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# A *DROSOPHILA MELANOGASTER* STRAIN FROM SUB-EQUATORIAL AFRICA HAS EXCEPTIONAL THERMOTOLERANCE BUT DECREASED Hsp70 EXPRESSION

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**Summary**

***Drosophila melanogaster*** **collected in sub-equatorial Africa in the 1970s are remarkably tolerant of sustained laboratory culture above 30 °C and of acute exposure to much warmer temperatures. Inducible thermotolerance of high temperatures, which in *Drosophila melanogaster* is due in part to the inducible molecular chaperone Hsp70, is only modest in this strain. Expression of Hsp70 protein and *hsp70* mRNA is likewise reduced and has slower kinetics in this strain (T) than in a standard wild-type strain (Oregon R). These strains also differed in constitutive and heat-inducible levels of other molecular chaperones. The lower Hsp70 expression in the T strain apparently has no basis in the activation of the heat-shock transcription factor HSF, which is similar in T and Oregon R flies. Rather, the reduced expression may stem from insertion of two transposable elements, *H.M.S. Beagle* in the intergenic region of the 87A7 *hsp70* gene cluster and *Jockey* in the *hsp70Ba* gene promoter. We hypothesize that the reduced Hsp70 expression in a *Drosophila melanogaster* strain living chronically at intermediate temperatures may represent an evolved suppression of the deleterious phenotypes of Hsp70.**

Key words: *Drosophila melanogaster*, evolutionary physiology, heat-shock protein, Hsp70, molecular chaperone, transposable element.

## Introduction

*Drosophila melanogaster* has been a key model system for the elucidation of mechanisms of thermotolerance and the evolution of thermotolerance. The heat-shock response was discovered in this model, a finding that eventually led to the discovery of heat-shock proteins, their encoding genes and molecular chaperones (Ritossa, 1996). In addition, the prominence of *Drosophila melanogaster* as a model system has encouraged numerous studies of its thermal biology and a comprehensive characterization of its thermal limits (David et al., 1983). Against this background, any exception is remarkably distinctive. In the early 1970s, L. Tsacas (C.N.R.S., Gif-Sur-Yvette, France) obtained *D. melanogaster* from Fort-Lamy (now N'Djamena), Chad, in the central semiarid tropical (Sahel) zone of Africa (latitude 12°N) (J. R. David, personal communication). The climate is seasonally warm, with daytime temperatures averaging above 38 °C between March and June. These flies were fertile in continuous culture at 30 °C and were eventually shared with investigators in St Petersburg and Moscow, Russia, where they were fertile in continuous culture at 31–32 °C. Insofar as *D. melanogaster* is generally acknowledged to be incapable of continuous culture above 30 °C (Parsons, 1973) and numerous attempts to breach this limit through laboratory selection have failed, this finding is remarkable and presents a rare opportunity to understand how a species may evolve enhanced thermotolerance.

In *D. melanogaster*, typically cultured at 18–25 °C, survival at high temperature is normally inversely related to the duration and severity of heat shock, with 1 h heat shocks of 38–39 °C sufficient to cause 50 % mortality (David et al., 1983; Feder and Krebs, 1997). Culture at higher temperatures, either constantly at slightly elevated temperatures (e.g. 28 °C) or cycling between typical and still higher temperatures, can increase thermotolerance above these basal levels (Bettencourt et al., 1999; Lansing et al., 2000). This enhanced thermotolerance can ensue both within the lifetime of an individual fly (i.e. phenotypic plasticity, thermal acclimation) or *via* genetic change among generations (i.e. evolution). Moreover, pretreatment with a mild heat shock (e.g. 1 h at 35–37 °C) can rapidly increase tolerance of a more severe heat shock; this is termed 'inducible thermotolerance'. Expression of the heat-shock proteins is one of the primary mechanisms underlying inducible thermotolerance in *D. melanogaster*. In this species, one such inducible protein, Hsp70, is encoded by five nearly identical genes at two chromosomal loci (87A7, two genes; 87C1, three genes) (for a review, see Feder and Hofmann, 1999). At least eight other members of the *hsp70*

gene superfamily, both inducible and constitutively expressed, are present in the genome (Burmester et al., 2000; Easton et al., 2000; Rubin et al., 1993). Hsp70 is the most abundant heat-shock protein induced by heat, and manipulation of its abundance (*via* antisense and engineering of *hsp70* copy number) is sufficient for large changes in inducible thermotolerance (Feder et al., 1996; Solomon et al., 1991; Welte et al., 1993). Activation of the heat-shock transcription factor HSF *via* trimerization, phosphorylation and nuclear localization is a key step in heat-shock protein expression. HSF is negatively regulated through interaction with Ku autoantigen, HSBP1 and Hsp70 family members (Cotto and Morimoto, 1999; Lerman and Feder, 2001; Tuteja and Tuteja, 2000; Zatsepina et al., 2000). When activated, HSF can bind heat-shock response elements (HSEs) in the *hsp70* promoter to induce transcription; the number and arrangement of the HSEs affect *hsp70* gene expression (Lis and Wu, 1994).

These findings for typical *D. melanogaster* led us to investigate basal and inducible thermotolerance in the 'T' (thermotolerant) strains descended from those *D. melanogaster* collected in Chad and their patterns of Hsp70 protein accumulation and its transcriptional regulation. Not only can the T strain survive prolonged culture at temperatures above 30 °C, it tolerates several high-temperature treatments (36 °C for 26 h; 38 °C for 4 h) better and low temperatures more poorly than does Canton-S, a standard wild-type line (Tikhomirova and Belyatskaya, 1980). When subjected to ionizing radiation and heat shock in combination, the T strain is less prone to mutagenesis and repairs radiation injuries more readily than wild-type and Hsp-deficient strains (Tikhomirova, 1980; Tikhomirova et al., 1993). In part, this resistance depends on culture temperature because both adults and oocytes of T flies reared at 25 °C (hereafter T25) show intermediate levels of resistance compared with those reared at 32 °C (hereafter T32) and Canton-S flies (Tikhomirova and Belyatskaya, 1980; Tikhomirova and Belyatskaya, 1993). Here, we differentiate between basal thermotolerance, which is presumably due to mechanisms other than heat-shock proteins, and inducible thermotolerance, which is due in part to heat-shock proteins, in the T32, T25 and a wild-type strain, and relate these differences to levels and varieties of heat-shock proteins and the regulation of their expression. Although we initially hypothesized that study of the T strain would disclose molecular and evolutionary mechanisms of increased thermotolerance, we report here several unique features of this strain that are apparently associated with decreased inducible thermotolerance.

## Materials and methods

### Drosophila melanogaster *strains and maintenance*

The T strain flies (see Introduction), maintained at 31–32 °C (T32) and 25 °C (T25) for many years (see Introduction), were compared with Oregon R wild-type flies. In some cases T32 flies were reared from egg to adulthood at 25 °C before analysis. For determinations of thermotolerance and for Hsp70-specific enzyme-linked immunosorbent assay (ELISA), T32 and T25 flies were transported to Chicago and maintained at their normal temperatures for at least one generation before use. These flies were compared with an Oregon R stock (A25) maintained at 25 °C in discrete generations for more than 20 years (Bettencourt et al., 1999; Cavicchi et al., 1995; Cavicchi et al., 1989). All other comparisons were with an Oregon R stock maintained at 25 °C in Moscow for many years. All flies were reared on a yeast, cornmeal, molasses and agar medium.

### *Thermotolerance*

Eclosing individuals were sequestered daily and, when 4 days old, were transferred by aspiration to a fresh glass vial containing 8 ml of medium. A circle of filter paper above the medium allowed flies access to it but prevented them from adhering to it. Each vial usually contained 16–20 animals, but occasionally contained as few as 10. On the next day, vials were capped with a moistened stopper above a cotton plug, placed in a rack with vials evenly spaced and immersed in a water bath at pretreatment and heat-shock temperatures. Some flies underwent direct exposure to heat shock (37–41 °C) for 30 min. Others underwent exposure to a pretreatment temperature (36–38 °C) for 30 min followed by 25 °C for 1 h, and then underwent heat shock. Thermotolerance was measured as the proportion of flies in a vial that were able to walk 24 h after heat shock.

### *Measurement of Hsp70 protein in entire flies*

Flies were heat-shocked as above but for different durations depending on the experimental design, immediately frozen in liquid nitrogen and then stored at −80 °C until analysis. Pairs of identically treated flies were lysed in 200–400 µl of ice-cold 1× Complete Protease Inhibitor (Boehringer-Mannheim Corp) in phosphate-buffered saline by grinding briefly with an ice-cold disposable pestle. Lysates were centrifuged at 14 000 ***g*** for 30 min at 4 °C, and the protein content of the supernatant was determined (BCA Assay, Pierce Chemical Co., Rockford, IL, USA). Supernatants prepared the same day were diluted to 20 µg ml$^{-1}$ protein in ice-cold coating buffer and used to coat micro-well plates (Falcon no. 3915 ProBind) for determination of Hsp70 content by ELISA (Feder et al., 1997; Feder et al., 1996). Plates were left overnight at 4 °C to allow proteins to adsorb. After extensive rinsing, bound Hsp70 was detected using a 1:5000 dilution of the *Drosophila* Hsp70-specific antibody 7FB (Velazquez et al., 1980; Velazquez et al., 1983) coupled to alkaline phosphatase *via* a secondary antibody (1:1000 rabbit anti-rat IgG; Cappel Organon Teknika) and a tertiary antibody (1:1000 alkaline-phosphatase-conjugated goat anti-rabbit IgG; Sigma). Plates were incubated at 37 °C with the phosphatase substrate *p*-nitrophenyl phosphate (1 mg ml$^{-1}$) prepared according to the manufacturer's instructions (Sigma), and the colored reaction product was measured at 405 nm in a micro-plate reader. For at least one replicate of each sample or standard, the primary antibody was omitted to allow correction for non-specific signal. The ELISA signal is proportional to Hsp70 concentration in the lysates and

is expressed as a percentage of a standard signal, that for a lysate of *D. melanogaster* S2 cells in tissue culture that had been exposed to 36.5 °C for 1 h and to 25 °C for 1 h before lysis.

*Protein labeling, gel electrophoresis and immunoblotting*

Ten salivary glands from third-instar larvae were labeled in 20 µl of Schneider's insect medium without methionine (Sigma) after the addition of 1 µl (1.85 MBq) of L-[$^{35}$S]methionine (Amersham) for 1 h at 25 °C after various treatments. Two-dimensional gel electrophoresis and other procedures applied were as described by O'Farrell et al. (O'Farrell et al., 1977) and Ulmasov et al. (Ulmasov et al., 1992). The position of major Hsps and actin was determined both by autoradiography (see above) and by subsequent staining of gels with silver (Creighton, 1990). Salivary glands were also labeled with $^{14}$C-labeled amino acids, which yielded essentially the same results (data not shown).

For immunoblotting, after sodium dodecyl sulfate/polyacrylamide electrophoresis (SDS–PAGE) of larval lysate prepared as above, the proteins were transferred to a nitrocellulose membrane (Hybond ECL; Amersham) according the manufacturer's protocol. Monoclonal antibodies specific to the entire *D. melanogaster* Hsp70 family (7.10.3) and to Hsp70 alone (7FB, see above) were obtained from Dr Susan Lindquist (The University of Chicago). Immune complexes were detected *via* chemoluminescence (ECL kit, Amersham) and 3,3′-diaminobenzidine (DAB) (Sigma) with corresponding peroxidase-conjugated anti-rat secondary antibodies.

*Preparation of RNA and northern hybridization*

RNA was prepared by the standard method using 4 mol l$^{-1}$ guanidine isothiocyanate (Chomczynsky and Sacchi, 1987). Blotting and northern hybridization with a *Cla*I-*Bam*HI fragment containing the *Drosophila melanogaster hsp70* gene cloned into the *Bam*HI site of pUC13 (McGarry and Lindquist, 1985) was performed (Sambrook and Fritsch, 1989) with slight modifications. Hybridization was overnight at 42 °C in 50 % formamide; this was followed by two 20 min washes in 2×SSC, 0.2 % SDS at 42 °C, two 20 min washes in 1×SSC, 0.2 % SDS at 42 °C and one 20 min wash in 0.2×SSC, 0.2 % SDS at 68 °C.

*Southern hybridization and restriction digests*

Genomic DNA from the different *D. melanogaster* strains (adult flies) was prepared as described previously (Zelentsova et al., 1986). Genomic DNA (20 µg) was used for a typical restriction enzyme digestion. Digests were prepared for hybridization by electrophoresis on 1 % agarose gels, denaturation and capillary-blotting onto nylon membranes according to the manufacturer's protocol. Fixation was by ultraviolet cross-linking with a UV Stratalinker 2400 (Stratagene). Standard high-stringency hybridization and wash conditions were used (Zelentsova et al., 1986). The probe was the same as that used in northern blots.

*Gel mobility-shift assay*

Flies were frozen and pulverized in liquid nitrogen, and the powder was suspended (1:5) in a buffer containing 20 mmol l$^{-1}$ Hepes, pH 7.9, 25 % v/v glycerol, 0.42 mol l$^{-1}$ NaCl, 1.5 mmol l$^{-1}$ $MgCl_2$, 0.2 mmol l$^{-1}$ EDTA, 0.5 mmol l$^{-1}$ phenymethylsulfonyl fluoride (PMSF) and 0.5 mmol l$^{-1}$ dithiothreitol and centrifuged at 100 000 ***g*** for 20 min. The supernatants were frozen in liquid nitrogen and stored at −70 °C. The protein concentration of the extracts was estimated with a modified Lowry method (Ulmasov et al., 1992).

Consensus HSE probe (Wu et al., 1988) was prepared by annealing partially complementary oligonucleotides (ATCCGAGCGCGCCTCGAATGTTCTAGAA and CTCGCGCGGAGCTTACAAGATCTTTTCCA) in 10 mmol l$^{-1}$ potassium phosphate buffer, pH 8.2, in the presence of 0.1 mmol l$^{-1}$ NaCl. Single-stranded termini were filled with Klenow polymerase and [$^{32}$P]ATP (Sambrook and Fritsch, 1989). For the gel mobility-shift assay, extracts containing 50 µg of protein were mixed with 0.5 ng of [$^{32}$P]HSE in the binding buffer (as described by Mosser et al., 1993). The binding-reaction mixture was incubated at room temperature (20 °C) for 20 min. Free probe was separated from HSE–HSF complexes by electrophoresis through 5 % polyacrylamide gels (Mosser et al., 1993). The gels were dried and exposed to X-ray film (Kodak X-Omat) at −70 °C.

To identify bands corresponding to HSF or Ku autoantigen, cell extracts of control or heat-shocked cells were preincubated for 20 min either with anti-*D. melanogaster* Ku antibodies (gift of D. Rio) or with anti-*D. melanogaster* HSF serum (gift of C. Wu, NIH) before incubation with the labeled HSE probe; the samples were then subjected to electrophoresis.

*Analyses of nucleotide sequences*

Genomic DNA from 75 adults per strain was obtained by standard phenol/chloroform extraction and was used as templates for amplification products to be cloned and sequenced. Single-fly DNA for polymorphism screens was prepared from individual flies by homogenizing adults in 50 µl of buffer with 0.2 µg µl$^{-1}$ Proteinase K (Gloor et al., 1993). Samples were incubated at 37 °C for 30 min, heated to 95 °C to inactivate Proteinase K and stored at –20 °C.

Polymerase chain reaction (PCR) of single-fly preparations was performed by adding 2 µl of template DNA to buffer (10 mmol l$^{-1}$ Tris-HCl, pH 9.0, 50 mmol l$^{-1}$ KCl, 0.1 % Triton X-100) with 1.5–3.0 mmol l$^{-1}$ $MgCl_2$, 0.2 mmol l$^{-1}$ each dNTP, 5 pmol of each primer and 1.25 units of Taq DNA polymerase (Promega) per 25 µl reaction mixture. For PCR amplification of fragments to be cloned and sequenced, 1 µl of template DNA from mass preparations was added to buffer (50 mmol l$^{-1}$ KCl, 50 mmol l$^{-1}$ Tris-HCl, pH 8.3) with 1.5 mmol l$^{-1}$ $MgCl_2$, 0.2 mmol l$^{-1}$ each dNTP, 5 pmol of each primer and 2.5 units of MasterAmp TAQurate DNA polymerase mix (Epicentre Technologies) per 100 µl reaction. Reaction conditions for all PCRs were 30 cycles of 1 min at 92 °C, 1 min at 54 °C and 1.5 min at 72 °C. To amplify a portion of the 87A7 locus, primers were: upper, 5′CATCCCAAAAATCTGTAAAGC3′; lower, 5′ACTGTGTTTCTGGGGTTCAT3′. These flank both an *H.M.S. Beagle* element insertion site and an approximately

140 base pair (bp) insertion characteristic of 56H8-type alleles (Bettencourt, 2001), if present. To amplify the *hsp70Ba* promoter, primers were: upper, 5′GCAAGCAATCATCATCCAAT3′; lower, 5′ACTGTGTTTCTGGGGTTCAT3′. These flank a *Jockey* element insertion site. Fig. 8A displays the sizes of the resultant amplification products, which were resolved *via* agarose gel electrophoresis. To screen individual flies for the *Jockey* element, primers were the foregoing *hsp70Ba* primers plus a *Jockey*-specific internal primer (lower, 5′AAGAAGACTCAAGCGACACC3′).

For cloning and sequencing, PCR products were amplified from bulk DNA template, gel-purified and/or cleaned with Qiagen spin columns, suspended in water and cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's recommendations. Individual plasmid clones were prepared with Qiagen Miniprep spin columns, suspended in water and sequenced. Sequencing reactions were performed with ABI Prism cycle sequencing kits (Perkin Elmer) according to the manufacturer's instructions. Sequencing was conducted on ABI 377 sequencers. All samples were sequenced using primers based on vector sequence. Internal sequencing primers (sequences available on request) were used to provide double-stranded coverage. Sequences were assembled manually and aligned using CLUSTAL X (Jeanmougin et al., 1998). Relevant sequence information has been deposited in GenBank and/or is available on request.

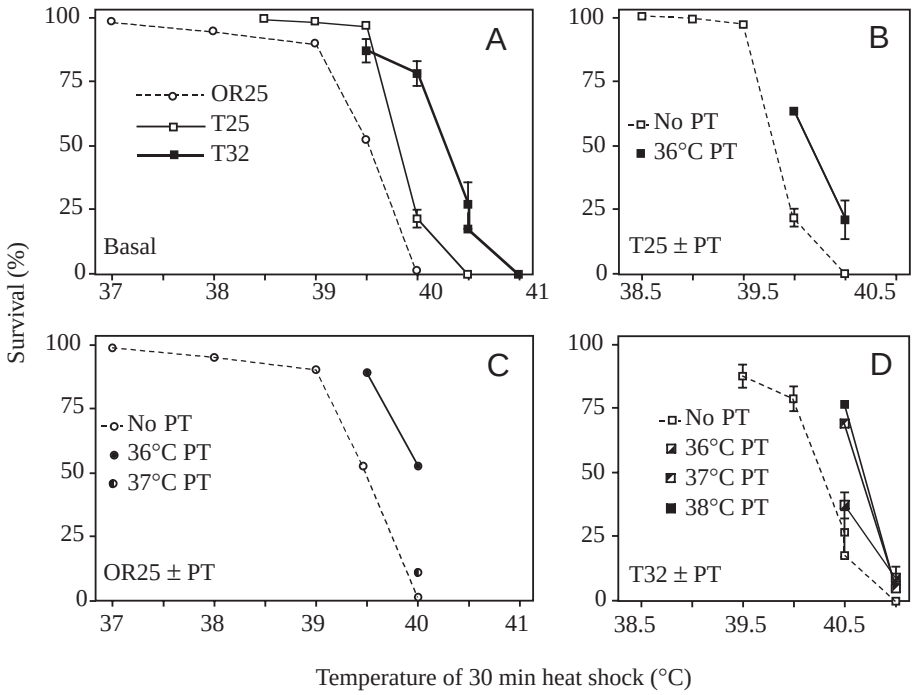


Fig. 1. Basal and inducible thermotolerance in the Oregon R (OR25), T25 and T32 strains of *Drosophila melanogaster*. Batches of 4-day-old adults were exposed to the indicated temperatures for 30 min either with or without a heat pretreatment (PT) as indicated. Means are plotted ±1 S.E.M. ($N$=2–5), which the plotted points often obscure. All axes use identical scales to facilitate comparison. (A) Thermotolerance in flies without pretreatment. (B–D) Thermotolerance of the three strains with and without a pretreatment. In B–D, without-pretreatment data are replotted from A.

## Results

### *Quantitative differences in thermotolerance and Hsp70*

The basal thermotolerance of T32 adults exceeded that of T25 adults, which in turn exceeded that of OR25 adults (Fig. 1A). The corresponding $LT_{50}$ values for 30 min heat shock, estimated by linear interpolation, are as follows: OR25, 39.5 °C; T25, 39.8 °C; T32, 40.3 °C. Of the T32 adults, 80 % survived a 40 °C heat shock, which killed 75 % of T25 adults and 100 % of OR25 adults.

In contrast, the inducible thermotolerance (quantified as the change in percentage survival with and without pretreatment) of T32 flies was equal to or less than that of T25 and OR25 flies (Fig. 1B–D). At the minimum temperature for 100 % mortality without pretreatment (40 °C for OR25, 40.5 °C for T25, 41 °C for T32), a 36 °C pretreatment improved survival by 50 % in OR25 flies, by 25 % in T25 flies and by less than 10 % in T32 flies. Inducible thermotolerance was not entirely absent in T32 flies; 37 °C and 38 °C pretreatments both improved survival at 40.5 °C (from 25 to 75 %) in the T32 strain.

Hsp70 levels after pretreatment and heat shock were likewise lower in the T32 strain than in the other strains (Fig. 2). In OR25 flies, Hsp70 levels increased during exposure to high temperature, continued to increase for 1–3 h afterwards, depending on temperature, and then decreased. In the T32 strain, the peak Hsp70 level was only 30–50 % of the level in OR25 flies. T25 flies were intermediate in this respect. Immunoblots of Hsp70 corroborate this finding (Fig. 3). Similarly, amounts of *hsp70* mRNA, determined 1 h after a 30 min exposure to 35 °C and 37.5 °C, were also greatest in OR25, intermediate in T25 and lowest in T32 (Fig. 4). After 39 °C heat shock, in contrast, *hsp70* mRNA levels in T32 were intermediate to those in the other strains (Fig. 4), and Hsp70 was detectable only in the T strains (Fig. 3). As with Hsp70 levels, *hsp70* mRNA abundance was least after the most intense heat shock in all strains. According to ELISA, the kinetics of Hsp70 variation were similar in the three strains, and the T32 strain did not accumulate more Hsp70 than the other strains at the highest temperature examined (Fig. 2, inset). Rearing eggs of the T32 strain to adulthood at 25 °C increased Hsp70 levels above those in both the T32 strain reared at 32 °C and the T25 strain (Fig. 2, arrow in inset).

Immunoblots with antibody 7.10, which detects all *D.*

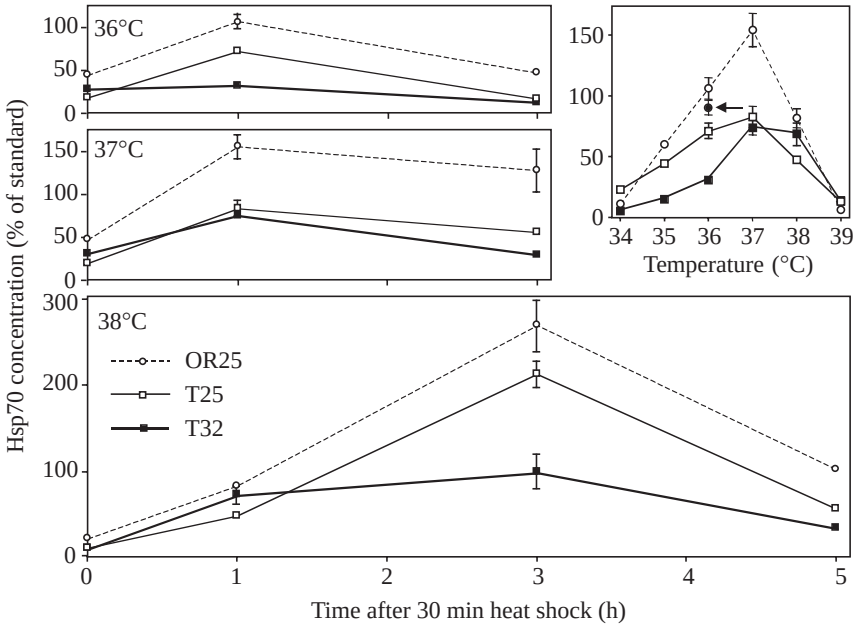


Fig. 2. Effects of heat-shock temperature and time after heat shock on normalized relative Hsp70 concentration in lysates of whole 4-day-old flies of the Oregon R (OR25), T25 and T32 strains of *Drosophila melanogaster*. Inset (top right): effects of heat-shock temperature on Hsp70 levels 1 h after a 30 min heat shock. Results are expressed relative to the signal obtained with a standard derived from heat-shocked *D. melanogaster* tissue culture cells (see Materials and methods). Each point is the mean of 5–9 independent measurements (except for OR25 at 34 °C, for which $N$=3). Means are plotted ±1 S.E.M., which the plotted points often obscure. The arrow in the inset indicates a mean for T32 strain flies reared at 25 °C. All axes use identical scales to facilitate comparison.

*melanogaster* Hsp70 family members, suggest that amounts of Hsp70 family members in non-heat-shocked flies are no greater in the T strains than in OR25 (Fig. 3). Although the aggregate Hsp70 family signal increases after heat shock, the increased level is less in the T strains than in OR25.

The T32 flies reared at 25 °C expressed much more *hsp70* mRNA after 35 °C heat shock than the T32 flies reared at 31–32 °C. This difference was absent or much less evident after 37.5 °C and 39 °C heat shock (Fig. 4).

### *Qualitative differences in the heat-shock proteins and genes of the T strains and OR*

Two-dimensional gel electrophoresis detected numerous differences between the protein patterns of OR25 and T flies, both in identifiable heat-shock proteins and in other proteins (Fig. 5, Fig. 6). A 45 kDa protein labeled conspicuously after heat shock in T but not in OR25 (Fig. 5, arrow c). Several small (22–27 kDa) heat-inducible proteins, presumably members of the *D. melanogaster* small Hsp family (Michaud et al., 1997), also differ in mobility between T and OR25 (Fig. 5, arrows 27,b,d,e). The Hsp70 family clearly differed between T and OR25 strains. Both strains expressed Hsp68 (Fig. 6), but this expression was more rapid in OR25 (Fig. 6C) than in T (Fig. 6E). Also, a third (i.e. in addition to Hsp68 and Hsp70) heat-inducible family member was evident in T but not in OR (low MW in Fig. 6E). This protein co-migrates with Hsp70 in the isoelectric focusing dimension and has a slightly lower molecular mass than Hsp68. It is also detected by antibody 7FB, which recognizes *D. melanogaster* Hsp70 but not other *D. melanogaster* Hsp70 family members (Palter et al., 1986) (data not shown).

Genomic Southern blots (Fig. 7), PCR and restriction digest of PCR products (Fig. 8) detected several presumptive polymorphisms at both the 87A7 and 87C1 *hsp70* loci in both strains. The nucleotide sequence at both loci reveals several noteworthy polymorphisms (Fig. 8). The 87A7 locus is polymorphic in natural populations; an approximately 140 bp insertion, presumably derived from a transposition event, is upstream of the coding sequence of the *hsp70Ab* gene in the '56H8' morph but absent from the '122' morph (Goldschmidt-Clermont, 1980). In both the T25 and T32 strains, 391 bp upstream of this insertion is an additional 265 bp insertion 98 %

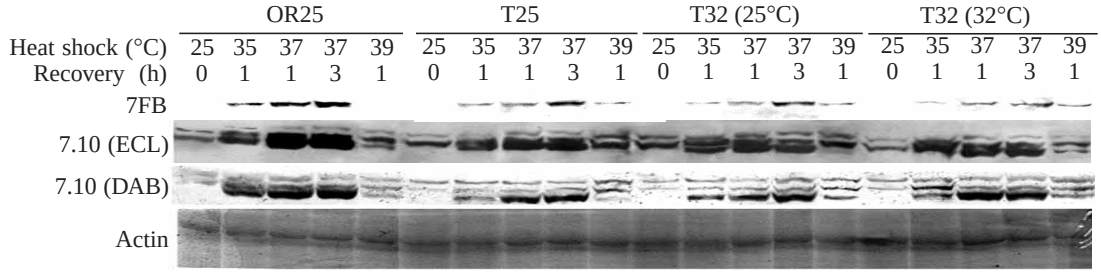


Fig. 3. Immunoblots of Hsp70 (detected with antibody 7FB) and all Hsp70 family members (detected with antibody 7.10) in salivary glands of third-instar larvae of the Oregon R (OR25), T25 and T32 strains of *Drosophila melanogaster* undergoing various temperature treatments. The blots were stripped and reprobed for actin as a standard for loading. DAB, detection by 3,3′-diaminobenzidine; ECL, detection by chemiluminescence.

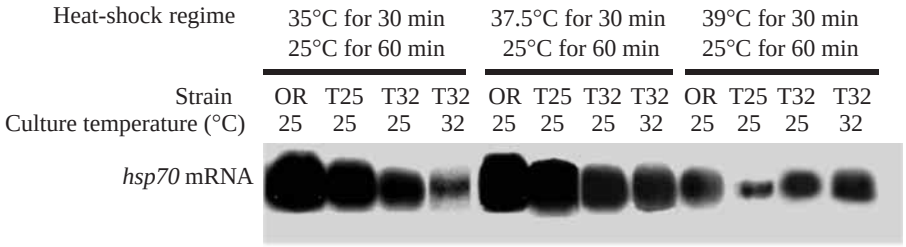


Fig. 4. Effects of strain, culture temperature and heat shock on *hsp70* mRNA levels.

identical to the long terminal repeat (LTR) of a full-length *H.M.S. Beagle* transposable element (Snyder et al., 1982). In each T strain, 36 individual flies were homozygous for the *H.M.S. Beagle* element. At the 87C1 locus, a 1.4 kilobase (kb) fragment of a *Jockey* element (Mizrokhi et al., 1985; Priimagi et al., 1988) disrupts the promoter of the *hsp70Ba* gene in some T strain flies. The first two HSEs (HSEs 1 and 2) are intact, but the *Jockey* element intervenes between these HSEs and HSEs 3 and 4. *Jockey* was fixed in 36 T25 flies sampled (all homozygous) and occurred at an allelic frequency of 0.611 in 36 T32 flies sampled.

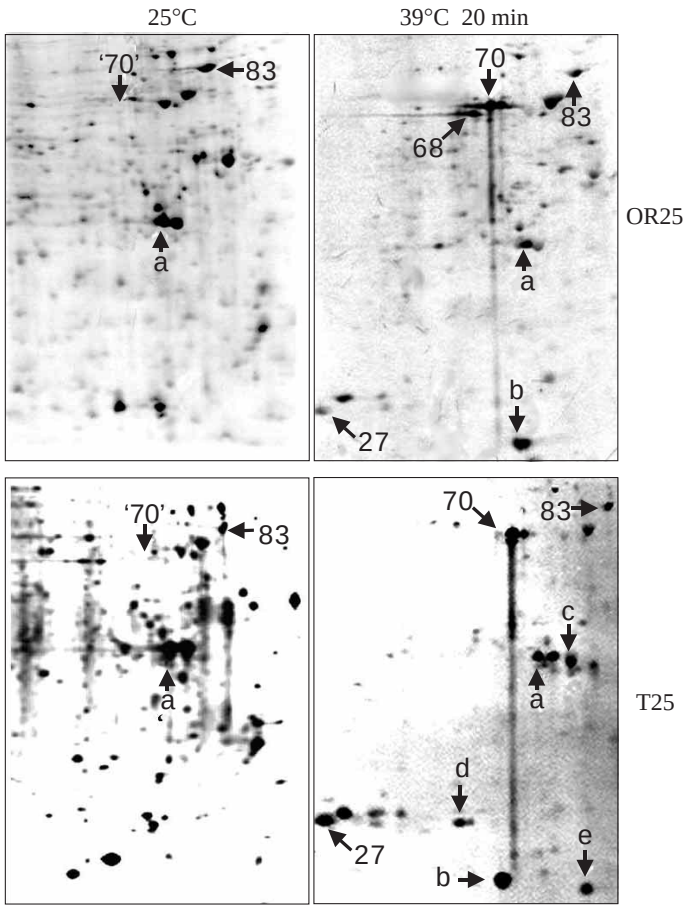


Fig. 5. Proteins synthesized in control conditions and during the hour after a 20 min 39 °C heat shock in salivary glands of OR25 and T25 strain larvae, as indicated by L-[$^{35}$S]methionine labeling, two-dimensional gel electrophoresis and autoradiography. Arrows indicate the positions of actin (a) and of identified or presumptive heat-shock proteins of interest. 27, Hsp27; 68, Hsp68; 70, Hsp70; 83, Hsp83; '70' indicates the position on the gel where Hsp70 should be detected. b, d, e, presumptive small Hsps; c, unknown presumptive Hsp.

*HSF activation in the T strains and OR*

In electrophoretic mobility-shift assays, three HSE complexes (*A–C*) are evident and differ in their characteristics (Fig. 9). These complexes are no longer detectable when lysates are incubated with a 200-fold molar excess of unlabeled HSE probe (Fig. 9A, lane 5). Corresponding supershifts after incubation with anti-HSF antibody (Fig. 9A, lane 3) and anti-Ku autoantigen antibody (lane 4) suggest that *A* is an HSE complex that includes HSF and that *B* is an HSE complex that includes Ku autoantigen. Neither antibody affected the mobility of complex *C*. Complexes *B* and *C* are constitutively present, except immediately after 40 °C heat shock (neither detectable) and 1 h after 40 °C heat shock (complex *B* not detectable) in the T strain. In both the OR25 and T strains, the HSF–HSE complex (complex *A*) is either not detectable or detectable only at low concentrations at 25 °C, and occurs at much higher concentrations after both 35 °C and 37 °C heat shock, corresponding to heat shocks at which Hsp70 level was maximally elevated. No qualitative differences between T and OR25 in complex *A* are evident that might be correlated with the lower levels of Hsp70 in the T strains. Although the T and OR25 strains differ in complex *A* after a 40 °C heat shock, this is a heat shock that is lethal to some (T) or all (OR25) flies (Fig. 1) and so may represent severe damage rather than a regulatory response.

## Discussion

The investigation of ecological and geographical variation in heat-shock protein expression, begun by Evgenev and colleagues (Evgenev et al., 1978; Evgenev et al., 1987; Lyashko et al., 1994; Ulmasov et al., 1993; Ulmasov et al., 1988; Zatsepina et al., 2000) and now widespread (Feder and Hofmann, 1999), clearly establishes that the magnitude, kinetics, threshold and molecular diversity of Hsp expression are correlated with the prevailing levels of stress that species, populations and developmental stages naturally undergo (Feder, 1999b; Feder and Hofmann, 1999). In addition, the work of Evgenev and colleagues suggests that species from high-temperature climates have higher constitutive levels of Hsp70 family members than related species from more moderate climates. At least two major questions are still unresolved: how do natural selection and other evolutionary processes create and maintain this covariation between the

heat-shock response and environment, and which genetic elements (e.g. *cis*- and *trans*-regulatory elements, gene copy number, coding sequence) does evolution manipulate to achieve this covariation? The answers to these questions may be most evident from intraspecific variation because variation among conspecifics is presumably the raw material upon which selection acts. To this end, we compared the T strain of *D. melanogaster* with another strain of the same species and found many noteworthy differences. Indeed, differences between the T strains and the Oregon R wild-type strain are much greater in some respects (e.g. in protein patterns revealed by two-dimensional electrophoresis, Fig. 5) than between different species of *Drosophila* (Sinibaldi and Storti, 1982).

At the outset, the extraordinary thermotolerance of the T strain was evident from its ability to thrive above temperatures previously considered to be the limit for continuous culture of this species (Parsons, 1973). This exceptional ability clearly extends to tolerance of acute high-temperature exposure (i.e. basal thermotolerance), in which the T strain in the present study outperforms other *Drosophila melanogaster* strains and compares favorably with many cactophilic *Drosophila* species (Krebs, 1999; Krebs and Loeschcke, 1995a; Krebs and Loeschcke, 1995b; Parsons, 1979; Stratman and Markow, 1998). In other strains and species, mechanisms of basal thermotolerance may include enhanced stability of cellular proteins through modification of primary structure and synthesis of thermoprotective osmolytes such as trehalose and sorbitol, constitutively expressed molecular chaperones, homeoviscous adaptation (i.e. adjustment in the lipid constituents of cells) and regulated depression of cellular function to moderate energy requirements. Although only semi-quantitative, the various determinations of constitutively expressed molecular chaperones reveal no obvious differences between T and OR25 flies (Fig. 3, Fig. 5, Fig. 6); if anything, levels are lower in T flies than in OR25 flies. Investigation of the other mechanisms of basal tolerance in the T

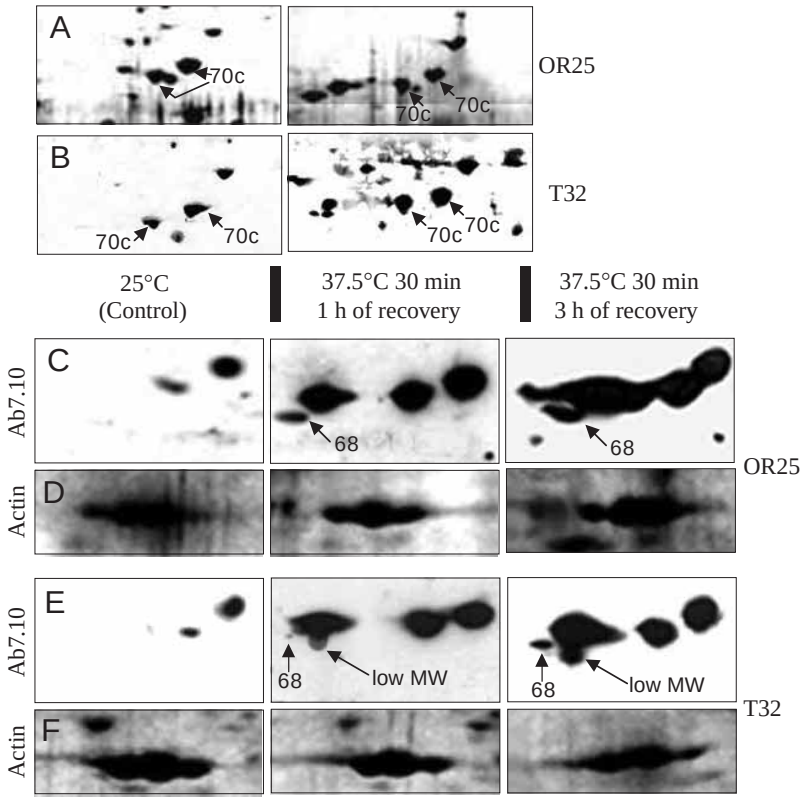


Fig. 6. Proteins present in control conditions and 1 and 3 h after a 30 min 37.5 °C heat shock in salivary glands of OR25 larvae and of T32 strain larvae acclimated for 3 days at 25 °C, as detected by silver staining and immunoblotting of two-dimensional electrophoresis gels. (A,B) Silver staining of the Hsp70 region in OR25 and T32 strains, respectively. 70c, Hsp70 cognates. (C,E) Hsp70 family members, as detected by antibody 7.10, in OR25 and T32 strains, respectively. Note the difference in Hsp68 (68) accumulation in the two strains and in the protein positioned immediately underneath the major Hsp70 spot (low MW). (D,F) Actin, as detected by silver staining, in OR25 and T32 strains, respectively.

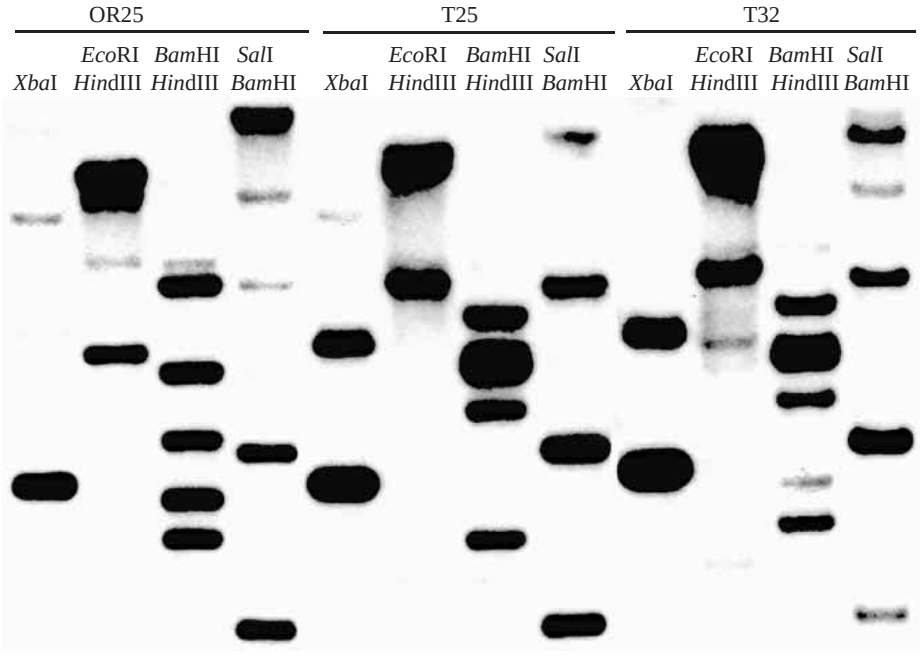


Fig. 7. Restriction digests of genomic DNA of the OR25, T25 and T32 strains of *Drosophila melanogaster* probed with the *Cla*I–*Bam*HI fragment of *hsp70*. Note the differences among strains.

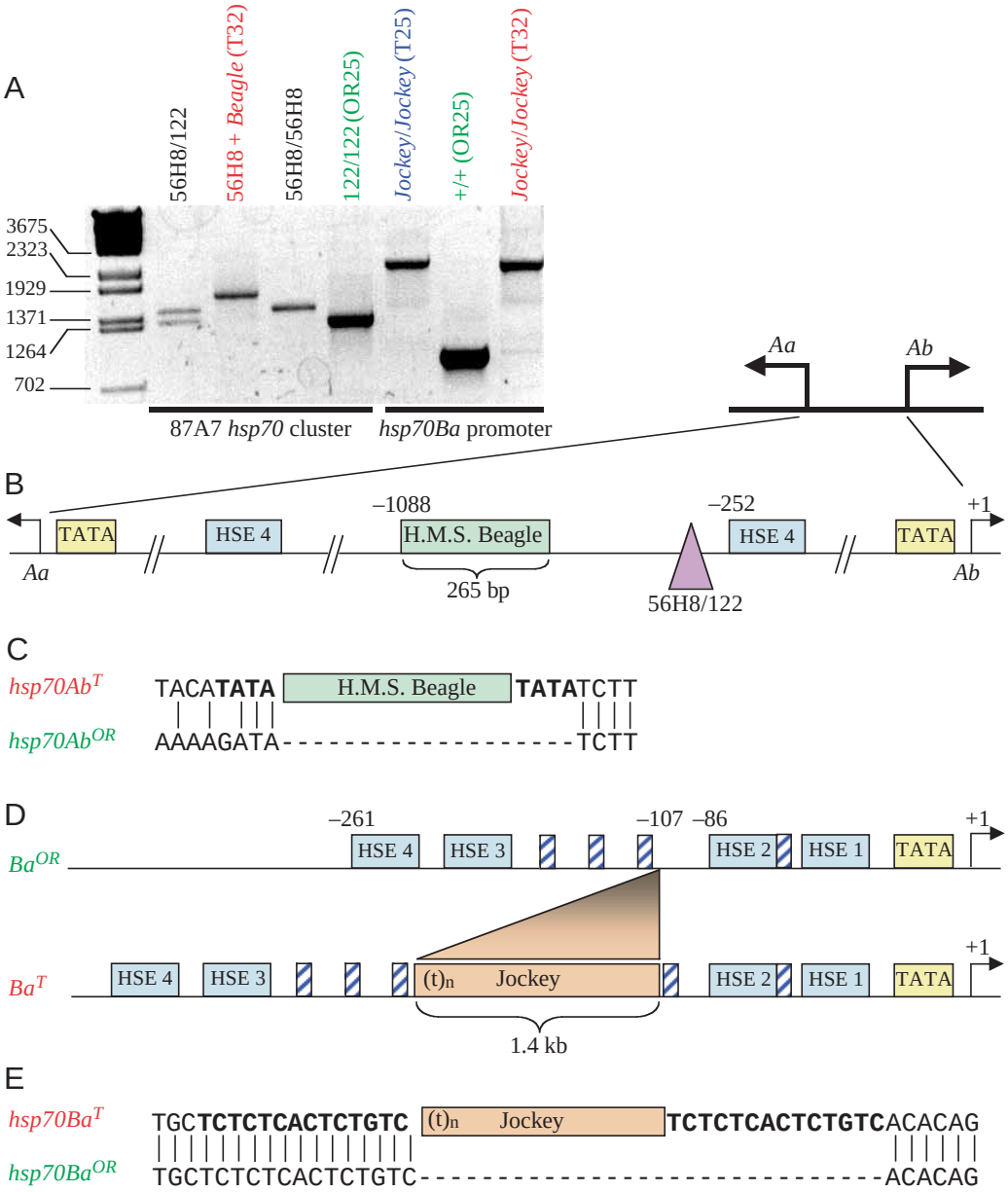


Fig. 8. Polymorphism at the 87A7 and 87C1 *hsp70* gene clusters. (A) PCR products from individual flies differing at the 87A7 and 87C1 *hsp70* gene clusters. Left-hand lane, DNA size markers; other lanes, PCR products from single flies of each genotype as indicated. (B) A 265 bp *H.M.S. Beagle* long-terminal repeat (LTR) is present in the 87A7 intergenic region of the T strains. The insertion lies 391 bp upstream of the 56H8/122 insertion/deletion polymorphism (triangle, 142 bp; see text). The nucleotide sequence begins 161 bp upstream of the *H.M.S. Beagle* insertion. The remainder of the 87A7 intergenic region was not sequenced: its size ranges from 1.6 kb in 122-type clusters to 3.6 kb in 56H8-type clusters (Bettencourt, 2001; Ish-Horowicz et al., 1979; Mason et al., 1982). (C) Site of insertion of *H.M.S. Beagle* element showing duplicated host DNA (bold). (D) A 1.4 kb fragment corresponding to the 3′ end of the *Jockey* element is inserted 107 bp upstream of the *hsp70Ba* transcription start site in the T strains. The orientation of *Jockey* is inverted with respect to the *hsp70Ba* gene, with the oligo(dT) at the distal end of the fragment. The insertion intervenes between HSE 2 and HSE 3, displacing HSE 3 and HSE 4 as well as three GAGA elements (hatched boxes; defined as the pentamer consensus sequence GAGAG; Omichinski et al., 1997; Wilkins and Lis, 1997). (E) Site of insertion of *Jockey*. Duplicated host DNA (bold) contains one of the putative GAGA elements. $(t)_n$, reverse complement of poly(A) tail.

strain, in which they are presently unknown, may well be fruitful.

In contrast, in terms of inducible thermotolerance, the T strain is unremarkable if not comparatively poor. Heat-shock protein expression is a principal mechanism of inducible thermotolerance in *D. melanogaster* (Feder et al., 1996; Solomon et al., 1991; Welte et al., 1993). Correspondingly, the T strain exhibits both lower amounts of most Hsps after heat shock and slower kinetics of Hsp70 and Hsp68 expression than the OR25 strain. The only obvious exceptions to this

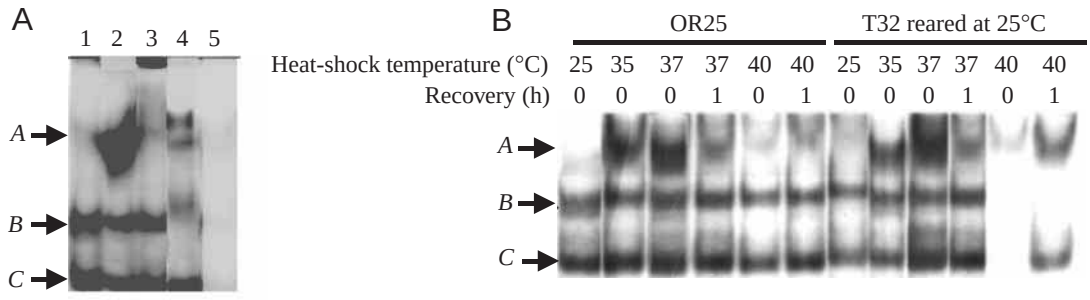


Fig. 9. Analysis of heat-shock element (HSE) binding activity in OR25 and T32 flies reared at 25 °C. The latter strain yielded the same results as for T25 and T32 flies reared at 32 °C; data not shown. (A) Effect of heat shock and identification of specific HSE complexes (*A–C*) in the OR25 strain. Lane 1, control (25 °C); lane 2, after a 30 min 37.5 °C heat shock; lane 3, a 30 min 37.5 °C heat shock plus anti-heat-shock-factor (HSF) antibody, with supershift implicating HSF as a component of complex 1; lane 4, a 30 min 37.5 °C heat shock plus anti-Ku autoantigen antibody, with supershift implicating Ku autoantigen as a component of complex 2; lane 5, a 30 min 37.5 °C heat shock plus 200-fold molar excess of unlabeled HSE. This image is a composite of several gels. (B) Effect of heat-shock intensity and recovery time on HSE complexes in the two strains.

conclusion are an apparent low-molecular-mass Hsp70 and several putative small Hsps (Fig. 5), the latter identified exclusively by electrophoretic mobility and heat inducibility. Small Hsps, related to α-crystallins, clearly protect against stress (including high temperature) in other species, and their encoding genes have evolved in response to environmental temperature in *D. melanogaster* (Frydenberg et al., 1999). Their role in thermotolerance has not been confirmed in *D. melanogaster*, however. In any event, decreased inducible thermotolerance and increased expression of small Hsps would seemingly be inconsistent. The low-molecular-mass Hsp70 (Fig. 6) is detected both by an antibody specific for all Hsp70 family members (7.10) and by one specific for *D. melanogaster* Hsp70 (7FB), and its electrophoretic mobility resembles that of Protein 38 in fig. 2B of Buzin and Petersen (Buzin and Petersen, 1982). Indeed, Buzin and Peterson detected isoforms of Hsp70 considerably more numerous than the Hsp70-encoding genes, and they hypothesized that this was due to alternative post-translational modifications. In the T strain, however, this particular Hsp70 is far more abundant than in other strains (Buzin and Petersen, 1982; Palter et al., 1986). Whether this Hsp70 is functional and the basis for its low molecular mass are presently unknown.

Part of the distinctive thermal phenotype of the T strain is due to its thermal history while in laboratory culture. The T25 strain is intermediate to OR25 and T32 in basal thermotolerance, in inducible thermotolerance, in Hsp70 expression (present study) and in several other aspects of resistance to heat and ionizing radiation (Tikhomirova, 1980; Tikhomirova and Belyatskaya, 1993; Tikhomirova et al., 1993). Laboratory evolution at the respective culture temperatures of the T25 and T32 strains is clearly sufficient to generate such differences in thermal phenotype and the heat-shock response (Bettencourt et al., 1999; Gilchrist and Huey, 1999; McColl et al., 1996; McKechnie et al., 1998; Sorensen et al., 1999), and the two strains differ in *hsp70Ba* allele frequency. Within a generation, culture at different temperatures (i.e. thermal acclimation) can also affect diverse traits in *D. melanogaster*, and these effects can persist for multiple generations (Crill et al., 1996; Hercus and Hoffmann, 2000). Thermal acclimation can affect the threshold temperature for induction of a heat-shock response (for a review, see Lerman and Feder, 2001), but several attempts to demonstrate a large effect in *D. melanogaster* (Bettencourt et al., 1999; Lerman and Feder, 2001) have failed.

This work adds to a growing list of *Drosophila* studies suggesting that, under certain conditions, evolution at high temperatures leads to decreased expression of Hsp70, a seemingly paradoxical outcome given the role of Hsp70 in thermotolerance in *Drosophila*. *D. melanogaster* undergoing laboratory natural selection at 28 °C (henceforth 'Cavicchi 28 °C lines') and *D. buzzatii* (a cactophilic species) reared for 6 h each day at 38.2 °C both express less Hsp70 than 25 °C controls (Bettencourt et al., 1999; Sorensen et al., 1999). Similarly, subtropical *D. buzzatii* collected from a low (i.e. warm) elevation express less Hsp70 than conspecifics collected from a high (i.e. cool) elevation (Sorensen et al., 2001). We suggest, as have others (Bettencourt et al., 1999; Krebs and Feder, 1997a; Krebs and Feder, 1997b; Krebs and Feder, 1998b; Krebs et al., 1998; Lansing et al., 2000; Sorensen et al., 2001; Sorensen et al., 1999), that this pattern is due (i) to the low levels of Hsp70 present in wild-type *D. melanogaster* transiently exposed to temperatures that these high-temperature lines chronically experience, (ii) to deleterious consequences of these low Hsp70 levels and (iii) to natural selection to reduce these low Hsp70 levels, which reduces Hsp70 levels at all temperatures as a correlated response to selection (Fig. 10).

Hsp70 is normally undetectable in *D. melanogaster* cells and whole *D. melanogaster* cultured at 25 °C and below (Velazquez et al., 1980; Velazquez et al., 1983), which are typical temperatures for *D. melanogaster* maintenance in the laboratory. Exposure to slightly higher temperatures (e.g. 28 °C) results in HSF activation (Lerman and Feder, 2001) and low, but non-zero, concentrations of Hsp70 (Lindquist, 1980). The constitutive presence of Hsp70 can be harmful in at least

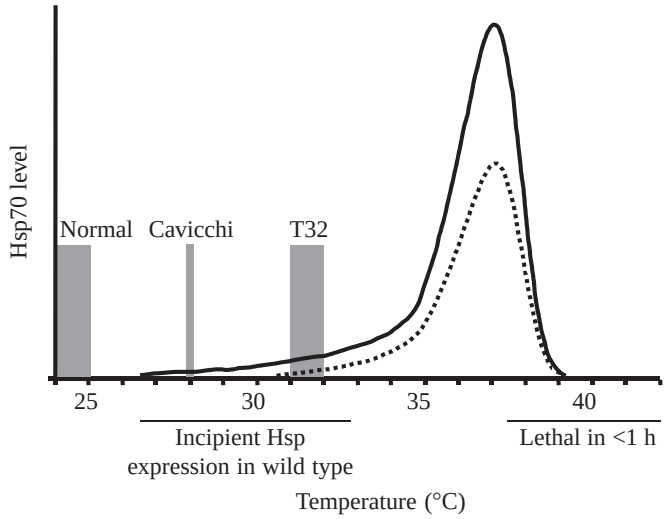


Fig. 10. Hypothesized effects of evolution at moderately high temperatures on the Hsp70 *versus* temperature norm of reaction. The solid line represents an idealized norm of reaction for wild-type *Drosophila melanogaster* reared at typical culture temperatures, 25 °C or below, and approximates diverse data for Hsp70 expression and heat-shock factor (HSF) activation in this species (Bettencourt et al., 1999; Dahlgaard et al., 1998; Feder et al., 1997; Lerman and Feder, 2001; Lindquist, 1980). Given this norm of reaction, *D. melanogaster* cultured at a constant 28 °C (Cavicchi 28 °C lines; Bettencourt et al., 1999) or 31–32 °C (T strain; T32) would constitutively undergo low levels of Hsp70 expression, incurring the deleterious consequences of Hsp70 (see Discussion) but none of the benefits realized only at higher temperatures. It is suggested that evolution at these moderately high temperatures acts to modify the norm of reaction (broken line) so that heat shocks at temperatures above 35 °C result in lower concentrations of Hsp70 than in the wild type.

three ways: by acting as a sink for substrate and cellular machinery that could be devoted to the synthesis of other proteins (Krebs and Feder, 1998a), as an impediment to normal processing and/or degradation of non-native proteins (Dorner et al., 1988; Dorner et al., 1992; Johnston et al., 1998; Newnam et al., 1999) and as an inappropriately regulated extra- and intracellular participant in diverse signaling pathways involving cell growth and death (Jaattela, 1999a; Jaattela, 1999b; Xie et al., 1999). Indeed, constitutive expression or overexpression of Hsp70 in *D. melanogaster* reduces growth, development and, potentially, fitness (Feder et al., 1992; Krebs and Feder, 1997a; Krebs and Feder, 1998b; Krebs and Loeschcke, 1994; Roberts and Feder, 2000), except in the context of inducible thermotolerance, where it clearly enhances fitness (Feder, 1999a; Roberts and Feder, 1999; Roberts and Feder, 2000). Strains evolving at moderately high but constant temperatures realize only the deleterious consequences of Hsp70, but none of the benefits, because these strains never encounter the extremely high temperatures against which Hsp70 is protective. In response, natural selection may have acted to reduce the entire Hsp70 *versus* temperature norm of reaction (Fig. 10), which results in decreased Hsp70 levels after exposure to temperatures commonly used in heat-shock studies. Interestingly, soil arthropods populating soils contaminated with high levels of heavy metals (another inducer of Hsp70) also evolve decreased Hsp70 expression (Kohler et al., 2000). Whether natural selection can result in an Hsp70 *versus* temperature norm of reaction that includes both suppression of Hsp70 expression at moderately high temperatures and increased Hsp70 expression at typical heat-shock temperatures or whether the levels of Hsp70 at moderately high and heat-shock temperatures are inextricably linked by a constraint of the Hsp70 autoregulatory apparatus (Lindquist, 1993) remains to be determined.

Regardless of its ultimate significance (*sensu* Mayr), the lower levels of Hsp70 expression in the T strains must have a mechanistic basis. Features determining the concentration of Hsp70 in *D. melanogaster* cells include *hsp70* copy number (Feder et al., 1996; Solomon et al., 1991; Welte et al., 1993), chromatin structure (Wu, 1980), transcription (Li et al., 1996; Mason and Lis, 1997; Morimoto et al., 1994), RNA processing (Lindquist, 1993; Yost et al., 1990), message stability (Petersen and Lindquist, 1989) and translation (Hess and Duncan, 1996; Zapata et al., 1991) and the sequestration and degradation of protein (Feder et al., 1992). As in prior studies (Bettencourt et al., 1999), we find no unequivocal evidence for genes other than the typical five *hsp70* genes in the T strain (data not shown). At the level of transcriptional activation, the T strain exhibits the same temperature-sensitivity of HSF–HSE interaction as the OR25 strain (Fig. 9). As in other organisms (Zatsepina et al., 2000), HSF constitutively forms additional complexes, one with Ku autoantigen and one of unknown composition, but both complexes are similarly abundant in both the T and OR25 strains. Presumably, therefore, the lower Hsp70 concentrations in the T strain have their basis downstream of HSF activation and binding to HSEs; indeed, the sizes of restriction fragments and PCR products indicate polymorphism in the *hsp70* loci, both in the strains examined here and worldwide (Bettencourt, 2001). One discrete candidate mechanism is disruption of the *hsp70* promoters by transposable elements, leading to altered transcription. It has been shown that both *H.M.S. Beagle* and *Jockey* elements may affect the expression of other genes whose promoters they disrupt (Kimbrell et al., 1988; Kimbrell et al., 1989; White and Jacobson, 1996a; White and Jacobson, 1996b). When present in the *hsp70Ba* promoter of the T strain, the *Jockey* element intervenes between the proximal and distal pairs of HSEs. Although the two proximal HSEs constitute the minimal Hsp70 promoter, the distal HSEs contribute to heat-inducible hsp70 transcription (Lee et al., 1992; Topol et al., 1985). The *H.M.S. Beagle* element is in a region of the 87A7 locus whose impact on *hsp70* transcription is unclear. Nonetheless, the *H.M.S. Beagle* sequence includes putative enhancer-like elements that may affect transcriptional regulation at some distance from the promoter (Kimbrell et al., 1988; Kimbrell et al., 1989). Transposable elements may be a significant evolutionary force in *D. melanogaster via* their alteration of the pre-existing genome, and it is tempting to conclude that these elements reduce Hsp70 levels in the T strain, which

natural selection has then favored in the native environment of the strain. Whether disruption of one or two *hsp70* genes out of five would be sufficient to reduce the overall transcription of *hsp70* mRNA is not known, however, and whether the *H.M.S. Beagle* and *Jockey* elements actually disrupt transcription as hypothesized requires further study.

The heat-shock genes and the proteins they encode are among the most ancient and highly conserved known, so much so that they have been considered useful in defining the phylogeny of major species groups in *Drosophila* (Bettencourt and Feder, 2001; Drosopoulou et al., 1996; Molto et al., 1994; Sinibaldi and Storti, 1982) and even phyla and kingdoms (Feder and Hofmann, 1999). Against this background, the variation among populations of a single species (i.e. T *versus* Oregon R) is remarkable. Evidently, adaptation *via* natural selection is sufficiently strong to overcome even the immense phylogenetic inertia of the heat-shock response.

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