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Thermal acclimation changes DNA-binding activity of heat shock factor 1 (HSF1) in the goby *Gillichthys mirabilis*: implications for plasticity in the heat-shock response in natural populations

Bradley A. Buckley and Gretchen E. Hofmann*

Department of Biology, Arizona State University, Tempe, AZ 85287-1501, USA

*Author for correspondence at Department of Ecology, Evolution and Marine Biology, University of California, Santa Barbara, CA 93106-9610, USA (e-mail: hofmann@lifesci.ucsb.edu)

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Summary

The intracellular build-up of thermally damaged proteins following exposure to heat stress results in the synthesis of a family of evolutionarily conserved proteins called heat shock proteins (Hsps) that act as molecular chaperones, protecting the cell against the aggregation of denatured proteins. The transcriptional regulation of heat shock genes by heat shock factor 1 (HSF1) has been extensively studied in model systems, but little research has focused on the role HSF1 plays in Hsp gene expression in eurythermal organisms from broadly fluctuating thermal environments. The threshold temperature for Hsp induction in these organisms shifts with the recent thermal history of the individual but the mechanism by which this plasticity in Hsp induction temperature is achieved is unknown. We examined the effect of thermal acclimation on the heat-activation of HSF1 in the eurythermal teleost *Gillichthys mirabilis*. After a 5-week acclimation period (at 13, 21 or 28°C) the temperature of HSF1 activation was positively correlated with acclimation temperature. HSF1 activation peaked at 27°C in fish acclimated to 13°C, at 33°C in the 21°C group, and at 36°C in the 28°C group. Concentrations of both HSF1 and Hsp70 in the 28°C group were significantly higher than in the colder acclimated fish. Plasticity in HSF1 activation may be important to the adjustable nature of the heat shock response in eurythermal organisms and the environmental control of Hsp gene expression.

Key words: Thermal acclimation, DNA-binding activity, heat shock factor 1 (HSF1), goby, *Gillichthys mirabilis*, heat-shock response.

Introduction

Eurythermal organisms adapted to variable thermal environments are faced with the challenge of maintaining biochemical and physiological performance across a range of temperatures. Despite the behavioral avenues of temperature control and stress avoidance available to many eurytherms, as environmental temperature begins to drive body temperature towards its stressful limits, survival can become dependent upon sub-organismal response mechanisms. Differential gene expression in response to environmental stress is one such mechanism (Pigliucci, 1996), and an increasing number of studies have focused on the gene-level effects of abiotic stressors, e.g. heat shock and oxidative stress (Gosslau et al., 2001) and oxygen availability (Gracey et al., 2001; Rees et al., 2001). In particular, the induction of heat shock proteins (Hsps) – a powerful tool for mediating the negative effects of thermal stress (Lindquist, 1986; Parsell and Lindquist, 1993) – has become important to ecologists and evolutionary biologists as a model of environmentally controlled gene expression and a source of phenotypic plasticity (for a review, see Feder and Hofmann, 1999). For instance, numerous recent studies have demonstrated that in organisms such as aquatic poikilotherms, which have body temperatures that are strongly influenced by their external conditions, the temperature at which Hsp genes are induced is not fixed for a given species but varies with the recent thermal history of the individual. In the field, higher threshold Hsp induction temperatures have been measured in summer-acclimatized mussels and fish (Dietz and Somero, 1992; Roberts et al., 1997; Buckley et al., 2001) than in their winter-acclimatized counterparts. In laboratory studies, acclimation to different temperatures resulted in intra-specific divergence of Hsp induction temperature in several species of marine invertebrates (Hofmann and Somero, 1996; Tomanek and Somero, 1999) and in various fishes, including the fathead minnow *Pimephales promelas* (Dyer et al., 1991), rainbow trout *Oncorhynchus mykiss* (Currie et al., 2000) and, the subject of the current study, the goby *Gillichthys mirabilis* (Dietz, 1994). However, it is currently unclear how this plasticity in the induction set point for Hsp genes is achieved mechanistically.

To investigate the processes underlying the adjustments to Hsp induction temperatures in eurytherms, we examined the activity of heat shock factor 1 (HSF1), the transcriptional

regulator of all inducible Hsp genes (for a review see Wu, 1995). Hsps function as molecular chaperones, interacting with polypeptides that have begun thermal denaturation during periods of heat stress, preventing their aggregation and degradation, and assisting in their eventual refolding (Fink, 1999; Hartl and Hayer-Hartl, 2002). Hsps can also control their own expression through interaction with HSF1 (reviewed in Morimoto, 1998; Pirkkala et al., 2001), which is maintained constitutively as a monomer in the cytosol. In the absence of thermal stress, HSF1 is complexed with 70 kDa and 90 kDa heat shock proteins (Hsp70 and Hsp90, respectively) (Baler et al., 1992; Rabindran et al., 1994; Zou et al., 1998; Ali et al., 1998). During heat stress, as the population of thermally damaged cellular proteins begins to increase, Hsp70 and Hsp90 dissociate from HSF1 to perform their chaperoning functions (Shi et al., 1998). HSF1 then acquires promoter-binding ability through homo-trimerization (Westwood et al., 1991; Westwood and Wu, 1993) and translocates to the nucleus (Sarge et al., 1993) where it binds to the heat shock element (HSE), a highly conserved sequence in the promoters of heat shock genes (Xiao and Lis, 1988). Further regulation of HSF1 trimerization and the final transactivation of Hsp genes is afforded through control of the phosphorylation state of HSF1 by members of the mitogen-activated protein kinases (MAPKs) (Sarge et al., 1993; Kim et al., 1997; Kline and Morimoto, 1997; Xia and Voellmy, 1997; He et al., 1998).

To date, there is no evidence that the temperature at which HSF1 becomes activated directly reflects the temperature of Hsp gene induction; indeed, HSF1 became activated in the gill tissue of the mussel *Mytilus californianus* at a temperature 9°C lower than the temperature at which Hsps began to appear in the cell (Buckley et al., 2001). Other studies have shown tissue-specific differences in HSF1 activation temperature within single individuals. The temperature of HSF1 activation is lower in germ cells than in somatic cells in mammals (Sarge, 1995) and rainbow trout (LeGoff and Michel, 1999), and the activation of the two isoforms of HSF1 identified in the zebrafish *Danio rerio* is temperature- and tissue-dependent (Råbergh et al., 2000). However, these studies did not focus on the effect of varying an organism's thermal environment on HSF1 activation. In a suggestive study on laboratory 'evolution', HSF1 activation temperature varied in *Drosophila* strains reared for 20 years in the laboratory at different temperatures (Lerman and Feder, 2001).

Here, we investigate the effect of thermal acclimation (5 weeks at a single temperature) on the activation temperature of HSF1 in individual organisms exposed to a subsequent, ecologically relevant heat stress. The intention was to approximate the type of adjustments to new temperature environments that are routinely made on a seasonal basis by natural populations. The goby *G. mirabilis* was chosen for the study because of its ability to tolerate a wide range of temperatures and the fact that its threshold Hsp induction temperature for Hsp70 and Hsp90 has already been shown to vary with acclimatization and acclimation (Dietz and Somero, 1992; Dietz, 1994). The acclimation-induced modification in HSF1 activation measured here in *G. mirabilis* is discussed in terms of its implications for the plasticity of the heat shock response (HSR) and its relevance to the environmental control of Hsp gene expression.

Materials and methods

Animal collection and acclimation conditions

Gillichthys mirabilis (Cooper, 1846) is an estuarine goby common to the Eastern Pacific and is found from central California to the Sea of Cortez, Mexico (Eschmeyer et al., 1983). All fish were collected by baited trap in the reverse estuary Estero Morua, at Puerto Peñasco, Sonora, Mexico (31.33°N, 113.60°W). Water temperature data were recorded with a submerged StowAway^{TM} (ONSET Computer Corporation) data logger permanently deployed in the estuary at the site of collection.

For the measurement of HSF1 activation and concentrations, fish were collected on June 16, 2001 and transported to the laboratory in an aerated cooler. After a 24 h adjustment period in re-circulating saltwater aquaria (at 21°C and salinity 32‰) five fish were sacrificed to serve as an initial 'time 0' group for the acclimation experiment. Five additional fish were kept in each of three re-circulating aquaria, set to 13, 21 and 28°C, for 5 weeks and were fed marine flake food (algae, krill, plankton) *ad libitum*. The water, which was continuously aerated, was changed weekly.

Fish used in the Hsp induction temperature experiments were from a second collection, which was made in April, 2000. Three fish from this sampling were acclimated to either 21°C or 28°C for 8 weeks before use in *in vivo* metabolic labeling experiments.

In vivo *metabolic labeling*

Metabolic labeling of liver tissue was conducted to detect the appearance of *de novo* synthesis of Hsps in response to heat shock. Dissected liver sections were held in 200 µl Eagles Minimum Essential Medium (MEM, adjusted with NaCl to 335 mosmol l^{-1}, the plasma osmolarity of *G. mirabilis*) in microcentrifuge tubes at a given exposure temperature for 1 h. Temperatures used for the 21°C-acclimated group were 15, 19, 22, 26, 29, 33 and 37°C; for the 28°C group, temperatures were 24, 26, 29, 32, 35, 39 and 42°C. After this exposure, all tubes were transferred to a 15°C waterbath, and tissue sections were incubated with 3.7×10^6 Bq of ^{35}S-labelled cysteine/methionine (specific activity 10 mCi ml^{-1}; NEN) for 2 h. Tissue sections were then rinsed twice with clean MEM buffer, and homogenized in 100 µl homogenizing buffer (HB) containing 50 mmol l^{-1} Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 1 mmol l^{-1} ethylenediaminetetraacetic acid (EDTA) and 1 mmol l^{-1} phenylmethylsulfonylfluoride (PMSF). Homogenates were heated at 100°C for 5 min, and centrifuged at 12 000 ***g*** for 10 min. Total counts of incorporated radiolabel in each extract were determined on a scintillation counter. Proteins were loaded onto 10% acrylamide gels, and separated

by SDS-polyacrylamide gel electrophoresis. An equal amount of radioactivity (75 000 c.p.m.) was loaded in each lane. Dried gels were exposed overnight to X-ray film (Kodak).

Electrophoretic mobility shift assays

Fish were killed *via* cervical transsection, and their livers removed and sectioned into pieces (approximately 100 μg). After immediately freezing a piece of tissue in liquid nitrogen for use in western blots, the remaining pieces of tissue were transferred to 200 μl ice-cold 335 mosmol l^{-1} MEM buffer in microcentrifuge tubes. Tubes were then held at control or heat shock temperatures for 1 h. The temperatures used were 13, 17, 21, 24, 27, 30 and 33°C for the 13°C-acclimated group, and 17, 21, 24, 27, 30, 33 and 36°C for the time 0, 21°C- and 28°C-acclimated groups. After the 1 h exposure, liver sections were immediately frozen in liquid nitrogen and stored at –80°C until used in subsequent assays.

For the electrophoretic mobility shift assays (EMSAs), frozen tissues were thawed in 200 μl extract buffer containing 25% (v/v) glycerol, 20 mmol l^{-1} Hepes, pH 7.9, 420 mmol l^{-1} NaCl, 1.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} EDTA, 0.5 mmol l^{-1} PMSF and 0.5 mmol l^{-1} dithiothreitol. Tissue extracts were centrifuged at 22 000 ***g*** at 4°C for 10 min. Pellets were discarded and the supernatants frozen at –80°C, after a sample was taken for total protein content determination by Bradford assay (Pierce). EMSAs were conducted using LightShift™ EMSA kits (Pierce). Tissue extracts (3 μg total protein) were incubated with 15 pmol of biotinylated HSE oligonucleotide probe (5′-GCC-TCGAATGTTCGCGAAGTTT-3′; Airaksinen et al., 1998) in 25 mmol l^{-1} Hepes, pH 7.6, 100 mmol l^{-1} NaCl, 15% (v/v) glycerol, 0.1% (v/v) NP-40, and 0.5 mmol l^{-1} PMSF in a final volume of 20 μl. Incubations were conducted for 20 min at 18°C. After incubation, HSF1–HSE complexes were separated from unincorporated HSE probe on 5% acrylamide non-denaturing gels and electrophoresed for approximately 2 h at 100 V. Proteins were then transferred to nylon membranes *via* electroblotting at 380 mA for 30 min. HSF1–HSE complexes were visualized by a chemiluminescent reaction with streptavidin–horseradish peroxidase according to the manufacturer's protocol and exposure of the blot to X-ray film (Kodak). Specificity of the HSE probe was confirmed in assays where unlabelled HSE probe was added in excess as a competitor.

Quantification of HSF1, Hsp70 and actin

Solid-phase immunochemistry

Western blotting analysis was used to quantify HSF1 and Hsp70 in the livers of gobies from each acclimation group. To test for the effect of thermal acclimation on overall protein synthesis, actin levels were also quantified. After heat shock, frozen tissue sections were thawed in 100 μl of HB. Homogenates were heated at 100°C for 5 min and centrifuged at 12 000 ***g*** for 10 min. Pellets were discarded, and total protein content of the supernatants was determined *via* Bradford assay (Pierce).

For the Hsp70 and actin western blots, proteins were separated on 10% gels by SDS-polyacrylamide gel electrophoresis. An equal amount of protein (10 μg) was loaded per lane. Proteins were then transferred to nitrocellulose membranes *via* electroblotting at 100 V for 30 min. To detect HSF1, 1 μg of each sample was directly blotted onto a nitrocellulose membrane using a BioRad Bio-Dot apparatus. All blots were dried for 45 min at 70°C.

Blots were blocked for 1 h in phosphate-buffered saline (PBS) (pH 7.5) containing 5% (w/v) non-fat dry milk (NFDM), followed by three 5 min washes in PBS and 0.1% (v/v) Tween-20. Immunodetection was accomplished by incubating all blots in primary antibody for 1 h with constant shaking. To quantify HSF1, blots were incubated for 1.5 h in a rabbit anti-

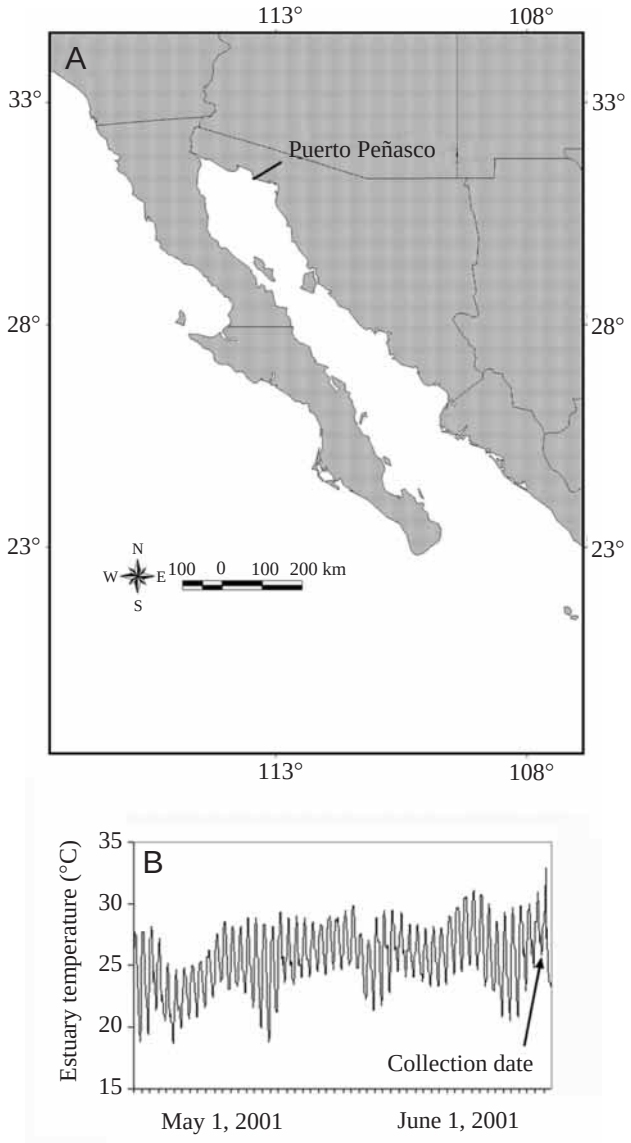

Fig. 1. Map of Estero Morua, Mexico and water temperature data. (A) Experimental animals were collected on June 19, 2001 by baited trap in an estuary (Estero Morua) near Puerto Peñasco, Sonora, Mexico. (B) Water temperatures at the collection site for 53 days prior to sampling. Temperature data were gathered *via* a submerged data recorder.

Drosophila HSF1 antibody (courtesy of Dr Carl Wu), diluted 1:10 000 in PBS containing 5% (w/v) NFDM, 20% (v/v) fetal bovine serum, 1 mmol l^{-1} PMSF and 0.02% (w/v) thimerosal. Incubation in primary antibody was followed by cross-reaction with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (BioRad 170-6515) diluted 1:50 000 in PBS containing 5% NFDM. Hsp70 levels were determined with an anti-Hsp70 rat monoclonal antibody (MA3-001, Affinity Bioreagents; diluted 1:2500). The secondary antibody was the same as in the HSF1 westerns, diluted 1:5000. For actin quantification, the primary antibody was a mouse anti-sea urchin actin antibody (courtesy of Dr JoAnn Otto) at a dilution of 1:10 000. The secondary antibody was a goat anti-mouse antibody conjugated to horseradish peroxidase (Sigma A-9044), used at a dilution of 1:10 000. Final detection of proteins was accomplished by exposing the blots to a solution of enhanced chemiluminescent (ECL) reagent (Amersham) for 5 min. Blots were then exposed to X-ray film (Kodak) for 15, 30 and 60 s to obtain exposures in the linear range of the detection signal. All samples were run in duplicate.

Statistics

Effects of acclimation and heat shock temperatures on HSF1 activity were determined by nested analysis of variance (ANOVA) (SYSTAT software). Differences between peak HSF1 activities were analyzed *via* paired Student's *t*-test. Effect of acclimation temperature on Hsp70, HSF1 and actin concentrations was determined by ANOVA and pairwise *post-hoc* comparison.

Results

Water temperature data

Water temperatures were recorded in the estuary at Puerto Peñasco, Mexico (Fig. 1A) in order to describe the recent thermal history of *G. mirabilis* in the field before the initiation of the acclimation experiment. Organisms at this location are routinely exposed to a variable thermal environment and can experience daily temperature changes of 12°C and an annual temperature range of 5–37°C (B. A. B. and G. E. H., unpublished data). Water temperature data for the 53 days prior to the date of collection are shown in Fig. 1B. The water temperature exceeded the study organism's preferred temperature of 23°C (Barlow, 1961) by several degrees for at least part of every day during this period, and in some cases (5/14–5/22 and 5/25–6/5) remained at or above that temperature for the entire day. The average daily temperature change during this period was 5.47±1.69°C, with a maximum temperature of 30.9°C recorded 9 days prior to collection.

Hsp induction temperatures

To confirm the plasticity in Hsp induction temperatures in *G. mirabilis* reported elsewhere (Dietz and Somero, 1992; Dietz, 1994), *in vivo* metabolic labeling experiments were performed on whole liver sections from fish acclimated to 21 and 28°C. Data from two representative fish are shown in Fig. 2. As in previous studies, Hsp induction temperature depended upon acclimation temperature. In the 21°C-acclimated fish, Hsp70 was induced at 22–26°C (Fig. 2A). In contrast, no induction of Hsps was observed in the 28°C-acclimation group until they were exposed to 32°C, with Hsp70 being the most strongly induced (Fig. 2B). Hsp90 appeared in the 21°C-acclimated group at 29°C, and in the warmer acclimated group at 35°C. It was noted that all protein synthesis was inhibited at 37°C in the 21°C-acclimated group, but protein synthesis was still robust in the 28°C-acclimated group at 39°C.

EMSA-determination of HSF1 activation

In order to use EMSAs to quantify the DNA-binding activity of HSF1, it was necessary to test the specificity of the cross-reaction of a biotinylated HSE-probe with HSF1 in *G.*

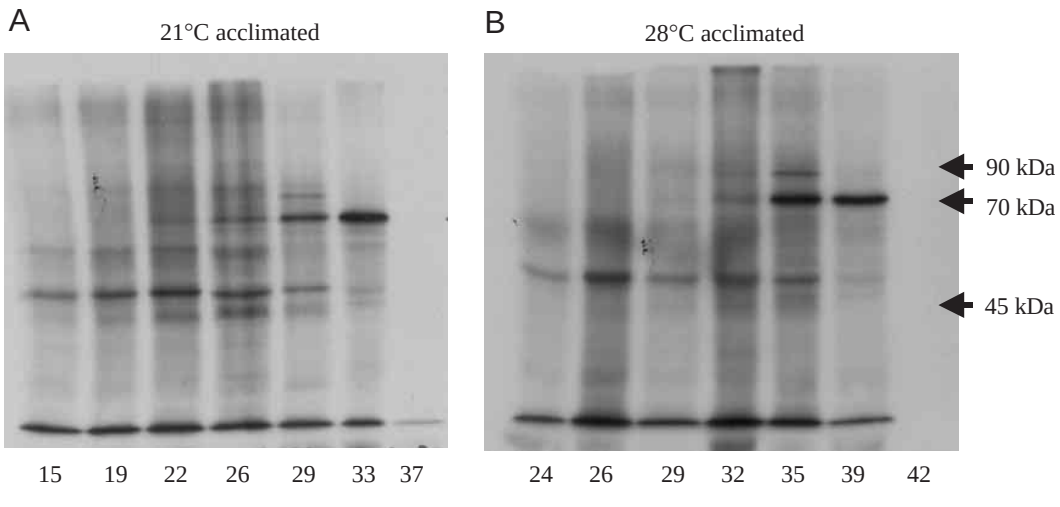

Fig. 2. Representative protein synthesis patterns in response to heat shock in the liver tissue of *Gillichthys mirabilis* (*N*=3) acclimated to (A) 21°C and (B) 28°C. Proteins were radiolabelled with ^{35}S-methionine/cysteine after heat shock for 1 h at the temperatures listed below each lane. After heat shock, proteins were separated on 7.5% polyacrylamide gels, which were dried and exposed to X-ray films. The positions of molecular mass markers are denoted on right (arrows). *N*=3 for both acclimation groups.

mirabilis. Specificity was confirmed with competitor/non-competitor assays (Fig. 3) in which the density of HSE-HSF1 bands did not decrease in the presence of excess unlabelled non-competitor DNA probe (AP2) but disappeared in the presence of an excess of unlabelled HSE probe.

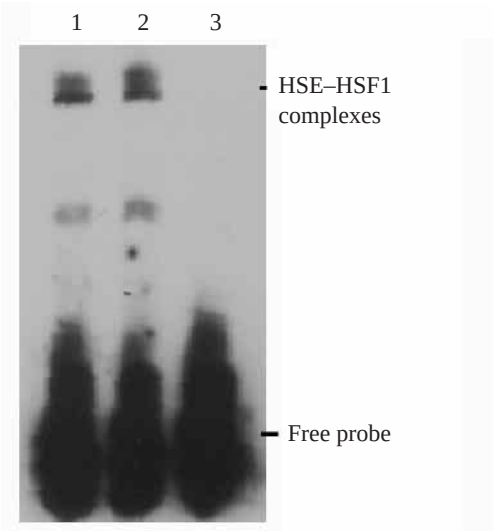

Fig. 3. HSF1–HSE complexes in the liver tissue of *G. mirabilis* visualized *via* electrophoretic mobility-shift assay (EMSA). A competition assay was run to determine HSE probe specificity. HSF-1 activation was visualized *via* EMSA, using a LightShift™ chemiluminescent EMSA kit (Pierce™). After a 1 h heat shock at the indicated temperature, 3 μg of homogenized liver tissue were incubated with 15 pmol of biotinylated-DNA protein for 20 min at 18°C and then separated on a 5% non-denaturing polyacrylamide gel. Protein was blotted to nylon membrane, and HSF1–HSE complexes were visualized through a chemiluminescence reaction. Lane 1, liver tissue and biotinylated HSE probe only; lane 2, identical to lane 1 except for the addition of a 200 mol l^{-1} excess of an unlabelled non-competitor DNA probe (AP2 from Promega); lane 3, identical to lane 1 except for addition of a 200 mol l^{-1} excess of unlabelled HSE probe. Intensities of HSF1–HSE bands were determined by scanning densitometry (BioRad™ Fluor S Multimager).

Effect of acclimation on HSF1 activation

After a 5-week acclimation period, the effect of heat shock on HSF1 activation in *G. mirabilis* liver was determined *via* EMSA. A representative HSF1 activation profile from each acclimation group (and time 0) is shown in Fig. 4 and the average HSF1 activation levels of the five fish from each group, as determined by scanning densitometry, are shown in Fig. 5. The effect of heat shock on the activation of HSF1 varied significantly with acclimation temperature (Fig. 5; ANOVA, $P<0.001$; Table 1), primarily because of the effects of heat shock on the 21°C and 28°C groups (Table 2). Within the time 0 group, there was no significant effect of heat shock temperature on HSF1 activation (Table 2), except for a decrease in HSF1 activation at 36°C (Fig. 5A; pairwise *t*-test $P<0.05$). Although there was not an overall effect of heat shock on HSF1 activity in the 13°C group (ANOVA, $P=0.388$; Table 2), there was a significant increase in activated HSF1 at 24–30°C in this group (Fig. 5; pairwise *t*-test, $P<0.05$; see below for more discussion of these results).

As opposed to the Hsp induction temperature, which is often a discrete set point at which the first appearance of Hsps is observed (see Fig. 2), we found rather a gradual increase in the level of activated HSF1 as a function of heat shock temperature, to a peak intensity level that was similar among all laboratory-acclimated groups (no differences in pairwise *t*-tests). In all cases, the value at the peak level of activated HSF1 was higher in the laboratory-acclimated groups than the peak level in the time 0 group ($P<0.001$; *t*-test). Across all groups, the most significant effects of acclimation temperature were observed at 33°C and 36°C (Table 3).

Among the acclimation groups (Fig. 5B-D), the heat shock temperature at which the activation of HSF1 reached a peak level varied with acclimation temperature. In the fish acclimated to 13°C, HSF1 DNA-binding activity increased steadily with heat shock temperature, peaking at 27°C (Fig. 5B). Activated HSF1 levels remained equivalent until 30°C, at which point a drop off similar to that seen in the time 0 group was measured. The 21°C-acclimated fish responded to heat shock similarly, with HSF1 activation increasing to a peak at 33°C before decreasing at 36°C (Fig. 5C). In contrast, HSF1 activation levels in the 28°C-acclimated group did not increase until exposure to 30°C and the intensity of HSF1 activation in this group continued to climb even at the highest exposure temperatures (Fig. 5D).

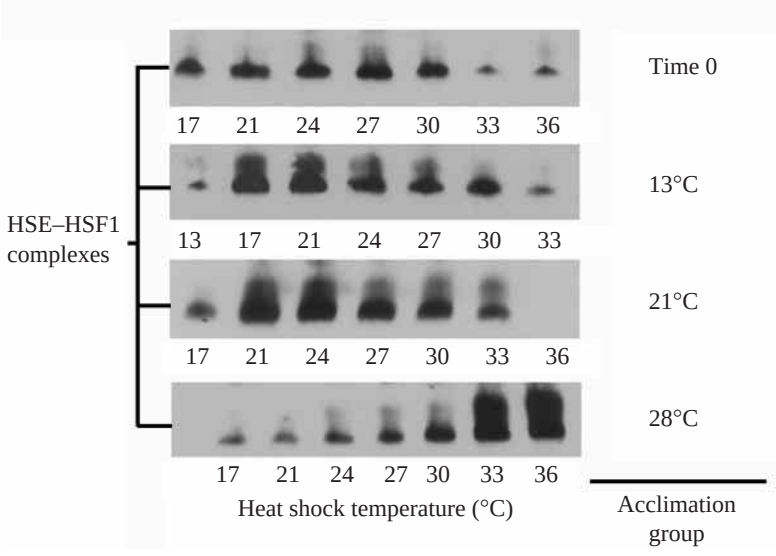

Fig. 4. Electrophoretic mobility-shift assay (EMSA) of HSF1 activation in liver tissue from *G. mirabilis*. Representative activation profiles of HSF1 in the liver tissues of differently acclimated *G. mirabilis* in response to heat shock (mean HSF1 activation levels for $N=5$ samples from each of the groups shown in Fig. 5). EMSAs were run as described in the legend to Fig. 3 and Materials and methods. Heat shock temperatures are labeled below each lane and acclimation groups are shown to the right of the figure.

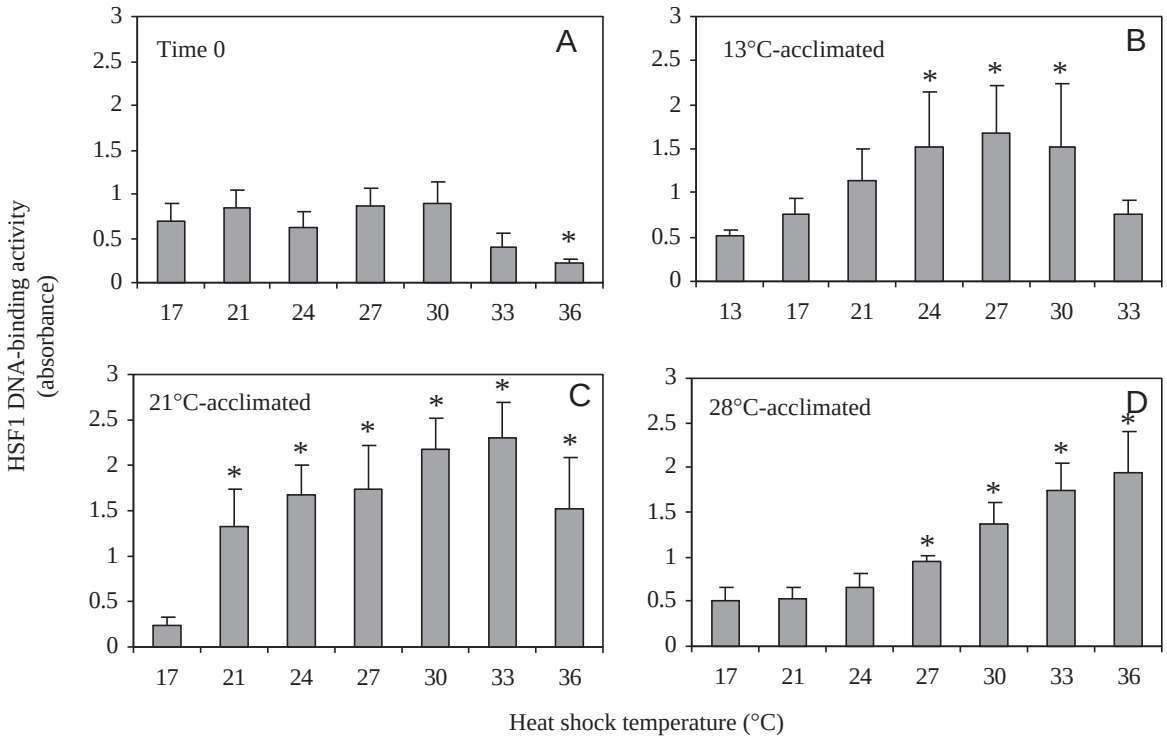

Fig. 5. The effect of thermal acclimation on the temperature sensitivity of HSF1 activation in liver tissue from *G. mirabilis*. Fish were collected in June, 2001 and either killed immediately (time 0), or acclimated for 5 weeks at 13°C, 21°C or 28°C. HSF1 activation levels were determined *via* electrophoretic mobility-shift assay. HSF1 activation levels are means ± S.E.M. (N=5). There was a significant effect of both heat shock temperature (P=0.011) and acclimation temperature (P<0.001) on HSF1 activation (ANOVA). Analyses of heat shock temperature effects within the acclimation groups are shown in Table 2. Analyses of the effect of acclimation group at a given heat shock temperature are listed in Table 3. *Within acclimation groups, heat shock temperature at which the mean HSF1 activity differed significantly (P<0.05; pairwise t-test) from that at the lowest heat shock temperature.

Table 1. *Analysis of variance of effects of heat shock temperature and acclimation temperature on HSF1 activation in* Gillichthys mirabilis *(pooled data)*

Source	d.f.	MS	*F*-value	*P*
Heat shock temperature (HST)	7	2.02	2.735	0.011
Acclimation temperature (AT)	3	4.905	6.871	<0.001
Nested: AT (HST)	20	1.86	2.945	<0.001
AT×HST	21	1.807	2.864	<0.001

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

Table 2. *Analysis of variance of effects of heat shock temperature on levels of activated HSF1 in differently acclimated* Gillichthys mirabilis

Acclimation group	d.f.	MS	*F*-value	*P*
Time 0	6	0.336	2.104	0.085
13°C-acclimated	6	1.071	1.098	0.388
21°C-acclimated	6	2.338	2.943	0.024
28°C-acclimated	6	2.085	5.577	<0.001

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

Concentrations of HSF1, Hsp70 and actin.

Concentrations of HSF1, Hsp70 and actin were determined for each acclimation group (Fig. 6). There was a significant increase in HSF1 with increasing acclimation temperature (ANOVA, P<0.001) (Fig. 6A). Levels of HSF1 were approximately 10% higher in 28°C-acclimated fish than in the time 0 and 13°C-acclimated fish.

Hsp70 levels also increased significantly with acclimation temperature (ANOVA, P=0.05) (Fig. 6B). Mean Hsp70 concentrations in the 28°C-group were 28% higher than in the 21°C-group and 47% higher than in the 13°C-group. However unlike HSF1, Hsp70 levels in the time 0 group were equal to those in the 28°C-group.

In contrast to Hsp70 and HSF1 levels, there was no difference in actin levels among acclimation groups (Fig. 6C), suggesting that overall protein synthesis did not increase at warmer temperatures.

Discussion

We investigated the effect of thermal acclimation on the acquisition of DNA-binding ability (‘activation’) of HSF1, the transcriptional regulator of all inducible heat shock genes, in the eurythermal goby *G. mirabilis*. We hypothesized that

Table 3. *Analysis of variance of effects of acclimation group on HSF1 activation in* Gillichthys mirabilis *at individual heat shock temperatures*

Heat shock temperature (°C)	d.f.	MS	*F*-value	*P*
13	NA	NA	NA	NA
17	3	0.284	2.000	0.152
21	3	0.658	1.576	0.232
24	3	1.605	2.488	0.095
27	3	1.12	1.703	0.205
30	3	1.411	1.541	0.240
33	3	3.93	10.196	<0.001
36	2	4.269	4.2	0.039

d.f., degrees of freedom; MS, mean squares; NA, not applicable.

plasticity in the activation of HSF1 could contribute to the observed plasticity in the temperature at which Hsps are induced in thermally stressed cells. The temperature sensitivity of HSF1 activation in *G. mirabilis* was determined to be dependent upon acclimation temperature, lending support to that hypothesis. To the best of our knowledge, this is the first evidence for acclimation-induced changes in the effect of heat shock on HSF1 activation.

As demonstrated here and elsewhere (Dietz and Somero, 1992; Dietz, 1994), the threshold temperature for Hsp70 and Hsp90 induction in the tissues of *G. mirabilis* varies considerably as a function of the recent thermal history of the individual (Fig. 2). In field-sampled individuals, Hsp90 was induced in brain tissue at 28°C in winter-acclimatized fish, and at 32°C in summer-collected fish (Dietz and Somero, 1992). Likewise, laboratory acclimation to warmer temperatures raised the Hsp induction temperature in the gill, brain and liver (Dietz, 1994). Here, the Hsp induction set point in liver tissue was several degrees higher in fish acclimated to 28°C than in those maintained at 21°C. Acclimation-related variability in Hsp induction thresholds has also been observed in the fathead minnow (Dyer et al., 1991), rainbow trout (Currie et al., 2000) and sea lamprey (Wood et al., 1999). In addition, fish species demonstrate a considerable amount of tissue-specificity in Hsp induction temperatures and intensities (reviewed in Iwama et al., 1998). The goal of the current study was to examine the plasticity in Hsp induction from a mechanistic perspective, specifically focusing on the temperature sensitivity of HSF1 activation.

Acclimation of *G. mirabilis* to different temperatures affected the activation of HSF1 upon subsequent heat shock (Fig. 5) in a manner similar to the changes observed in Hsp induction temperature (Fig. 2). In contrast to the time 0 group (Fig. 5A), in which heat shock did not significantly increase levels of activated HSF1, in the laboratory-acclimated groups, heat shock resulted in a significant increase in HSF1 activation in all cases (Fig. 5B–D). However, the temperatures at which peak HSF1 activation was observed were strongly dependent upon acclimation temperature. The highest HSF1 activity was

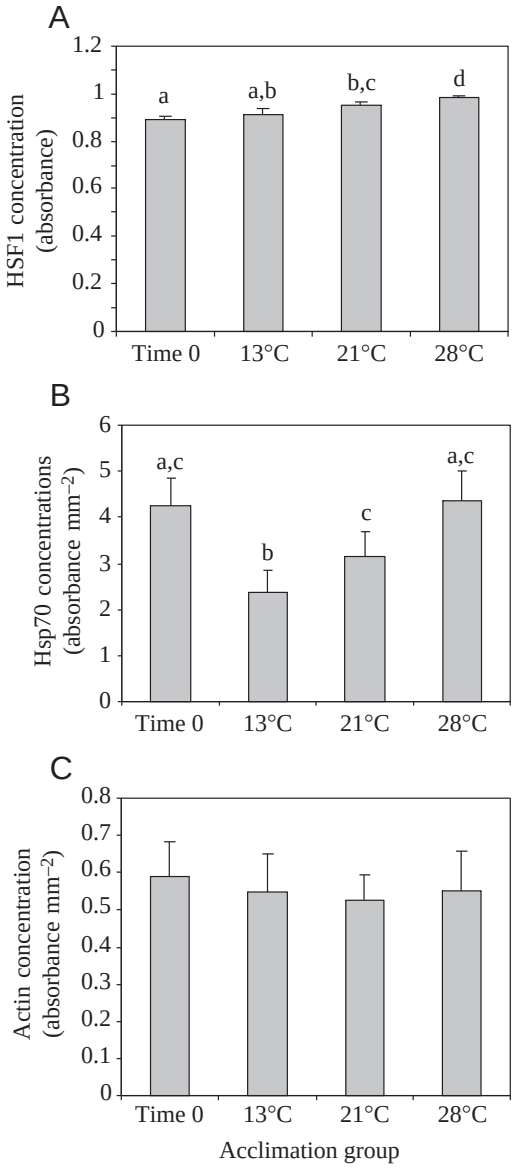

Fig. 6. Concentrations of (A) HSF1, (B) Hsp70 and (C) actin in liver tissue from *G. mirabilis*. Levels of HSF1 and actin were determined by western blotting. Briefly, liver tissue was homogenized in 50 mmol l^{-1} Tris-Cl, pH 6.8, 4% sodium dodecyl sulfate (SDS) and 1 mmol l^{-1} phenylmethylsulfonylfluoride (PMSF). Homogenates were boiled for 5 min and centrifuged at 12 000 ***g*** for 10 min before loading on 7.5% polyacrylamide gels. After electrophoresis, proteins were blotted onto nitrocellulose membranes. Blots were incubated in primary antibody (for antibodies used, see Materials and methods) for 1.5 h and secondary HRP-conjugated antibody for 1 h. Bands were visualized by a chemiluminescence reaction and their densities determined by scanning densitometry. Values are means ± S.E.M. (*N*=5). Within a graph, values labeled with different letters differ significantly from one another ($P<0.05$: pairwise *t*-test).

observed at 27°C in the 13°C group, at 33°C in the 21°C group and at 36°C in the 28°C group (although this was the highest experimental temperature and so may not represent the true temperature of peak HSF1 activation for the 28°C group).

Furthermore, in the time 0, 13°C- and 21°C-acclimated groups, a decrease in HSF1 activation was observed at the highest heat shock temperature. Although HSF1 activation in the 28°C-acclimated group continued to climb at these same temperatures (mirroring the robust Hsp synthesis observed at 35 and 39°C in warm-acclimated *G. mirabilis* as opposed to cold-acclimated fish; Fig. 2), we postulate that a similar decrease in HSF1 activation in this group could have been observed at a sufficiently high heat shock temperature. The decrease in HSF1 activation that occurred at high temperatures is interesting in light of the break down in total protein synthesis observed in this, and many other, species at temperatures near their upper thermal lethal limit (Hochachka and Somero, 2002). It is possible, therefore, that acclimatory adjustments made in the machinery of gene expression and protein synthesis may play an important role in determining the upper thermal limit of Hsp induction.

The effect of thermal acclimation on HSF1 activation observed here is broadly consistent with other related studies. Clos et al. (1993) demonstrated that transfection of a human HSF1 gene into *Drosophila* cells reprogrammed the activation temperature of HSF1, illustrating the importance of cellular context on the regulation of Hsp genes. In addition, HSF1 activation temperature was found to differ among warm- and cold-adapted lizard species (Zatsepina et al., 2000). However, the species chosen for this study were from different families and so it is not possible to separate out those differences in HSF1 activation temperature that were caused by thermal acclimatization and those that were solely owing to phylogenetic distance.

The changes in the temperature profiles of HSF1 activation measured in the current study occurred concomitantly with a significant increase in the concentration of HSF1 with warm acclimation (Fig. 6A). Concentrations of actin – chosen for its status as the product of a non-inducible housekeeping gene – were also quantified to control for any direct effects of temperature on protein synthesis. There was no significant acclimatory change in actin levels (Fig. 6C). As the peak HSF1 activation intensities among acclimation groups were similar (no significant differences in pairwise *t*-tests), the impact that the additional HSF1 measured in the warm-acclimated fish had on HSF1 activation measurements is unclear. The HSF1 gene is under the control of general transcription factors and is not itself heat-inducible and HSF1 concentrations have been shown to be poor indicators of Hsp gene expression rates (Victor and Benecke, 1998). Evidence for acclimatory changes in HSF1 concentrations in poikilotherms under ecologically relevant conditions is equivocal. In one study, laboratory-acclimation did not alter HSF1 concentrations in *Mytilus trossulus* compared to levels in field-collected individuals (Buckley et al., 2001), whereas in another, HSF1 concentrations were higher in laboratory-acclimated snails (*Tegula* congeners) compared to levels in the field (Tomanek and Somero, 2002). However in laboratory-acclimated *Tegula* congeners, acclimation temperature itself had no effect on HSF1 concentration (Tomanek and Somero, 2002). In a field study, HSF1 levels were higher in desert lizards than in non-desert species (Zatsepina et al., 2000).

One factor that might have affected the HSF1 activation temperature in this study is the observed increase in Hsp levels with warm acclimation (Fig. 6B). Increases in standing stock Hsp concentrations in response to warm acclimation and acclimatization have also been observed in other taxa and are probably the outcome of repeated gene induction events occurring in response to increasing temperatures and the build up of damaged proteins (Hofmann and Somero, 1995; Maloyan et al., 1999; Buckley et al., 2001; Tomanek and Somero, 2002). The concentrations of Hsps in the 28°C group were nearly twice those in the 13°C group and 28% higher than those acclimated to 21°C. The amplified level of Hsps in the 28°C-acclimated fish in this study is correlated with a higher temperature of peak HSF1 activation compared to the colder-acclimated groups. This is consistent with a scenario in which the high levels of chaperone in the livers of the 28°C group maintained a greater proportion of the HSF1 pool in the inactive monomeric state at heat shock temperatures that were sufficient to cause significant HSF1 activation in the colder-acclimated groups.

Why the equally-elevated levels of Hsp70 in the time 0 group were not correlated with a similar temperature profile of HSF1 activation to that of the 28°C group is unknown. The HSF1 levels in the time 0 group were the lowest of any of the treatments, coupled with high Hsp70 levels. According to the current model of HSF1 regulation, this would result in the greatest repression of HSF1 activity by Hsp70 of any of the groups. However, there were only relatively minor differences in HSF1 levels among groups compared to the differences in Hsp70 levels and so it is difficult to explain the HSF1 activity in the time 0 fish in terms of the HSF1/Hsp70 ratio alone.

It is worth noting that the time 0 fish were freshly collected from the field, where they are exposed to variable conditions. This species inhabits burrows in the muddy substrate of the estuary where water temperatures may diverge from those of the water column (Eschmayer et al., 1983). Furthermore, hypoxia and salinity stress are also routine daily challenges in this habitat. Therefore, the previous stress exposure of these fish is unclear. It is possible that the effect of a subsequent heat shock on these field-collected fish may have differed from the effect of heat shock on the fish acclimated to a single temperature under controlled conditions.

While much has been gained from the accumulation of studies on Hsp gene regulation in the cells of homeothermic organisms, it is becoming increasingly clear that the HSR in poikilotherms cannot be discussed solely in terms of mammalian regulatory models derived from organisms with body temperatures that have become more or less independent of their environmental temperatures. For eurythermal organisms, a constantly adjustable regulatory scheme to control Hsp production would be favorable in order to tailor the response to the current thermal conditions. Here, we have demonstrated the potential for acclimation-induced variability in the activation temperature of HSF1, which supports

hypotheses linking plasticity in promoter binding events to downstream changes in the temperature at which Hsp levels increase and decrease in the stressed cell.

This study and similar projects which illuminate the role that the HSR, and other molecular stress responses, might play in ecological processes will contribute to our understanding of the organismal 'black box', which is common to many current ecological models of community structure. Futhermore, describing the scope of phenotypic plasticity conferred by the HSR and other mechanisms of acclimatization will greatly inform predictions concerning the impact of global climate change on natural populations.

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