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LABORATORY SELECTION AT DIFFERENT TEMPERATURES MODIFIES HEAT-SHOCK TRANSCRIPTION FACTOR (HSF) ACTIVATION IN *DROSOPHILA MELANOGASTER*

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Summary

The magnitude and time course of activation of the heat-shock transcription factor (HSF) differ among *Drosophila melanogaster* lines evolving at 18 °C, 25 °C or 28 °C for more than 20 years. At lower heat-shock temperatures (27–35 °C), flies from the 18 °C population had higher levels of activated HSF (as detected by an electrophoretic mobility shift assay) than those reared at 25 °C and 28 °C. At higher temperatures (36 and 37 °C), however, the 28 °C flies had the highest levels of HSF. These differences persisted after one generation of acclimation at 25 °C, suggesting that phenotypic plasticity was limited. In addition, larvae from the 28 °C lines activated HSF less rapidly after a 35 °C heat shock than those from the 18 °C and 25 °C populations. These results are similar but not identical to previously reported differences in expression of Hsp70 (the major heat-inducible stress protein in *Drosophila melanogaster*) among the experimental lines. We conclude that HSF activation evolves rapidly during laboratory culture at diverse temperatures and could play an important role in the evolution of the heat-shock response.

Key words: heat-shock factor, *Drosophila melanogaster*, laboratory evolution, acclimation, Hsp70.

Introduction

In response to heat and other stresses, all organisms express heat-shock proteins (Hsps), molecular chaperones that confer stress tolerance by preventing the cytotoxic aggregation of misfolded proteins and facilitating their reactivation after stress (Parsell and Lindquist, 1994; Feder et al., 1995). Although the coding sequences of Hsp genes are highly conserved (Gupta and Singh, 1994), the magnitude, kinetics and thermal threshold of Hsp expression covary with the thermal regimes experienced by populations, species and higher taxa (Feder and Hofmann, 1999). Less well established, however, is how this variation has arisen. Presumably, natural selection has altered one or more of the mechanisms that regulate Hsp expression. These regulatory mechanisms include specializations of chromatin structure (Wu, 1980), transcription (Morimoto et al., 1994; Li et al., 1996; Mason and Lis, 1997), RNA processing (Yost et al., 1990; Lindquist, 1993), message stability (Peterson and Lindquist, 1989) and translation (Zapata et al., 1991; Hess and Duncan, 1996). Of these, the transcriptional activation of the heat-shock transcription factor (HSF) differs in thermal threshold among lizard species according to environment (Zatsepina et al., 2000), and *hsp70* message stability may account for contrasting Hsp expression in *Hydra* species from variable and constant environments (Brennecke et al., 1998). Apart from these findings, the extent to which mechanisms controlling Hsp expression vary and whether these mechanisms are susceptible to natural selection within populations are largely unknown. Here, we investigate the evolution of HSF activation in populations of *Drosophila melanogaster* that have undergone sustained laboratory culture at different temperatures, and show that HSF activation temperature can evolve rapidly.

In the absence of stress, HSF is an inactive monomer. After heat shock, HSF converts to a trimer that can bind to heat-shock elements (HSEs) located within the Hsp promoter to induce transcription of *hsp* genes (Westwood and Wu, 1993; Morimoto, 1998). HSF oligomerization and DNA binding, which can occur within 30 s of heat shock, result in vigorous transcription (Zimarino, and Wu, 1987; Fernandes et al., 1994). Attenuation of the heat-shock transcriptional response entails negative regulation of HSF by Hsps themselves and by heat-shock factor binding protein 1 (HSBP1), which interacts with the HSF trimerization domain to downregulate its activity (Satyal et al., 1998; Cotto and Morimoto, 1999).

Diverse experiments have established that the temperature threshold for HSF activation is phenotypically malleable. For example, altering the growth temperature of HeLa cells modifies the sensitivity and pattern of HSF activation (Abravaya et al., 1991). When human HSF (HSF1) is expressed in *Drosophila melanogaster* cells, the temperature of HSF induction decreases to 32 °C (Clos et al., 1993).

Conversely, *Drosophila melanogaster* HSF is constitutively active when expressed in human cells at 37 °C, a temperature at which human HSF1 is normally quiescent. The threshold for HSF activation can also vary among tissues within a single organism: while 42 °C activates HSF in mouse liver, the testes, which normally have a lower temperature, exhibit HSF–HSE binding at 36 °C (Sarge et al., 1995; Sarge, 1995). The question we have addressed here, however, is whether laboratory evolution at diverse temperatures can achieve similar evolutionary change among members of a species.

We have investigated this question in *D. melanogaster* populations that were founded from a single ancestral population and have been undergoing laboratory evolution at 18 °C, 25 °C and 28 °C for more than 20 years. These populations have now diverged in several traits (Cavicchi et al., 1985; Cavicchi et al. 1991; Cavicchi et al. 1995; Bettencourt et al., 1999). Importantly, the 28 °C population has evolved both lower levels (30–50 % less than other lines) of Hsp70 (the major inducible heat-shock protein of *Drosophila* spp.; Parsell and Lindquist, 1994) expression and reduced inducible thermotolerance. Here, we perform electrophoretic mobility shift assays to investigate whether the thermal threshold and kinetics of HSF activation have also co-evolved and whether phenotypic plasticity (i.e. response to developmental temperature rather than evolution temperature) contributes to this variation.

Materials and methods

Experimental fly strains

The experimental populations were founded from a single Oregon R stock more than 20 years ago. Pairs of replicate lines have been maintained at 25 °C (‘A25’ and ‘B25’) and 28 °C (‘A28’ and ‘B28’), and one line at the ancestral 18 °C, in discrete generations (Bettencourt et al., 1999; for details, see Cavicchi et al., 1985; Cavicchi et al., 1995). The three thermal selection populations are referred to here as 18 °C, 25 °C and 28 °C.

To distinguish between phenotypic plasticity and evolutionary change, we reared 18 °C and 28 °C populations at either their ‘native’ temperatures or for one generation at 25 °C. A single generation of acclimation was utilized to minimize both cross-generational effects and *de novo* laboratory adaptation (Quintana and Prevosti, 1990; Jenkins and Hoffmann, 1994).

Heat-shock treatment

To estimate activation temperature, we measured HSF levels in third-instar larvae of all lines after heat shock at a series of temperatures. Five larvae were placed in 1.5 ml cryotubes with 10 µl of phosphate-buffered saline (PBS) (to humidify) and submerged in water baths at constant temperatures from 25 to 38 °C for 1 h. To investigate the time course of HSF activation, we heat-shocked four third-instar larvae at 35 °C for 0.5–50 min. Immediately after heat shock, the tubes were frozen in liquid nitrogen and stored at –80 °C.

Preparation of cellular extracts and gel-shift assays

Frozen larvae were homogenized in 300 µl of extract buffer [20 mmol l^{-1} Hepes, pH 7.9, 25 % glycerol, 0.42 mol l^{-1} NaCl, 1.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} EDTA, pH 8.0, 0.5 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF), 0.5 mmol l^{-1} dithiothreitol] on ice with a motorized plastic pestle, and lysates were pelleted by centrifugation at 20 000 ***g*** for 20 min at 4 °C. Supernatants were stored at –80 °C. Gel shift assays were performed with a double-stranded ideal HSE oligonucleotide probe (5′-CTAGAAGCTTCTAGAAGCTTC-TAG-3′), which contains four inverted repeats of the HSE consensus sequence nGAAn (a gift from Dr Richard Morimoto). Binding reactions were performed by adding 15 µg of total protein extract (quantified using the BCA protein assay, Pierce; extract volumes ranged from 3 to 6 µl) to 0.1 µg of γ^{32}P-labelled probe, and incubating for 15 min at room temperature (25 °C) in 20 µl of binding buffer (20 mmol l^{-1} Tris, pH 7.8, 100 mmol l^{-1} NaCl, 1 mmol l^{-1} Na-EDTA, 10 % glycerol) with 1 µg $µl^{-1}$ bovine serum albumin (BSA) and 1 µg $µl^{-1}$ poly[d(I-C)]. Loading dye (2 µl) (0.2 % Bromophenol Blue, 0.2 % xylene cyanol, 50 % glycerol) was added to samples after completion of the binding reaction. Competition reactions contained a 100-fold excess of either unlabelled HSE oligonucleotide or unlabelled oligonucleotide containing the binding sequence for nuclear factor (NF)-κB. Free and bound DNA were separated by electrophoresis in a 4 % native polyacrylamide gel in 0.5× TBE (0.045 mol l^{-1} Tris-borate, 0.001 mol l^{-1} EDTA) at 150 V for 1.5 h at room temperature.

Quantification and analysis of active HSF levels

Gels were dried, opposed to a PhosphorScreen (Molecular Dynamics) for 1–3 days, and scanned with a Molecular Dynamics PhosphorImager. Control reactions with activated recombinant HSF confirmed the size of HSF–HSE bands (data not shown). Active HSF levels were determined by quantifying the average intensity of pixels within equivalent rectangles bordering HSE–HSF bands using ImageQuant v1.11 software (Molecular Dynamics). Background density was subtracted, and statistical analyses were performed on raw values. For the kinetics assay, all raw values were standardized relative to a control binding reaction included on each gel, and statistical analyses were performed on these standardized values after background correction. Four binding reactions were performed for each experimental line for a total of 20 binding reactions per gel. Samples from a given heat-shock temperature or duration were always analyzed on the same gel, facilitating comparisons among populations and precluding strict comparisons across heat-shock temperatures or durations. For the kinetics assay, one of the eight 25 °C samples was randomly replaced with the control binding reaction.

Statistical analyses

Statistical analyses used StatView 4.5 (Abacus Concepts). The lack of a replicate 18 °C line precluded a nested analysis; therefore, both ‘line’ (four samples per line) and ‘temperature’ (four 18 °C samples, and a total of eight 25 °C and 28 °C samples) effects were tested at each heat-shock temperature/duration. Factorial analyses of variance (ANOVAs) were

performed to test for the effect of thermal evolution temperature. For the kinetics assay, which contained standardized HSF activity values, the evolution temperature × heat-shock duration interaction was also tested.

Results

Heat-shock temperature affected the level of activated HSF (Fig. 1). At 25 °C, levels of HSF-DNA binding were low but detectable. HSF activation increased with temperature between 30 and 36 °C, then decreased with increasing temperature. HSF levels did not differ between the replicate lines (i.e. A25 *versus* B25 and A28 *versus* B28; *post-hoc* Scheffe test for line effects, $P>0.05$ at each heat-shock temperature; data not shown) in all experiments performed. Thus, data for replicate lines were pooled.

Laboratory evolution affected the magnitude of HSF activation (Fig. 2; Table 1). At lower heat shock temperatures (27–35 °C), larvae evolving at 18 °C had higher levels of activated HSF than those reared at 25 °C and 28 °C. These differences were statistically significant at heat-shock temperatures of 27 °C, 30 °C, 32 °C and 33 °C. At a higher heat-shock temperature (36 °C), however, the 28 °C flies had higher HSF levels than the other two populations.

Differences in HSF activation among populations reared at their native temperatures persisted after one generation of acclimation to a common temperature of 25 °C, suggesting that phenotypic plasticity is limited (Fig. 3). These differences were significant at heat-shock temperatures of 27 °C, 30 °C and 36 °C (Table 2). While a direct densitometric comparison of native and acclimated HSF levels is not possible because individual gels differed in signal intensity, Fig. 3 makes clear that differences in HSF activation maintained the same rank order as in non-acclimated lines. Thus, acclimation did not change the relative responses of the experimental populations to heat shock.

Laboratory evolution affected the kinetics of HSF activation (Fig. 4; Tables 3, 4). HSF activation was less rapid in the 28 °C population than in the 25 °C and 18 °C populations, and the 28 °C flies activated significantly less HSF after 1 min, 5 min, 10 min and 40 min of heat shock at 35 °C (Table 3). Analysis of variance shows significant effects of heat-shock duration and evolution temperature and of the heat-shock duration × evolution temperature interaction (Table 4).

Discussion

Heat-shock protein expression covaries with thermal habitat in a wide variety of species (Feder and Hofmann, 1999). However, while a few studies have examined the regulatory

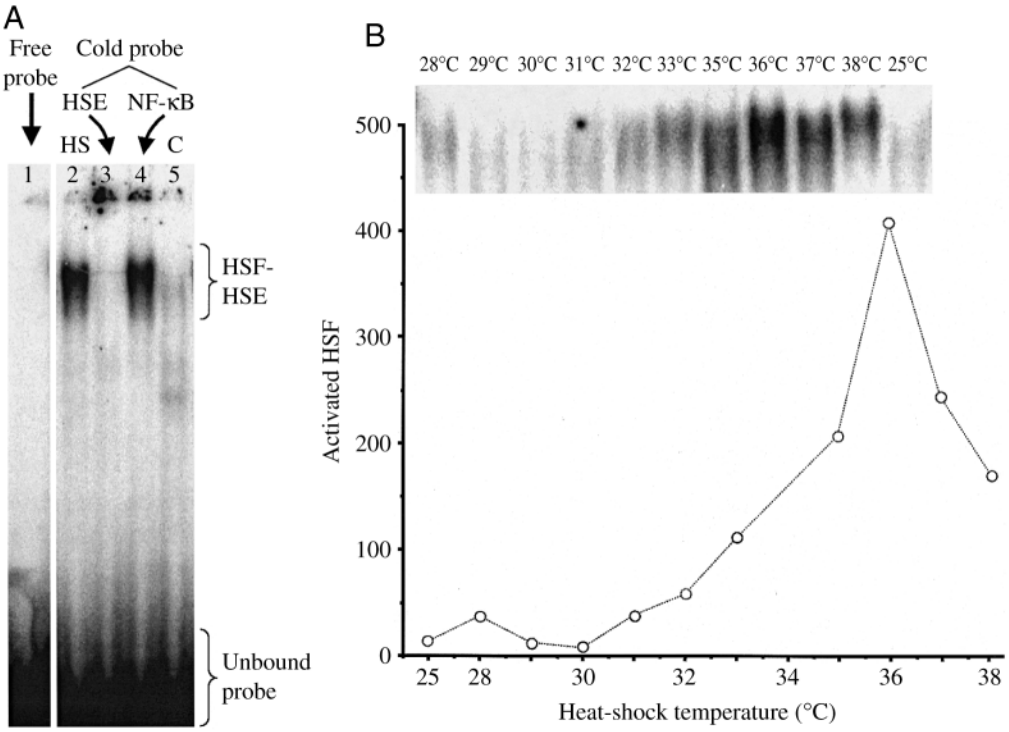

Fig. 1. Electrophoretic mobility shift assay. (A) A ^{32}P-labelled heat-shock element (HSE) oligonucleotide probe has retarded mobility on a polyacrylamide gel when bound to trimerized heat-shock factor (HSF) [compare lanes 2 (heat shock HS) and 5 (control, C)]. The addition of excess unlabelled HSE (lane 3) competes with ^{32}P-labelled HSE for HSF binding, while the addition of unlabelled probe corresponding to the recognition sequence for NF-κB (lane 4) does not. Lanes 2–4, 15 μg of total protein extract from larvae heat-shocked at 36 °C. Lane 5, 15 μg of protein extract from larvae at room temperature. Lane 1, no protein extract added. (B) Gels were scanned (top), and HSF levels were quantified with a Molecular Dynamics PhosphorImager and plotted on an arbitrary scale (one binding reaction per heat-shock temperature; bottom). Five third-instar larvae from the 18 °C population were heat-shocked at the temperatures indicated and analyzed on a single gel (A and B).

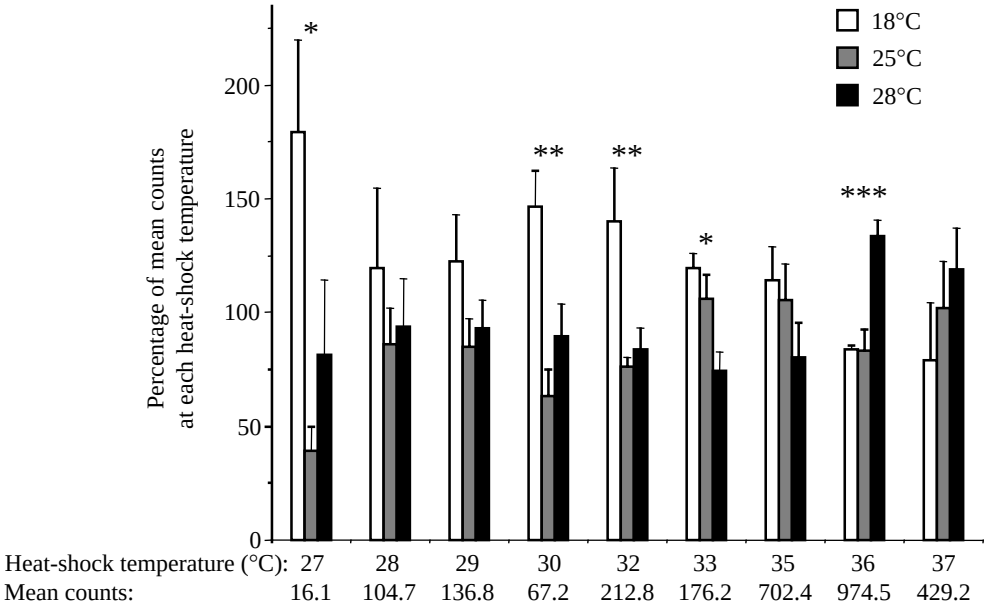

Fig. 2. Activated heat-shock factor (HSF) levels in third-instar larvae from the thermal selection populations after a 1 h heat-shock treatment. Larvae were collected from their native rearing temperature (18 °C, 25 °C or 28 °C). HSF levels (*y*-axis) were determined by gel-shift assay and are mean PhosphorImager counts for each selection population expressed as a percentage of the mean counts at each heat-shock temperature (*x*-axis). Statistical tests (ANOVA) were performed on raw counts. Asterisks denote a significant effect of evolution temperature (*P<0.05; **P<0.01; ***P<0.001) at a given heat-shock temperature. Values are standardized means + standardized S.E.M.

basis of Hsp variation between species (Brennecke et al., 1998; Zatsepina et al., 2000), the molecular mechanisms that underlie variation in Hsp expression and thermotolerance within species are unknown. The present study found that laboratory selection modifies the magnitude and kinetics of HSF activation in *D. melanogaster* populations derived from the same ancestral stock and is the first demonstration that experimental evolution can vary this step of the Hsp regulatory

Table 1. *Analyses of variance of effects on activated heat-shock factor levels of evolution temperature at the heat-shock temperatures indicated in flies reared at their evolution temperatures*

Heat-shock temperature (°C)	Source	d.f.	MS	*F*-value	*P*
25	Evolution temperature	2	728.56	2.384	0.1241
	Residual	16	305.553		
27	Evolution temperature	2	677.15	5.231	0.017
	Residual	17	129.441		
28	Evolution temperature	2	1664.794	0.491	0.6206
	Residual	17	3392.904		
29	Evolution temperature	2	3595.728	1.427	0.2673
	Residual	7	2519.467		
30	Evolution temperature	2	4173.739	7.288	0.0052
	Residual	17	572.651		
32	Evolution temperature	2	25 733.799	7.289	0.0056
	Residual	16	3530.712		
33	Evolution temperature	2	10 278.211	5.406	0.0152
	Residual	17	1901.387		
35	Evolution temperature	2	96 043.554	1.091	0.3584
	Residual	17	88 066.486		
36	Evolution temperature	2	531 305.974	11.84	0.0008
	Residual	15	44 873.813		
37	Evolution temperature	2	38 812.313	0.712	0.5049
	Residual	17	54 547.833		

MS, mean square; d.f., degrees of freedom.

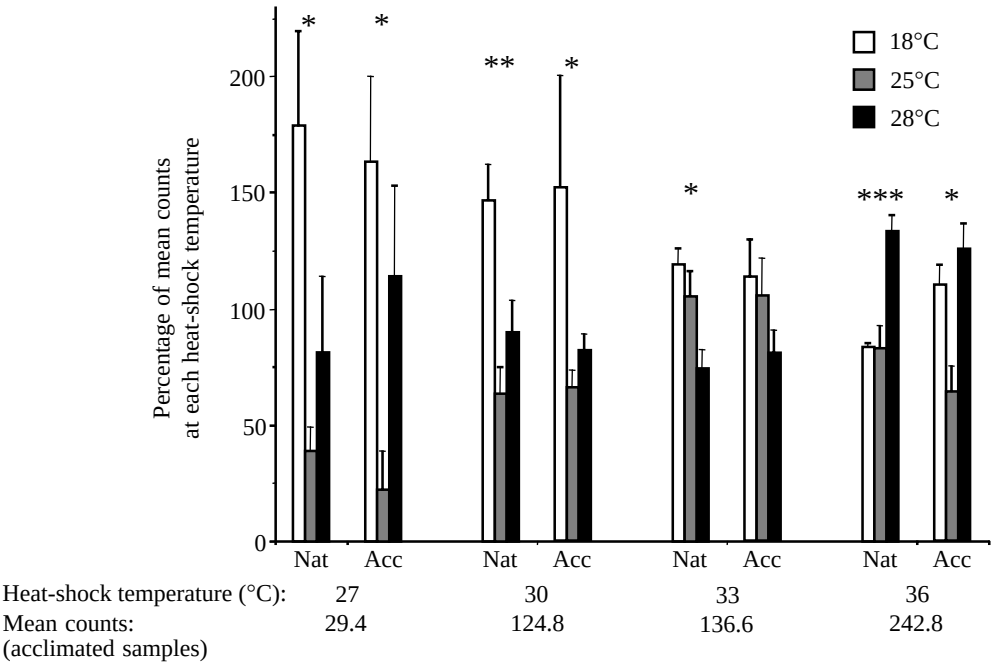

Fig. 3. Activated heat-shock factor (HSF) levels in third-instar larvae induced by a 1 h heat-shock treatment after one generation of acclimation at 25 °C. HSF levels (*x*-axis) are mean PhosphorImager counts for each selection population expressed as a percentage of the mean counts at each heat-shock temperature (*x*-axis). Values from flies reared at their 'native' evolutionary temperature (Nat; replotted from Fig. 2) are shown alongside those from acclimated flies (Acc) for comparison. The 25 °C Acc and Nat values are from different samples. Statistical tests (ANOVA) were performed on raw counts. Asterisks denote a significant effect of evolution temperature (**P*<0.05; ***P*<0.01; ****P*<0.001) at a given heat-shock temperature for either the acclimated or native samples. Values are standardized means + standardized S.E.M.

pathway among members of the same species. Our findings, moreover, are consistent with the general observation that Hsp induction occurs at lower temperatures in organisms inhabiting colder environments than in those from warmer environments (Feder and Hofmann, 1999).

An alternative explanation for the differences observed in HSF activation, which our findings exclude, is that these differences result from phenotypic plasticity rather than evolutionary change. Parental environment and thermal acclimation, for example, can influence heat resistance and other thermal traits in *Drosophila* species (Stephanou and Alahiotis, 1983; Jenkins and Hoffmann, 1994; Crill et al., 1996). Hence, in one experiment, we acclimated the evolving populations to a common temperature for one generation to examine whether differences remained. Our finding, that differences in HSF activation among the experimental populations persist after acclimation, is consistent with those for Hsp70 expression (Bettencourt et al., 1999) and indicates that the modification of HSF was an evolutionary response to laboratory selection at different temperatures. While a single generation of acclimation may not be sufficient to eliminate all cross-generational effects (Crill et al., 1996), more lengthy acclimation periods can themselves result in evolved differences (Quintana and Prevosti, 1990). However, differences in thermotolerance among the experimental lines persist or even increase after five generations of acclimation

Table 2. *Analyses of variance of effects on activated heat-shock factor levels of evolution temperature at the heat-shock temperatures indicated after acclimation for one generation at 25 °C*

Heat-shock temperature (°C)	Source	d.f.	MS	*F*-value	*P*
25	Evolution temperature	2	1352.042	1.882	0.1844
	Residual	16	718.236		
27	Evolution temperature	2	2703.631	4.544	0.0262
	Residual	17	594.975		
30	Evolution temperature	2	16024.81	5.283	0.0164
	Residual	17	3033.027		
33	Evolution temperature	2	3425.376	1.378	0.2788
	Residual	17	2484.996		
36	Evolution temperature	2	22446.389	6.271	0.0105
	Residual	17	3579.338		

MS, mean square; d.f., degrees of freedom.

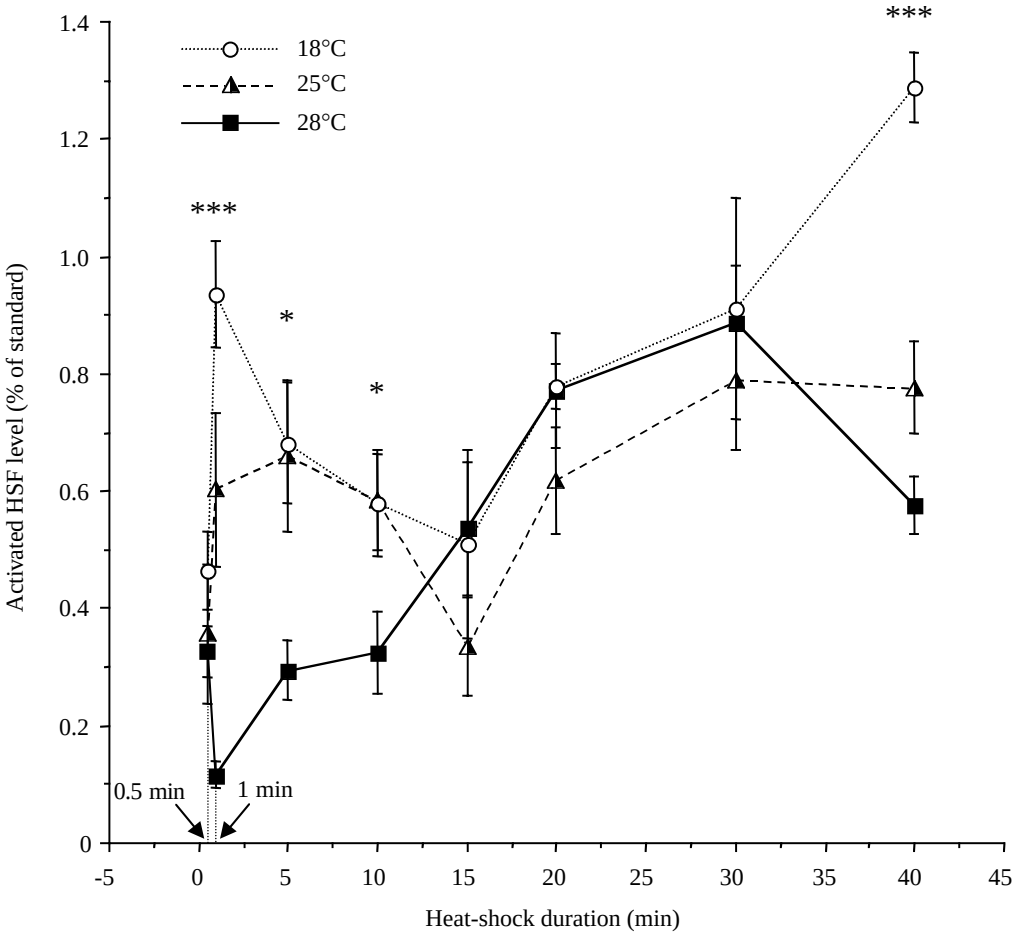

Fig. 4. Activated heat-shock factor (HSF) levels in third-instar larvae after a heat shock of 35 °C for the durations indicated. Larvae were collected from their native rearing temperature (18 °C, 25 °C or 28 °C). Raw PhosphorImager counts were standardized to a control binding reaction. Asterisks denote a significant effect (ANOVA) of evolution temperature (*P<0.05; ***P<0.001) at a given heat-shock duration. Values are means ± S.E.M.

compared with differences after a single generation (Cavicchi et al., 1995), suggesting that a single generation of acclimation was appropriate.

Heat-shock factor activation had a distinctive relationship to both heat-shock temperature and evolution temperature. After heat shocks at lower temperatures (27–35 °C), flies evolving at 18 °C activated more HSF than those reared at 25 °C and 28 °C (Fig. 2). While the absolute differences between the populations were minimal at lower heat-shock temperatures, by virtue of the lower raw counts, these difference were statistically significant at several heat-shock temperatures and were consistent across this range of temperatures. At higher heat-shock temperatures, the pattern reversed, with the 28 °C population having the highest levels of activated HSF. Thus, while HSF activation differs among the populations, it is not consistent across all heat-shock temperatures. Hsp70 expression differs consistently among the populations from 33 to 37 °C (Bettencourt et al., 1999). The time course of HSF activation also showed a response to selection: the 28 °C flies responded less rapidly to heat shock than the others (Fig. 4), a result consistent with the findings that the 28 °C population expresses lower levels of Hsp70 at lower heat-shock temperatures and has reduced inducible thermotolerance (Cavicchi et al., 1995; Bettencourt et al., 1999).

The lower HSF activation after 27–35 °C heat shocks in *Drosophila melanogaster* evolving at moderate to high temperatures may be due to an evolutionary trade-off between the benefits and disadvantages of Hsp expression. These benefits include inducible thermotolerance and other fundamental chaperone functions (Solomon et al., 1991; Welte et al., 1993; Feder et al., 1996; Krebs and Feder, 1997; Dahlgaard et al., 1998). The role of HSF is equally critical: mutation of HSF in *Drosophila melanogaster* abrogates HSF–DNA binding and *hsp70* transcription after heat shock and dramatically reduces basal and inducible thermotolerance (Jedlicka et al., 1997). However, excessive Hsp expression or expression in the absence of stress can have deleterious effects on growth and development (Feder et al., 1992; Krebs and Feder, 1997). After evolution at 28 °C for over 20 years, cellular components other than Hsp70 may have responded to selection, rendering 28 °C a non-stressful temperature. Flies evolving at a constant 28 °C may, therefore, incur the penalties

Table 3. *Analyses of variance of effects on activated heat-shock factor levels of heat shock at 35 °C for the durations indicated*

Duration of heat-shock (min)	Source	d.f.	MS	*F*-value	*P*
0.5	Evolution temperature	2	0.059	3.022	0.0789
	Residual	15	0.019		
1	Evolution temperature	2	0.989	22.93	<0.0001
	Residual	15	0.043		
5	Evolution temperature	2	0.324	5.352	0.0166
	Residual	16	0.061		
10	Evolution temperature	2	0.154	3.756	0.0460
	Residual	16	0.041		
15	Evolution temperature	2	0.083	0.988	0.3938
	Residual	16	0.084		
20	Evolution temperature	2	0.052	1.013	0.3868
	Residual	15	0.052		
30	Evolution temperature	2	0.026	0.265	0.7702
	Residual	16	0.098		
40	Evolution temperature	2	0.68	25.149	<0.0001
	Residual	16	0.027		

MS, mean square; d.f., degrees of freedom.

without ever realizing any benefits of Hsp expression. Selection may thus lead to a depression of HSF activation and Hsp70 expression at lower heat-shock temperatures in the 28 °C populations. Interestingly, *D. melanogaster* and *D. buzzatii* larvae subjected to a laboratory selection regime consisting of daily heat shocks responded similarly to selection: thermally selected flies expressed less Hsp70 than controls (Lansing et al., 2000; Sorensen et al., 1999). Thus, the fitness trade-offs of Hsp70 expression may result in decreased Hsp70 expression in flies subject to thermal selection at both constant and cycling temperatures.

The hypothesized trade-off calls for at least some Hsp expression, however little, at warm evolution temperatures (e.g. 28 °C). Yet Bettencourt et al. (Bettencourt et al., 1999) found low levels of Hsp70 expression at 33 °C (although they did not test expression below this temperature), Krebs and Feder (Krebs and Feder, 1997) found only negligible Hsp70 expression at 33 °C, and Velazquez et al. (Velazquez et al., 1983) did not detect significant levels of *hsp70* message at 27 °C (although a slight induction was observed at 29 °C). Our results, however, show that larvae from all three experimental populations activate some HSF after a heat shock of 27 °C, and Jedlicka et al. (Jedlicka et al., 1997) found substantial HSF–HSE binding at 30 °C that was correlated with *hsp70* transcription (and some constitutive HSF–DNA binding at 25 °C that was not correlated with *hsp70* transcription). Other studies have found Hsp70 induction at low temperatures. In cells of *Drosophila* cultured at 23 °C, for example, Hsp70 synthesis was apparent after heat shock at 26 °C and peaked at 37 °C (Lindquist, 1980), and Gehring and Wehner (Gehring and Wehner, 1995) found that Hsp70 is first induced weakly in *Drosophila* at 27–29 °C, with stronger expression beginning at 31 °C (in brain tissue). Thus, the activated HSF levels after heat shocks of 27–30 °C may correspond to low levels of Hsp70 activation. If so, selection may have resulted in reduced HSF activation at these and other low heat-shock temperatures in the 28 °C population to minimize the deleterious effects of unnecessary Hsp70 expression. Alternatively, the low levels of activated HSF may not correspond to Hsp70 expression levels significant enough to elicit such a hypothesized trade-off. However, HSF controls the transcription of, and is regulated by, other genes in addition to *hsp70*. It is possible that changes in the expression of these genes have contributed to, or result from, changes in HSF activation.

Heat-shock factor activation is one of many possible targets on which selection may have acted to modify Hsp expression. Others include promoter sequences as well as those affecting Hsp message degradation, translation and other traits (see

Table 4. *Analysis of variance of effects on activated heat-shock factor levels of heat-shock duration and evolution temperature after heat shock at 35 °C*

Source	d.f.	MS	*F*-value	*P*
Heat-shock duration	7	0.888	15.264	<0.0001
Evolution temperature	2	0.603	10.37	<0.0001
Duration × Temperature	14	0.203	3.492	<0.0001
Residual	126	0.058		

MS, mean square; d.f., degrees of freedom.

Introduction). Indeed, recent studies have found variation in putative *hsp* regulatory sequences. Frydenberg et al. (Frydenberg et al., 1999), for example, have found that the *Drosophila hsp23* promoter region is variable for a repeated tetranucleotide sequence, Favatier et al. (Favatier et al., 1999) have detected a bi-allelic polymorphism within the HSE of the human *hsp70-1* gene and Bettencourt and Feder (Bettencourt and Feder, 1999) have identified nucleotide polymorphisms within the 3′ and 5′ *hsp70* untranslated regions of *Drosophila melanogaster*. However, the phenotypic consequences of these variants remain to be determined. In addition, other cellular targets, including membrane structure and protein stability, may have been modified by laboratory selection. While other mechanisms may contribute to the evolved differences in Hsp70 expression, HSF activation clearly evolves in response to thermal selection and probably plays an important role in the evolution of the heat-shock response.

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