were prepared from each selected line ($HN_{1–4}$ and $LN_{1–4}$) according to the following protocol. On day 13 or 14 following the egg stage, adults were CO_2-anesthetized and separated according to sex. Six groups of 20–25 males/group, and six groups of 20–25 females/group, were each placed in fresh food vials and allowed to recover from anesthesia for ~24 h. One sample (i.e. 20–25 flies) from each sex was transferred to a 1.5 ml screw-top tube, quick-frozen and stored at –80°C. The remaining samples (5 groups from each sex) were each transferred to an empty food bottle containing moistened filter paper (~2 cm^2) and placed in a 36°C water bath. One sample from each sex was removed from the water bath after 10 min, 20 min, 30 min or 60 min. Upon removal, each sample was transferred immediately to a 1.5 ml screw-top tube, quick-frozen and stored at –80°C. [Quick-freezing adults at –70°C does not affect HSP70 levels compared to flash-freezing adults in liquid nitrogen (Dahlgaard et al., 1998).]

Each sample was prepared for protein gel electrophoresis (12% SDS-PAGE) by homogenizing the flies in 200–400 μl of ice-cold, 2 mmol l^{-1} PMSF (phenylmethylsulphonyl fluoride, protease inhibitor, MP Biomedicals, Inc., Aurora, OH, USA) in 1× phosphate-buffered saline (PBS) with a hand-held Kontes homogenizer (Kimble/Kontes, Vineland, NJ, USA). Homogenized samples were centrifuged at 14 000 ***g*** for 30 min at 4°C. Samples of the supernatant (40–60 μl) were transferred to microcentrifuge tubes, quick-frozen and stored at –80°C.

Western blot analysis

Total protein concentration of the samples (supernatants) was measured using a Bradford Protein Assay according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). Samples were denatured by heating at 95°C for 5 min in SDS sample buffer (final concentrations: 12.5% glycerol, 0.5% Bromophenol Blue, 1.25% sodium dodecylsulfate, 0.5 mol l^{-1} Tris-Cl, pH 6.8, 1.25% β-mercaptoethanol). 30–50 μg of protein was loaded into each well for electrophoresis, and 5 ng of HSP70 protein (Product # NSP-555, Stressgen Biotechnologies Corp., San Diego, CA, USA) was included as a standard. Proteins were separated on 12% polyacrylamide gels (mini-Protean II, Bio-Rad Laboratories) for ~45 min at 180 V, according to standard methods. Separated proteins were transferred onto a PVDF (polyvinylidene difluoride) membrane (Hybond™-P, GE Healthcare Life Sciences, Waukesha, WI, USA; Protean II system, Bio-Rad Laboratories). Following transfer, blots were incubated in 5% non-fat dried milk in phosphate-buffered saline (1.9 mmol l^{-1} $NaH_2PO_4.H_2O$, 8.1 mmol l^{-1} $Na_2HPO_4.7H_2O$, 137 mmol l^{-1} NaCl, 2.6 mmol l^{-1} KCl, pH 7.4) + 0.1% Tween® 20 (PBS-T) either overnight at 4°C or for 1 h at 23°C, followed by a 1-h incubation using two primary antibodies simultaneously: (1) rabbit anti-rat HSC70/HSP70 polyclonal antibody (in whole rabbit antiserum) against amino acid residues 446–641 (Stressgen Biotechnologies Corp., San Diego, CA, USA, Product # SPA-757; 1:100 000 in blocking solution); and (2) rabbit anti-actin polyclonal IgG antibody against amino acid residues 20–33 of invertebrate actin (Sigma, St Louis, MO, USA, Product# A5060, 2.46 μg ml^{-1} in blocking solution). Following two 10-min washes in PBS-T, blots were incubated for 1 h with the secondary antibody: goat anti-rabbit IgG polyclonal antibody conjugated to HRP (horseradish peroxidase; Stressgen Biotechnologies Corp., Product #SAB-300, 1:10 000 in blocking solution). Blots were washed 3× for 10 min each wash in PBS-T. Chemifluorescence of immunoreactions was detected using ECL Plus™ Western Blotting Detection Reagents according to the manufacturer's instructions (GE Healthcare Life Sciences). Heat-shock proteins and actin were quantified with a Molecular Dynamic STORM Phosphorimager (GE Healthcare Life Sciences) and software, ImageQuant, version.5.2. Quantification of actin was used to normalize the protein concentrations of the samples.

Statistical analyses

All statistical analyses were performed using *R*, version 2.2.1 (*R* Development Core Team, 2006). Estimation of the parameters for the unimodal and bimodal distributions for T_{KD} was by restricted maximum likelihood (Gilchrist and Huey, 1999). The hypothesis that a bimodal distribution provided a better description of the data than the unimodal distribution was tested using a log-likelihood ratio test. Additional statistical analyses, all performed in *R* (version 2.2.1), are described in the Results.

Results

Response to knockdown selection

All the original lines displayed a bimodal distribution for T_{KD} at the onset of selection, as detailed elsewhere (Gilchrist and Huey, 1999). Bimodality has been observed in ~50 populations of *D. melanogaster* for which T_{KD} has been measured (G.W.G., unpublished), except in the T_{KD} selected lines. While the

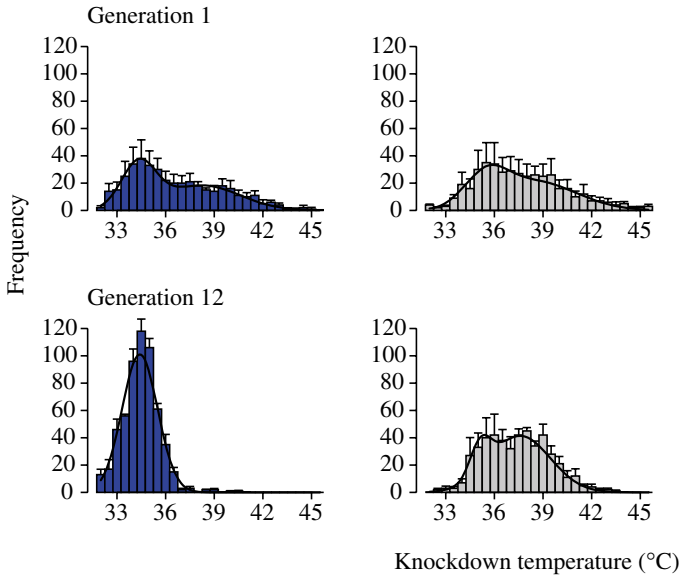

Fig. 2. Histograms of T_{KD} for the newly established LN, CN and HN lines in generations 1 (top) and 12 (bottom). At generation 12, the flies had undergone 9 generations of knockdown selection. The error bars show s.e.m. values for groups of flies fractionated at 0.5°C intervals. Each LN, CN and HN group comprises four replicate populations. During knockdown, ~1000 flies from each line are assayed for T_{KD}; thus, each graph represents T_{KD} frequency distribution for ~4000 flies. The fitted line on each graph shows the maximum likelihood fit for a bimodal distribution. The fitted parameters for the distributions are listed in Table 1.

bimodal distribution for T_{KD} was retained in the original Control lines, the original High T_{KD} lines lost most of the lower mode and displayed an increase in the mean of the upper mode. Conversely, the original Low T_{KD} lines lost most of the upper mode and displayed a decrease in the mean of the lower mode.

The mean distributions for T_{KD} in the newly established lines (i.e. HN, LN and CN) for the 1st and 12th generations are shown in Fig. 2, with parameters given in Table 1. Both the LN and the CN lines show bimodality in Generation 1, after combining the original lines. In contrast, the HN lines are more nearly unimodal, although there was some regression towards a lower mean T_{KD} after combining the original lines. By Generation 12 (after 9 generations of selection: selection was not imposed for 3 of the 12 generations), the new HN and LN lines more closely resembled the original T_{KD} lines at Generation 46 (Gilchrist and Huey, 1999). In the LN lines, both females and males have nearly lost the upper mode. The proportions of LN flies that fell under the lower mode ranged from 0.96 to 1.00 (mean=0.98) and 0.83 to 1.00 (mean=0.93) for the females and males, respectively (Table 1). The mean temperature of the lower mode for both sexes was lower in the LN lines (34.7°C) than in the HN lines (37.1°C). Interestingly, the HN lines somewhat increased in bimodality for T_{KD}. Nonetheless, a high proportion of the HN flies fell under the upper mode (females ranged from 0.47 to 0.80, mean=0.66; males ranged from 0.83 to 1.00, mean=0.90). The mean temperature of the upper mode for both sexes was higher in the HN lines (41.1°C) than in the LN lines (38.1°C).

Shifts in knockdown temperature following pretreatment heat-shock

The T_{KD} distribution of males and females of the CN, HN and LN lines was measured immediately following pretreatment and was compared to T_{KD} distribution for flies that were not pretreated (Fig. 3). Paired *t*-tests were used to compare mean T_{KD} of pretreated flies with mean T_{KD} of non-pretreated flies for each sex from each CN, HN and LN group (e.g. LN females were compared only with non-pretreated LN females). The only group showing a significant increase in T_{KD} following the pretreatment was the LN females (P=0.0236). Both females and males from all HN lines had a significant drop in T_{KD} following the pretreatment (P=0.0002; P=0.0007,

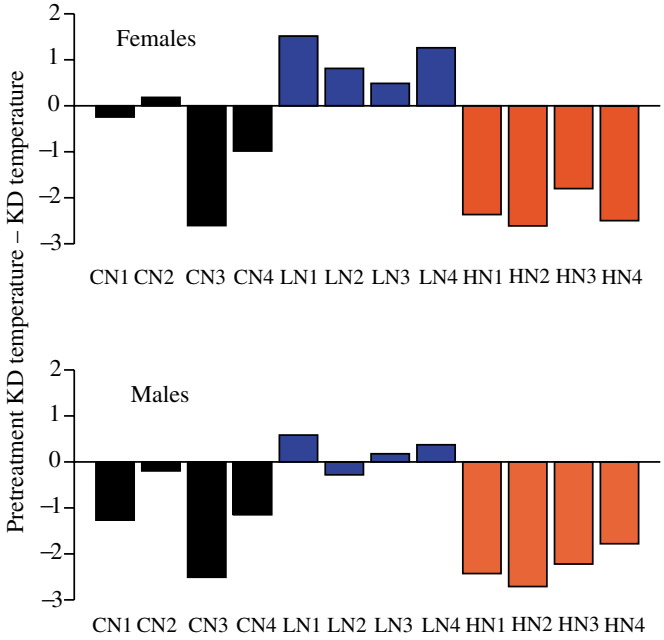

Fig. 3. The effect of a heat-shock pretreatment on T_{KD}. Each bar represents the difference (in °C) between the mean T_{KD} of pretreated flies and the mean T_{KD} of non-pretreated flies for the $CN_{1–4}$, $LN_{1–4}$, and $HN_{1–4}$ lines. The values for the females are shown at the top, males at the bottom. Mean difference in T_{KD} for females (and for males) from each line was estimated using ~500 pretreated flies and ~500 non-pretreated flies. Black bars, values for $CN_{1–4}$; blue bars, $LN_{1–4}$; red bars, $HN_{1–4}$.

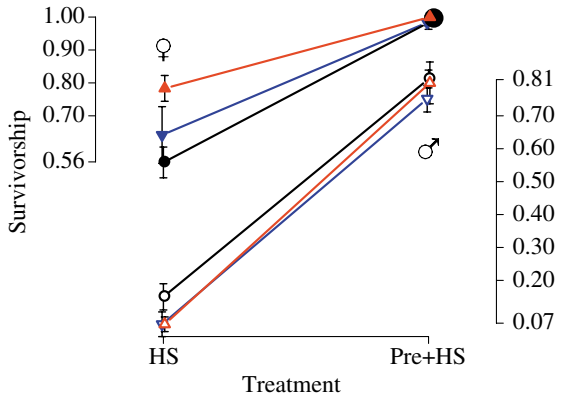

Fig. 4. Pretreatment of flies (heat hardening) increases survivorship, measured by proportion of flies that are alive 24 h after thermal stress. HS=38.5°C heat shock for 1 h, with no pretreatment. Pre+HS=36°C heat hardening pretreatment for 1 h followed by a resting period (25°C for 1 h) prior to heat shock at 38.5°C for 1 h. Each data point represents the mean survivorship of ~300 males or females (i.e. ~75 flies from each of the four CN, LN or HN lines). Values are means ± s.e.m. Red lines depict change in survivorship in HN flies, with red filled triangles (pointing upward) for females; open triangles, males. Blue lines depict change in survivorship in LN flies, with blue filled triangles (pointing downward) for females; open triangles, males. Black lines depict changes in survivorship in the CN flies, with black filled circles for females; open circles, males.

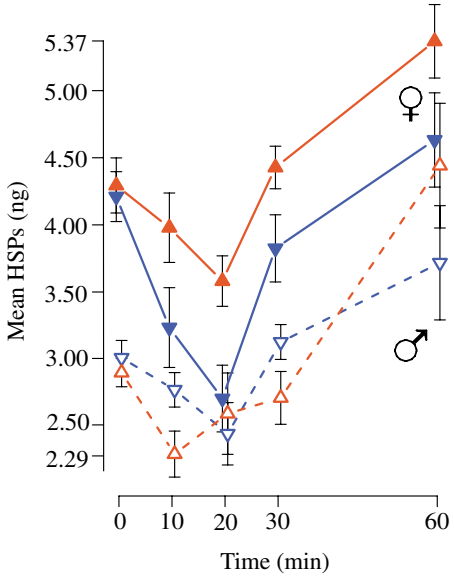

Fig. 5. Total HSC70/HSP70 protein content prior to (0 min) and at 10, 20, 30 and 60 min following a 36°C heat stress in female and male flies. Results are expressed relative to 5 ng HSP70 protein included as a standard (see Materials and methods). The data were adjusted for block effects of running western blots in groups. Solid red lines and red filled triangles, values for the HN females; solid blue lines and blue filled triangles, LN females; broken red lines and open red triangles, HN males; broken blue lines and open blue triangles, LN males. Values are mean ± s.e.m. of 8 samples (2 samples from each $HN_{1–4}$ or $LN_{1–4}$ line).

respectively). Control males and females, as well as LN males, had no significant change in T_{KD} distribution in response to the pretreatment.

Pretreatment heat-shock induces thermal survival

Males and females from all CN, LN and HN lines demonstrated induced thermal survivorship, with the females showing the highest induced (and basal) survivorship (Fig. 4). We used generalized linear modeling for a binomially distributed response variable and a logit link function to test for differences in thermal survivorship between non-pretreated and pretreated flies. Because the females had much higher survivorship overall, the data for males and females were analyzed separately. The analyses indicated that: (1) the pretreatment significantly increased survivorship in both sexes ($D_{[1,118]}$=349.65, P=5.050e^{-78}; and $D_{[1,118]}$=906.94, P=3.047e^{-199}, for females and males, respectively); and (2) there was a weak selection × treatment interaction in both sexes ($D_{[2,105]}$=6.51, P=0.04; $D_{[2,105]}$=6.22, P=0.04, for females and males, respectively). Most of the variation among the selected lines was in the non-pretreated group (Fig. 4): the HN females had a slightly higher survivorship than the CN or LN females, and the CN males had a slightly higher survivorship than the LN or HN males. The survivorship of pretreated females from all lines was very similar; likewise for the males.

Profiles of HSC70/HSP70 protein after heat-stress

HSC70/HSP70 protein levels were measured at multiple time intervals in T_{KD} selected females and males held at 36°C. HSC70/HSP70 values from the samples on each gel were normalized using relative proportions of actin from the same samples. We used linear regressions to test for a functional relationship between actin proportions and the time intervals (data not shown). Actin proportions were log transformed prior to analyses. The relative proportions of actin did not change significantly over time in both the HN and LN groups ($F_{[1,115]}$=3.21, P=0.0758 and $F_{[1,120]}$=1.366, P=0.2448, respectively).

The data were analyzed for the effect of sex and selection treatment on HSP levels over time, with the time of heat exposure treated as an orthogonal polynomial. (The data were adjusted for block effects of running western blots in groups.) A third-order orthogonal polynomial provided only a marginally better fit ($F_{[64,68]}$=2.2247, P=0.07612) than a second-order, so we used the second-order to avoid over-fitting the model. We tested for homogeneity of the orthogonal polynomial coefficients among the sex × selection groups and could not reject the null hypothesis (homogeneity: $F_{[2,68]}$=0.93, P=0.401). We then used an analysis of covariance, fitting common second-order orthogonal polynomial coefficients to the sex × selection groups, and tested for homogeneity among the intercepts. A significant interaction between sex and selection ($F_{[1,74]}$=4.84, P=0.031) suggests that males and females responded differently to thermal stress. The HN females have significantly higher levels of the HSPs than LN

Table 1. *Maximum likelihood estimates for unimodal and bimodal KD temperature (T_{KD}) distributions for Control lines, High KD lines and Low KD lines in generation 12*

Treatment	Sex	Replicate	M_u (S_u)	P_{low}	M_{low} (S_{low})	M_{high} (S_{high})	G	P
Control	F	1	35.23 (1.35)	1.00	35.23 (1.35)	NA	0.00	1.000
		2	34.85 (1.62)	0.86	34.45 (1.04)	37.27 (2.30)	161.50	0.000
		3	35.86 (1.89)	0.83	35.17 (1.10)	39.13 (1.33)	176.18	0.000
		4	35.51 (1.88)	0.85	34.93 (1.27)	38.87 (1.24)	79.38	0.000
Control	M	1	36.40 (1.88)	0.57	35.33 (0.82)	37.84 (1.94)	162.07	0.000
		2	36.19 (2.19)	0.73	35.37 (1.29)	38.44 (2.54)	124.64	0.000
		3	37.29 (2.40)	0.60	35.72 (1.18)	39.67 (1.73)	104.91	0.000
		4	36.78 (2.71)	0.62	35.30 (1.30)	39.19 (2.68)	142.39	0.000
High	F	1	40.40 (2.15)	0.20	37.08 (1.18)	41.23 (1.39)	97.33	0.000
		2	39.24 (2.21)	0.41	37.22 (2.06)	40.67 (0.60)	328.92	0.000
		3	39.57 (2.55)	0.53	38.29 (2.93)	40.99 (0.61)	295.80	0.000
		4	39.79 (2.12)	0.23	36.78 (1.12)	40.69 (1.41)	68.39	0.000
High	M	1	40.80 (1.98)	0.00	NA	40.80 (1.98)	0.00	1.000
		2	40.38 (1.90)	0.17	36.92 (1.37)	41.10 (0.97)	215.00	0.000
		3	40.96 (2.19)	0.12	36.39 (1.11)	41.57 (1.45)	144.42	0.000
		4	40.77 (1.96)	0.12	37.21 (1.28)	41.27 (1.46)	48.94	0.000
Low	F	1	34.42 (1.17)	0.99	34.38 (1.10)	38.86 (0.65)	28.59	0.000
		2	34.33 (1.05)	1.00	34.33 (1.03)	38.50 (0.00)	63.59	0.000
		3	34.77 (1.18)	0.96	34.64 (0.95)	38.08 (1.71)	105.32	0.000
		4	34.47 (1.07)	0.98	34.41 (0.93)	38.68 (1.13)	79.63	0.000
Low	M	1	35.21 (1.51)	0.90	34.86 (1.06)	38.38 (1.34)	118.39	0.000
		2	34.86 (1.01)	0.97	34.77 (0.89)	37.57 (0.66)	28.01	0.000
		3	35.27 (1.42)	1.00	35.27 (1.42)	NA	0.00	1.000
		4	35.10 (1.33)	0.83	34.74 (0.78)	36.87 (1.91)	220.11	0.000

A significant result for the log-likelihood test suggests that the bimodal distribution provides a better description of T_{KD}. M_u and S_u are maximum likelihood estimates (MLEs) for means and standard deviations (s.d.) assuming a unimodal model. M_{low} and S_{low} are the mean and s.d. of the low mode and M_{high} and S_{high} are the mean and s.d. of the high mode, assuming a bimodal distribution. P_{low} is the proportion of the flies that fall under the lower mode.

High, high KD lines; and Low, low KD lines; G, G statistic.

females (Tukey HSD, P=0.0175), whereas there is no significant difference in protein levels between the HN and LN males (Tukey HSD, P=0.9991).

The data were also analyzed for the effect of selection treatment on HSP levels over time separately for the sexes. We used a REML (restricted maximum likelihood estimation) fit of a mixed-effects model, with 'line within selection treatment' treated as a random factor. The results from these analyses show concurrence with those from the analyses described above: the females from the LN and HN lines differed in HSP content over time (Likelihood Ratio Test: $\chi^2_{[1]}$=6.36, P=0.001165), while the LN and HN males showed no significant difference (LRT: $\chi^2_{[1]}$=0.76, P=0.383).

T_{KD} selection did not affect constitutive levels of HSC70, as shown at '0 min' for both sexes (Fig. 5). The only difference in constitutive HSC70 was between the sexes: the females had ~40% more HSC70 than the males. In all groups, protein levels dropped initially and then began to climb after 20 min (10 min in HN males). By 60 min, the HSP content had surpassed constitutive levels in all groups, suggesting that HSP70 induction had occurred (Palter et al., 1986). The HN and LN males showed no statistically significant difference in HSC70/HSP70 content at 60 min, although the average level in HN males surpassed that of the LN males by ~25%. The HN females, relative to the LN females, had significantly higher HSP levels at 60 min.

Discussion

Responses to selection for high and low resistance to T_{KD}

Through artificial selection for high T_{KD}, we generated replicate populations of *D. melanogaster* that maintain locomotor function at ~40°C (Fig. 2 and Table 1). Selection for low T_{KD} generated replicate populations with a mean T_{KD} of ~35°C, resembling T_{KD} of the Control lines (mean T_{KD}: ~36°C). As a consequence of selection, the bimodal

distribution for T_{KD} consistently observed in many populations of *D. melanogaster* was largely diminished in both the High and Low T_{KD} lines (HN and LN, respectively). In the LN lines, both sexes generally lost the upper mode, while in the HN lines both sexes tended to lose the lower mode. Loss of the lower mode in HN males is particularly striking: only ~10% of the males fell under the lower mode. More of the HN females (~33%) fell under the lower mode, suggesting that despite strong selection for high T_{KD}, the lower mode in HN females was moderately less responsive to selection. The biological significance of the widespread bimodal distribution for T_{KD} is unclear, but its persistence in natural populations of *D. melanogaster* suggests that there may be an adaptive advantage to retaining both modes and that the fixation of either mode may entail some significant trade-off in fitness. The bimodality of wild (and unselected) flies may reflect a polymorphism regulating thermotolerance, or a multilocus system for dealing with heat, cold, or levels of heat-shock proteins.

Influence of pretreatment on T_{KD}

Prior to measuring T_{KD} in this experiment, LN, HN and CN flies were subjected to a pretreatment heat-shock known to induce HSP70 in many temperate populations of *Drosophila melanogaster*. We predicted that pretreatment would improve knockdown performance in LN (and CN) flies. This prediction proved accurate for LN females only (Fig. 3). During a typical knockdown experiment (no pretreatment), an average LN female is heat-stressed for ~13 min, during which their HSC70 content drops precipitously (Fig. 5). When the flies are pretreated just prior to measuring knockdown, the HSP profile during knockdown may be quite different due to the heat-shock response elicited by the pretreatment. We propose that the improved knockdown performance in LN females may be due to HSP70 induction, which appears to improve locomotor function in adults from some populations of *D. melanogaster* (Roberts et al., 2003; Newman et al., 2004; Klose and Robertson, 2004). It is unclear why the pretreatment had no significant effect on knockdown performance in LN males. Our data indicate that HSP70 induction occurs in the males also. This discrepancy underscores the difference in stress responses between males and females.

The pretreatment heat-shock had an adverse effect on T_{KD} in both HN males and females: T_{KD} dropped $\geqslant$2°C for most of the HN lines (Fig. 3). We speculate that energetic trade-offs may contribute to the reduction in T_{KD}. During a typical knockdown experiment, HN flies are heat-stressed for ~25 min at temperatures approaching $\geqslant$40°C. During a typical knockdown experiment, HSC70 levels drop and HSP70 induction is initiated (Fig. 5). As discussed above, when flies are pretreated just prior to knockdown, HSP70 has accumulated at the onset of knockdown. As a result, the pretreated HN flies may face strong energetic challenges during knockdown, such as: (1) the ATP-driven activities of HSP70 (and perhaps other molecular chaperones), coupled with (2) rising metabolic demands associated with increasing body temperature. The metabolic rate of HN flies may increase ~70% during knockdown as temperature reaches T_{KD} (data not shown). We propose that at very high temperatures metabolic trade-offs may perturb cellular processes fundamental to locomotor function in the pretreated HN flies, resulting in lower than normal T_{KD}.

Basal and induced thermal survivorship

We hypothesized that the induction of thermal survivorship through a pretreatment would be evident in LN (and CN) flies, and not HN flies. Our results indicate that enhanced thermal survivorship was induced in both LN and CN flies, as well as in HN flies (Fig. 4).

All males had low basal thermal survivorship (<10%, on average), with CN males faring slightly better than the others, whereas all females had relatively high basal thermal survivorship (~65%, on average). High levels of HSC70 in females may contribute to their high basal thermotolerance. The HN females had significantly higher basal survivorship (~80%) than LN females (~63%); yet 'basal' HSC70 (i.e. HSC70 of non-stressed flies) did not differ between the HN and LN females. Thus, the difference in survivorship between the females cannot be ascribed to a dosage-effect of 'basal' HSC70. When heat-stressed for 60 min at 36°C, the HN females consistently maintained higher levels of the HSPs (Fig. 5), so perhaps the maintenance of high levels of the one (or both) of these proteins results in higher basal thermal survivorship.

Adaptive change in the heat-shock response

Our findings did not support the hypothesis that the lines selected for high knockdown thermotolerance have relatively high levels of constitutive HSC70. The only difference in constitutive HSC70 was between the sexes: non-stressed females had, on average, ~40% more HSC70 than the males (Fig. 5). This is not surprising, given that the ovaries and embryonic tissues are enriched with HSC70 (Palter et al., 1986). These findings re-emphasize the importance of quantifying HSC70 levels separately in females and males.

At stressful temperatures in *D. melanogaster*, the synthesis of many proteins is repressed (Storti et al., 1980; Lindquist, 1981), but HSC70 exhibits translational thermotolerance and continues to be synthesized, albeit at lower levels than in non-stressed flies (Palter et al., 1986). For several hours following heat-stress, cognate proteins, including HSC70, are the most abundant non-inducible HSPs. Yet our data indicate that after only a few minutes at 36°C, HSC70 levels of males and females from all T_{KD} lines dropped significantly. After 20–30 min, the HSP levels increased above the nadir, which may reflect some degree of recovery of HSC70, as well as the induction of HSP70.

During the initial 20 min of heat-stress, the LN females showed a significantly greater decline in HSC70 compared to the HN females. This early drop in protein levels represented a ~35% reduction in the LN females, but only ~18% in the HN females. One explanation for the significantly steeper drop observed in the LN females is that HSC70 degradation

proceeds at a higher rate. HSC70 is a key molecular chaperone that binds to unfolded and otherwise impaired proteins, and returns them to their native conformation or assists in their degradation *via* the ubiquitination system (Bercovich et al., 1997). Degradation of proteins through this system proceeds by tagging of proteins with ubiquitin followed by degradation by 26S proteasomes (Wickner et al., 1999). Although HSC70 is known to conscribe the ubiquitination system for degradation of its substrates (Bercovich et al., 1997), it is possible that HSC70 itself is also targeted for proteolysis through the same system. In support of this putative mechanism, a recent study showed that a ubiquitin ligase, CHIP (carboxy terminus of HSP70-binding protein), ubiquinates HSC70 *in vivo*, thus tagging it for proteolysis (Qian et al., 2006) and reducing $t_{1/2}$ (half life) of HSC70 *in vitro*. We hypothesize that the heat-shock response in HN females involves blunted degradation of HSC70 through changes in the activity of one or more ubiquitin-proteasome factors.

The differential drop in HSC70 protein during heat-stress may be linked to regulation of HSP70 induction. In *Drosophila*, HSP70 induction is controlled post-translationally through the activity of heat-shock transcription factor, HSF1 (Jedlicka et al., 1997). In non-stressed cells, HSF1 is an inactive monomer. Following exposure to stress, HSF1 trimers translocate to the nucleus and bind to the heat-shock element (HSE) on the *HSP70* promotor, activating transcription. Both induced HSP70 and HSC70 proteins act as negative feedback regulators of HSP70 transcription (Morimoto, 1998) by physically binding to HSF1 and attenuating the heat-shock response through repression of transcription (Shi et al., 1998). Conversely, depletion of HSP70/HSC70 (in conjunction with depletions of HSP40 and HSP90) promotes transcriptional activation (Marcher and Wu, 2001).

The idea that ‘a common stress signal activating HSF is the *relief of repression imposed by the HSPs*’ is not novel (Marcher and Wu, 2001). While others have proposed that such relief of HSP70 repression results from the titration of HSPs by impaired proteins (Lindquist and Craig, 1988; Cotto and Morimoto, 1999), we propose that it may also be a consequence of HSC70 degradation. According to our model, HN females display reduced HSC70 degradation during heat-shock. Because HSC70 protein inhibits the activity of HSF1 (Morimoto, 1998), less HSC70 degradation in HN females results in increased ability (at least partially) to inhibit HSF1 activity. This would explain both the smaller drop in HSC70 (0–20 min) in HN females, as well as the reduced increase in inducible HSP70 after 20 min. We speculate that retention of HSC70 in the HN females during heat-stress may reduce induction of HSP70, thus protecting reproductive capacity following exposure to high temperatures (Silbermann and Tatar, 2000). We are currently examining this idea.

Lastly, an alternative hypothesis would be that HSP70 induction takes place earlier in the HN females compared to the LN females, obscuring a drastic drop in HSC70. Because our antibody detects both HSC70 and HSP70 protein levels, we were unable to determine when the initiation of HSP70 induction takes place. However, a study by Feder et al. suggests that this is not necessarily a problem (Feder et al., 1997). Feder et al. measured induction of HSP70 in *Drosophila* cell cultures using ELISA. The authors were unable to detect induced HSP70 protein in cells continuously exposed to 36°C for 15 min, and they detected only minor levels after 30 min. This suggests that in our study the initial drop in protein content is showing degradation of HSC70 solely, and that the observed increase in HSP70 at 30 min is a result of HSP70 induction, perhaps coupled with synthesis of HSC70.

Conclusions

The HSP profile and thermal sensitivities of the High T_{KD} lines did not resemble those previously noted in desert and warm-climate *Drosophila*, in which high basal thermotolerance is associated with high levels of constitutive HSC70 and the temperature set-point for HSP70 induction is shifted upward. Our findings show a different heat-shock response in the thermotolerant flies, as well as complex patterns of thermotolerance.

We found no evidence that the HSPs studied support locomotor function during a typical knockdown experiment; yet they appear to have an impact when induced prior to knockdown. For example, when HN flies were pretreated just prior to knockdown, T_{KD} was reduced significantly. Although induction of HSP70 may reduce T_{KD} thermotolerance in the HN flies, it appears to increase thermal survivorship. Given these contrasting findings, we surmise that any conclusions concerning the ‘effects of HSP70 induction on thermotolerance’ are contingent upon the methods used to assay thermotolerance.

The patterns of thermotolerance in the HN lines discussed above indicate a negative relationship between basal and inducible T_{KD} thermotolerance. Furthermore, high basal T_{KD} thermotolerance in HN flies was not correlated with a similarly high level of inducible thermal survivorship. (Inducible thermal survivorship in the HN males did not differ from that of LN or CN males, which have lower basal T_{KD} thermotolerance.) Taken together, these results are in disagreement with other findings (Kellett et al., 2005), which suggest that basal thermotolerance is positively related to inducible thermotolerance in *Drosophila.* We suggest that the physiological mechanisms supporting high basal T_{KD} thermotolerance in our HN lines differ from those required for high inducible thermotolerance.

This work was supported by NSF DEB-0344273 to G.W.G. and D.M.R. We wish to thank Katie Provost for technical assistance and two anonymous reviewers for their insightful comments.

References

Bercovich, B., Stancovski, I., Mayer, A., Blumenfeld, N., Laszlo, A., Schwartz, A. and Ciechanover, A. (1997). Ubiquitin-dependent degradation of certain protein substrates *in vitro* requires the molecular chaperone HSC70. *J. Biol. Chem.* **272**, 9002-9010.

Bettencourt, B. R., Kim, I., Hoffmann, A. A. and Feder, M. E. (2002).

Response to natural and laboratory selection at the *Drosophila* HSP70 genes. *Evolution* **56**, 1796-1801.

Cotto, J. J. and Morimoto, R. I. (1999). Stress-induced activation of the heat-shock response: cell and molecular biology of heat-shock factors. *Biochem. Soc. Symp.* **64**, 105-118.

Dahlgaard, J. and Loeschcke, V. (1997). Effects of inbreeding in three life stage of *Drosophila buzzatii* after embryos were exposed to a high temperature stress. *Heredity* **78**, 410-416.

Dahlgaard, J., Loeschcke, V., Michalak, P. and Justesen, J. (1998). Induced thermotolerance and associated expression of the heat-shock protein HSP70 in adult *Drosophila melanogaster*. *Funct. Ecol.* **12**, 786-793.

Feder, M. E. and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.* **61**, 243-282.

Feder, M. E. and Krebs, R. A. (1998). Natural and genetic engineering of the heat-shock protein HSP70 in *Drosophila melanogaster*: consequences for thermotolerance. *Am. Zool.* **38**, 503-517.

Feder, M. E., Rossi, J. M., Solomon, J., Solomon, N. and Lindquist, S. (1992). The consequences of expressing HSP70 in *Drosophila* cells at normal temperatures. *Genes Dev.* **6**, 1402-1413.

Feder, M. E., Cartaño, N. V., Milos, L., Krebs, R. A. and Lindquist, S. L. (1996). Effect of engineering *hsp70* copy number on Hsp70 expression and tolerance of ecologically relevant heat-shock in larvae and pupae of *Drosophila melanogaster*. *J. Exp. Biol.* **199**, 1837-1844.

Feder, M. E., Blair, N. and Figueras, H. (1997). Natural thermal stress and heat-shock protein expression in *Drosophila* larvae and pupae. *Funct. Ecol.* **11**, 90-100.

Georgopoulos, C. and Welch, W. J. (1993). Role of the major heat-shock proteins as molecular chaperones. *Annu. Rev. Cell Biol.* **9**, 601-634.

Gilchrist, G. W. and Huey, R. B. (1999). The direct response of *Drosophila melanogaster* to selection on knockdown temperature. *Heredity* **82**, 1-16.

Gong, W. J. and Golic, K. G. (2006). Loss of HSP70 in *Drosophila* is pleiotropic, with effects on thermotolerance, recovery from heat-shock and neurodegeneration. *Genetics* **172**, 275-286.

Hartl, F. U. (1996). Molecular chaperones in cellular protein folding. *Nature* **381**, 571-580.

Hoffmann, A. A., Anderson, A. R. and Hallas, R. (2002). Opposing clines for high and low temperature resistance in *Drosophila melanogaster*. *Ecol. Lett.* **5**, 614-618.

Huey, R. B., Crill, W. D., Kingsolver, J. G. and Weber, K. E. (1992). A method for rapid measurement of heat or cold resistance of small insects. *Funct. Ecol.* **6**, 489-494.

Jedlicka, P., Mortin, M. A. and Wu, C. (1997). Multiple functions of Drosophila heat-shock transcription factor in vivo. *EMBO J.* **16**, 2452-2462.

Kellett, M., Hoffmann, A. A. and McKechnie, S. W. (2005). Hardening capacity in the *Drosophila melanogaster* species group is constrained by basal thermotolerance. *Funct. Ecol.* **19**, 853-858.

Klose, M. K. and Robertson, R. M. (2004). Stress-induced thermoprotection of neuromuscular transmission. *Integr. Comp. Biol.* **44**, 14-20.

Krebs, R. A. and Feder, M. E. (1997a). Natural variation in the expression of the heat-shock protein HSP70 in a population of *Drosophila melanogaster* and its correlation with tolerance of ecologically relevant thermal stress. *Evolution* **51**, 173-179.

Krebs, R. A. and Feder, M. E. (1997b). Deleterious consequences of HSP70 overexpression in *Drosophila melanogaster* larvae. *Cell Stress Chaperones* **2**, 60-71.

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

Lerman, D. N. and Feder, M. E. (2001). Laboratory selection at different temperatures modifies heat-shock transcription factor (HSF) activation in *Drosophila melanogaster*. *J. Exp. Biol.* **204**, 315-323.

Leung, S.-M., Senisterra, G., Ritchie, K. P., Sadis, S. E., Lepock, J. R. and Hightower, L. E. (1996). Thermal activation of the bovine HSC70 molecular chaperone at physiological temperatures: physical evidence of a molecular thermometer. *Cell Stress Chaperones* **1**, 78-89.

Lindquist, S. (1980). Translational efficiency of heat-induced messages in *Drosophila melanogaster* cells. *J. Mol. Biol.* **137**, 151-158.

Lindquist, S. (1981). Regulation of protein synthesis during heat-shock. *Nature* **293**, 311-314.

Lindquist, S. and Craig, E. A. (1988). The heat-shock proteins. *Annu. Rev. Genet.* **22**, 631-677.

Marcher, G. and Wu, C. (2001). Modulation of *Drosophila* heat-shock transcription factor activity by the molecular chaperone DROJ1. *EMBO J.* **20**, 499-509.

McColl, G., Hoffmann, A. A. and McKechnie, S. W. (1996). Response of two heat-shock genes to selection for knockdown heat resistance in *Drosophila melanogaster*. *Genetics* **143**, 1615-1627.

Michalak, P., Minkov, I., Helin, A., Lerman, D. N., Bettencourt, B. R., Feder, M. E., Korol, A. B. and Nevo, E. (2001). Genetic evidence for adaptation-driven incipient speciation of *Drosophila melanogaster* along a microclimatic contrast in 'Evolution Canyon', Israel. *Proc. Natl. Acad. Sci. USA* **98**, 13195-13200.

Morimoto, R. I. (1998). Regulation of the heat-shock transcriptional response: cross talk between a family of heat-shock factors, molecular chaperones, and negative regulators. *Genes Dev.* **12**, 3788-3796.

Morimoto, R. I., Tissieres, A. and Georgopoulos, C. (ed.) (1994). *Biology of Heat-shock proteins and Molecular Chaperones*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Newman, A. E. M., Xiao, C. and Robertson, R. M. (2004). Synaptic thermoprotection in a desert-dwelling *Drosophila* species. *J. Neurobiol.* **64**, 1-11.

Palter, K. B., Watanabe, M., Stinson, L., Mahowald, A. P. and Craig, E. A. (1986). Expression and localization of *Drosophila melanogaster* HSP70 cognate proteins. *Mol. Cell. Biol.* **6**, 1187-1203.

Parsell, D. A. and Lindquist, S. (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* **27**, 437-496.

Qian, S.-B., McDonough, H., Boellmann, F., Cyr, D. M. and Patterson, C. (2006). CHIP-mediated stress recovery by sequential ubiquitination of substrates and HSP70. *Nature* **440**, 551-555.

***R* Development Core Team** (2006). *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.

Roberts, S. P., Marden, J. H. and Feder, M. E. (2003). Dropping like flies: environmentally induced impairment and protection of locomotor performance in adult *Drosophila melanogaster*. *Physiol. Biochem. Zool.* **76**, 615-621.

Shi, Y., Mosser, D. D. and Morimoto, R. I. (1998). Molecular chaperones as HSF1-specific transcriptional repressors. *Genes Dev.* **12**, 654-666.

Silbermann, R. and Tatar, M. (2000). Reproductive costs of heat-shock protein in transgenic *Drosophila melanogaster*. *Evolution* **54**, 2038-2045.

Sørensen, J. G., Dahlgaard, J. and Loeschcke, V. (2001). Genetic variation in thermal tolerance among natural populations of *Drosophila buzzatii*: down regulation of HSP70 expression and variation in heat-stress resistance traits. *Funct. Ecol.* **15**, 289-296.

Sørensen, J. G., Kristensen, T. N. and Loeschcke, V. (2003). The evolutionary and ecological role of heat-shock proteins. *Ecol. Lett.* **6**, 1025-1037.

Storti, R. V., Scott, M. P., Rich, A. and Pardue, M. L. (1980). Translational control of protein synthesis in response to heat-shock in *D. melanogaster* cells. *Cell* **22**, 825-834.

Sung, D. Y. and Guy, C. L. (2003). Physiological and molecular assessment of altered expression of Hsc70-1 in *Arabidopsis*. Evidence for pleiotropic consequences. *Plant Physiol.* **132**, 979-987.

Ulmasov, K. A., Shammakov, S., Karaev, K. and Evgen'ev, M. B. (1992). Heat-shock proteins and thermoresistance in lizards. *Proc. Natl. Acad. Sci. USA* **89**, 1666-1670.

Velazquez, J. M., Sonoda, S., Bugaisky, G. and Lindquist, S. (1983). Is the major *Drosophila* heat-shock protein present in cells that have not been heat-shocked? *J. Cell Biol.* **96**, 286-290.

Weber, K. E. (1988). An apparatus for measurement of resistance to gas-phase agents. *Drosoph. Inf. Serv.* **67**, 91.

Welte, M. A., Tetrault, J. M., Dellavalle, R. P. and Lindquist, S. L. (1993). A new method for manipulating transgenes: engineering heat tolerance in a complex, multicellular organism. *Curr. Biol.* **3**, 842-853.

Wickner, S., Maurizi, M. R. and Gottesman, S. (1999). Posttranslational quality control: folding, refolding, and degrading proteins. *Science* **286**, 1888-1893.

Zatsepina, O. G., Ulmasov, K. A., Beresten, S. F., Molodtstov, V. B., Rybtsov, S. A. and Evgen'ev, M. B. (2000). Thermotolerant desert lizards characteristically differ in terms of heat-shock system regulation. *J. Exp. Biol.* **203**, 1017-1025.

Zatsepina, O. G., Velikodvorskaia, V. V., Molodtsov, V. B., Garbuz, D., Lerman, D. N., Bettencourt, B. R., Feder, M. E. and Evgenev, M. B. (2001). A *Drosophila melanogaster* strain from sub-equatorial Africa has exceptional thermotolerance but decreased HSP70 expression. *J. Exp. Biol.* **204**, 1869-1881.