Adjusting the thermostat: the threshold induction temperature for the heat-shock response in intertidal mussels (genus *Mytilus*) changes as a function of thermal history

Bradley A. Buckley[1], Marie-Eve Owen[2] and Gretchen E. Hofmann[1,*]

[1]*Department of Biology, Arizona State University, Tempe, AZ 85287-1501, USA* and [2]*Department of Biology, Bishops University, Lennoxville, Quebec, Canada J1M 1Z7*

*Author for correspondence (e-mail: ghofmann@asu.edu)

Summary

Spatio-temporal variation in heat-shock gene expression gives organisms the ability to respond to changing thermal environments. The temperature at which heat-shock genes are induced, the threshold induction temperature, varies as a function of the recent thermal history of an organism. To elucidate the mechanism by which this plasticity in gene expression is achieved, we determined heat-shock protein (Hsp) induction threshold temperatures in the intertidal mussel *Mytilus trossulus* collected from the field in February and again in August. In a separate experiment, threshold induction temperatures, endogenous levels of both the constitutive and inducible isoforms of Hsps from the 70 kDa family and the quantity of ubiquitinated proteins (a measure of cellular protein denaturation) were measured in *M. trossulus* after either 6 weeks of cold acclimation in the laboratory or acclimatization to warm, summer temperatures in the field over the same period. In addition, we quantified levels of activated heat-shock transcription factor 1 (HSF1) in both groups of mussels (HSF1 inducibly transactivates all classes of Hsp genes). Lastly, we compared the temperature of HSF1 activation with the induction threshold temperature in the congeneric *M. californianus*. It was found that the threshold induction temperature in *M. trossulus* was 23 °C in February and 28 °C in August. This agreed with the acclimation/acclimatization experiment, in which mussels acclimated in seawater tables to a constant temperature of 10–11 °C for 6 weeks displayed a threshold induction temperature of 20–23 °C compared with 26–29 °C for individuals that were experiencing considerably warmer body temperatures in the intertidal zone over the same period. This coincided with a significant increase in the inducible isoform of Hsp70 in warm-acclimatized individuals but no increase in the constitutive isoform or in HSF1. Levels of ubiquitin-conjugated protein were significantly higher in the field mussels than in the laboratory-acclimated individuals. Finally, the temperature of HSF1 activation in *M. californianus* was found to be approximately 9 °C lower than the induction threshold for this species.

Key words: mussel, *Mytilus trossulus*, *Mytilus californianus*, heat shock, heat shock response, heat shock protein, Hsp70, temperature, thermal acclimation, gene expression.

Introduction

To persist in a particular habitat, organisms must be responsive to the changes in their physical environment. Although these responses are often behavioral or metabolic in nature, one powerful mechanism employed by organisms is the adjustment of gene expression in response to environmental change (Pigliucci, 1996). Such adjustments in gene expression afford the organism a source of physiological plasticity, an outcome that may be especially significant in eurythermal ectotherms that regularly experience broadly fluctuating environmental conditions. A well-described molecular response that provides a degree of physiological plasticity is the heat-shock response. During a heat-shock response, a discrete set of proteins, the heat-shock proteins

(Hsps), is induced in response to various stressors that are denaturing to the cellular protein pool (Lindquist, 1986; Parsell and Lindquist, 1993); these stresses are not limited to extreme temperature and include a range of other environmental and chemical perturbations (for a review, see Feder and Hofmann, 1999). In stressed cells, denaturing proteins may enter one of two pathways: either degradation by a cellular protease or refolding facilitated by molecular chaperones (Wickner et al., 1999). Because heat-shock proteins (Hsps) act as molecular chaperones and stabilize thermally denaturing proteins (Hartl, 1996; Fink, 1999; Mogk et al., 1999), the induction of Hsps is thought to have adaptive value for organisms faced with heat stress (Parsell and

Lindquist, 1993; Feder et al., 1996). Not surprisingly, Hsp expression patterns have been shown to have ecologically significant ramifications for many organisms (for a review, see Feder and Hofmann, 1999), and these patterns are directly related to thermotolerance at the organismal level (e.g. Feder et al., 1997; Tomanek and Somero, 1999).

One significant development in the pursuit of the ecological importance of Hsps has been the demonstration that the temperature at which these genes are activated, the threshold induction temperature, varies within the lifetime of a single organism and is subject to thermal acclimation and acclimatization. In particular, studies on marine fish and invertebrates have shown that there is a great deal of plasticity in the threshold induction temperature for Hsps as a function of season (Dietz and Somero, 1992; Roberts et al., 1997) and laboratory acclimation (Dietz, 1994; Hofmann and Somero, 1996; Tomanek and Somero, 1999). These observations have particular significance because intertidal organisms often face extreme variations in environmental temperature (see Helmuth, 1999) that may call for commensurately variable protein chaperoning.

However, despite the growing database regarding expression patterns of Hsps in nature, we know very little about how Hsp genes are regulated in response to variations in environmental temperature. What processes account for the season- or temperature-dependent manner in which the gene activation temperatures change? Furthermore, how is environmental temperature 'sensed' and then transduced to the nucleus? The key to answering these questions is undoubtedly related to how the Hsp genes are transcriptionally activated. During gene expression, transactivation of heat-shock genes is mediated by the interaction between heat-shock transcription factor 1 (HSF1) and the heat-shock element (HSE), a series of pentameric units of the sequence 5′-nGAAn-3′ found in the promoter of all Hsp genes (Xiao and Lis, 1988; Wu, 1995). Although several HSFs have been characterized in eukaryotic cells, HSF1 is the factor responsive to conditions and treatments that induce the heat-shock response (Sarge et al., 1993; Wu, 1995). It has been shown that the temperature of HSF1 activation, specifically the acquisition of promoter-binding ability, is influenced by evolutionary temperature in *Drosophila melanogaster* reared at different temperatures for more than 20 years in the laboratory (Lerman and Feder, 2001) and in phylogenetically distant lizards adapted to different habitat temperatures (Zatsepina et al., 2000). However, in *D. melanogaster*, the HSF1 activation temperature was found not to differ between strains with different thermotolerances and Hsp synthesis profiles (Zatsepina et al., 2001). At present, it is unclear what role HSF1 plays in the plasticity of the heat-shock response as a whole, especially within a single species during acclimatization in nature or during short-term acclimation in the laboratory. Particularly lacking are studies in non-model organisms that integrate simultaneous measurements of protein denaturation, molecular chaperone expression and the behavior of HSF1 in the face of realistic temperature stresses. With regard to HSF1, specific unanswered questions are (i) does the amount of activated HSF1 change during thermal acclimation/acclimatization and (ii) what is the relationship between the temperature of HSF1 activation and the threshold induction temperature of Hsp genes in organisms experiencing temperature stress in nature?

To begin to address these questions, the present study extends our examination of the heat-shock response in two species of intertidal mussel from the genus *Mytilus*. First, using established metabolic labeling techniques, we tested the plasticity of the threshold induction temperature in gill tissue both by comparing winter- and summer-acclimatized *M. trossulus* and through comparisons of laboratory-acclimated mussels with mussels simultaneously acclimatized in the field. Second, in the latter comparisons, western blotting was used to monitor levels of the constitutive and inducible isoforms of the molecular chaperones from the 70 kDa Hsp gene family and levels of ubiquitin-conjugated proteins (a direct measure of cellular protein denaturation) post-acclimation/acclimatization. Finally, concentrations of HSF1 were quantified in gill from the same mussels. In addition, a separate experiment was conducted in which we used electrophoretic mobility shift assays (EMSAs) to examine HSF1 DNA-binding activity in relation to the threshold induction temperature for the Hsp gene products in *M. californianus*.

The results presented here show that the threshold induction temperature for Hsp70 (i.e. the temperature at which the heat-inducible hsp70 gene was expressed) in gill tissue was subject to thermal acclimation/acclimatization and varied significantly with the thermal history of the organism. In addition, the shift in the set-point for the induction of Hsp70 genes was correlated with a concomitant sevenfold increase in levels of the inducible isoform of Hsp70 but no change in the levels of the constitutive cognate isoform of Hsp70 or HSF1. Finally, the temperature of HSF1 activation (i.e. acquisition of DNA-binding ability) in *M. californianus* was approximately 9 °C lower than the threshold temperature of Hsp gene induction, which has important implications for our understanding of how these genes are regulated during adaptation to a given thermal regime.

Materials and methods

Study organism and acclimation conditions

Mytilus trossulus (Gould) were collected from the British Camp study site in Garrison Bay on San Juan Island, Washington (Puget Sound; 48°34′N, 123°9′W). Specimens for the acclimation experiment were collected in June 1998 and transported in a chilled cooler (at approximately 10 °C) to the Friday Harbor Laboratories (University of Washington) on San Juan Island. For the acclimation treatment, mussels were maintained in a flow-through seawater table where water temperature was 10–11 °C for the entire acclimation period; seawater temperature at the time of collection was 10 °C. During the course of the acclimation, the mussels were never aerially exposed until use in experiments. Specimens for the summer-acclimatized sampling were collected in mid-July

1998. Mussels were collected before they had been exposed to air by the receding tide and were returned immediately to the Friday Harbor Laboratories for use in experiments. For the electrophoretic mobility shift assays, summer-acclimatized *Mytilus californianus* were shipped overnight in a chilled cooler from the Oregon coast to Arizona State University, where they were held in the laboratory in recirculating seawater tanks prior to use.

Preparation of gill tissue

Gill lamellae were dissected from individual mussels, and approximately 100 mg of tissue was homogenized in 5 volumes of homogenization buffer (HB) consisting of 50 mmol l^{-1} Tris-HCl, pH 6.8, 4 % sodium dodecyl sulfate (SDS) and 1 mmol l^{-1} phenylmethylsulfonylfluoride (PMSF). The homogenate was heated at 100 °C for 5 min and then centrifuged at 12 000 g for 15 min at room temperature (approximately 23 °C). The resulting supernatant was analyzed for protein content using a modified Bradford assay (Pierce Coomassie Plus) and stored at −20 °C prior to electrophoresis.

Heat-shock protein induction experiments: metabolic labeling with ^{35}S-labelled amino acids

Metabolic labeling was conducted according to the protocol described by Hofmann and Somero (Hofmann and Somero, 1996). Dissected pieces of gill tissue (approximately 100 mg) were placed in 0.5 ml of Hepes-buffered artificial sea water [20 mmol l^{-1} Hepes, 7.57 mmol l^{-1} (NH$_4$)$_2$SO$_4$, 375 mmol l^{-1} NaCl, 9.35 mmol l^{-1} KCl, 2.7 mmol l^{-1} NaHCO$_3$, 17.95 mmol l^{-1} Na$_2$SO$_4$, 37.7 mmol l^{-1} MgCl$_2$.6H$_2$O, 8 mmol l^{-1} CaCl$_2$.2H$_2$O, 10 mmol l^{-1} glucose] containing 3.7×10^6 Bq of ^{35}S-labelled methionine/cysteine amino acid mixture (NEN). Each 1.5 ml microcentrifuge tube was pre-equilibrated to the desired incubation temperature prior to the addition of the tissue; the incubation temperatures used were 14, 17, 20, 23 26, 29, 32 and 35 °C. After the 2 h incubation period, tissues were washed twice with 1 ml of Hepes-buffered sea water at 4 °C, homogenized in 200 µl of HB and processed using the protocol described in the sample preparation section above. Samples from the induction experiments were not assayed for protein concentration; instead, a sample was analyzed in a liquid scintillation counter to determine the amount of radioactivity per milliliter for each gill extract.

Electrophoresis and fluorography

Patterns of protein synthesis during the temperature exposures were examined using SDS–polyacrylamide gel electrophoresis in combination with fluorography, as described by Hofmann and Somero (Hofmann and Somero, 1996). Proteins were separated on 10 % polyacrylamide gels, and each lane was loaded to give equivalent amounts of radioactivity (250 000 cts min^{-1}). Following electrophoresis, gels were treated with an autoradiographic enhancer (EN^{3}HANCE; DuPont NEN), dried and exposed to X-ray film (Kodak X-OMAT AR5) at −70 °C for an empirically determined period.

Solid-phase immunochemical measurement of Hsp70, HSF1 and ubiquitin conjugates

Immunochemical assays and scanning densitometry were employed to determine the levels of both the constitutive and inducible isoforms of Hsp70, HSF1 and ubiquitin-conjugated protein in gill tissue from laboratory-acclimated and field-acclimatized specimens of *Mytilus trossulus*. Hsp70 western blots were performed as described by Hofmann and Somero (Hofmann and Somero, 1995) except that wet electrophoretic transfer at 30 V for 15 h was used during the western protocol (transfer buffer: 20 mmol l^{-1} Tris, 192 mmol l^{-1} glycine, 20 % methanol). Equal amounts of protein (10 µg total protein) were separated on 7.5 % polyacrylamide gels. The immunodetection was performed using an anti-Hsp70 rat monoclonal antibody that crossreacts with the constitutive and inducible forms of Hsp70 (Affinity Bioreagents; MA3-001). For HSF1 western blots, the blot was sequentially incubated with an anti-HSF1 antibody (mouse anti-*Drosophila*; courtesy of Dr Carl Wu) diluted 1:10 000 in 0.5 % bovine serum albumin in phosphate-buffered saline (PBS)/0.1 % Tween 20 followed by a horseradish-peroxidase-conjugated secondary antibody (goat anti-mouse) diluted 1:20 000 in 5 % non-fat dry milk in PBS. Western blots were ultimately developed using an enhanced chemiluminescence (ECL) protocol according to the manufacturer's instructions (Amersham). Ubiquitin conjugate analysis was performed as described previously (Hofmann and Somero, 1995). In all cases, the linear relationship between the chemiluminescent signal and the quantity of antigen was tested with known quantities of purified, commercially purchased protein and was further optimized for the gill extracts such that the amount of protein loaded was within the linear range of the ECL signal.

Electrophoretic mobility shift assay

For electrophoretic mobility shift assays (EMSAs), gill tissue (approximately 100 mg) was incubated in 1.5 ml microcentrifuge tubes containing 0.5 ml of Hepes-buffered artificial sea water. From each individual mussel, a piece of gill tissue was exposed to the desired temperature for 2 h; the temperature range was 13–32 °C. Following the temperature exposures, individual pieces of tissue were flash-frozen in liquid nitrogen prior to being processed for EMSA. To prepare tissue extracts, the gill samples were thawed on ice and lysed in a stabilization buffer containing 25 % 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. EMSAs were conducted using LightShift chemiluminescent EMSA kits (Pierce). Briefly, gill extracts (7 µg total protein) were incubated with approximately 15 pmol of biotinylated HSE oligonucleotide probe (5′-GCCTC*GAAT*G*TTC*G*CGAA*TTT-3′; Airaksinen et al., 1998) in 25 mmol l^{-1} Hepes (pH 7.6), 100 mmol l^{-1} NaCl, 15 % glycerol, 0.1 % NP-40 and 0.5 mmol l^{-1} PMSF in a final volume of 20 µl. Incubations were conducted for 30 min at 23 °C. After incubation, assay mixtures were applied to 5 % acrylamide non-denaturing gels and electrophoresed for

approximately 2 h at 100 V. Gels were then transferred to nylon membranes *via* electroblotting at 380 mA for 30 min. HSF1–HSE complexes on each blot 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). The specificity of the HSE probe was confirmed in assays in which unlabelled HSE probe was added in excess as a competitor; in these assays, the intensity of the HSE–HSF1 complex band was reduced on the resulting X-ray film.

Air and body temperature data

Mussel body temperatures were measured in the field during a low-tide aerial exposure. A digital thermometer was inserted into the shell of five mussels (of a similar size to those used in experiments), and their temperature was recorded every 20 min for 7 h. Ambient air temperature was also recorded over the same period.

Results

Hsp induction profiles

Overall, the induction threshold temperature for Hsp production in *Mytilus trossulus* was found to be strongly influenced by the organism's thermal history. Fig. 1 shows protein synthesis patterns from metabolic labeling experiments conducted after exposure to heat shock in isolated gill tissue from *M. trossulus* collected in February and August. The induction threshold temperature for Hsps was 23 °C in the mussels collected in winter and 28 °C in the mussels collected in summer; these findings were consistent amongst all individuals tested (*N*=5 for each month).

To test further the effects of thermal history on the induction of Hsps, a second experiment compared mussels acclimated in the laboratory to 10 °C for 6 weeks with mussels that remained in the intertidal for the same time period. Fig. 2A shows the pattern of protein synthesis during similar metabolic labeling experiments on the laboratory-acclimated mussels *versus* that of the summer, field-acclimatized mussels. *De novo* protein synthesis, visualized here as radioactive bands, occurred readily at all temperatures in the thermal gradient except at 32 °C in the laboratory-acclimated animals (synthesis was robust at that temperature, however, in the warm-acclimatized field animals). In the individual acclimated to 10–11 °C in the laboratory for 6 weeks, noticeable expression of heat-shock proteins in the 70–90 kDa molecular mass range occurred after exposure to 20 °C, whereas in the mussel allowed to acclimatize to summer field conditions the induction threshold temperature, at 26 °C, was considerably higher.

Fig. 2B shows just those lanes that illustrate the temperature of Hsp induction in separate individuals from both the laboratory and field groups (*N*=4 additional mussels for each), demonstrating the consistency of the upward shift in induction temperature. In laboratory-acclimated

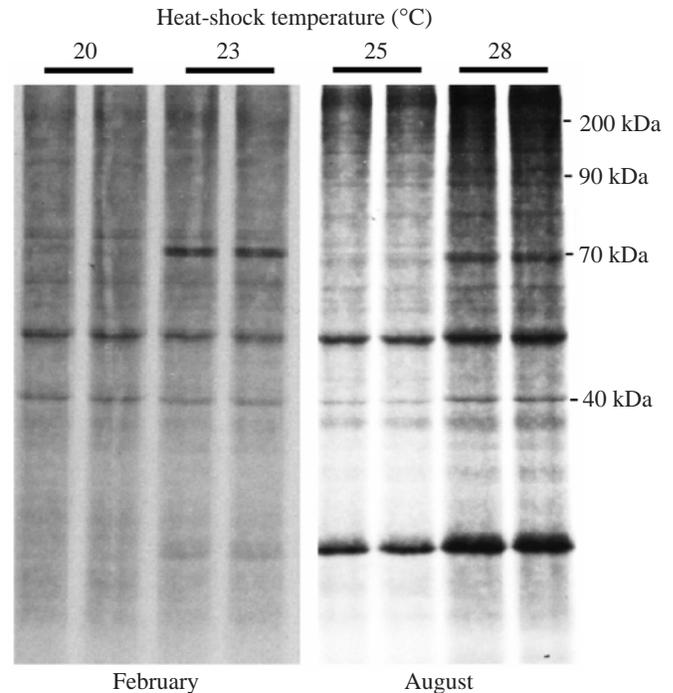Heat-shock temperature (°C)

Fig. 1 Seasonal plasticity in the induction threshold temperature in the intertidal mussel *Mytilus trossulus*. Pieces of dissected gill tissue from mussels collected in the field in February and August were incubated at the indicated temperatures for 2 h and then metabolically labeled at (13 °C) for 2 h in the presence of ^{35}S-labelled methione/cysteine. Following metabolic labeling, proteins were separated on 10 % SDS–polyacrylamide gels and processed using standard fluorographic techniques. Individual lanes were loaded with equal counts of radioactivity (3.7×10^7 Bq), and the locations of molecular mass markers (not shown) are indicated on the right. The threshold temperature of heat-shock protein induction for each gel is given above the right-hand pair of columns.

individuals, the induction temperature was 20–23 °C, while that of the field mussels was 26–29 °C. In addition, an Hsp of low molecular mass (approximately 26 kDa) was induced at 20–23 °C in the laboratory-acclimated mussels, but was constitutively expressed in the field-acclimatized mussels (Fig. 2B).

There was an appreciable increase in the levels of both the 70 kDa class of Hsps and the low-molecular-mass class at 20–23 °C in laboratory-acclimated mussels, but in the field-acclimatized mussels these peaks occurred at 26–29 °C. Field-acclimatized mussels saw daily body temperatures fluctuations of 15–34 °C during tidal emersion, temperatures considerably higher than air temperature as a result of direct solar exposure (Fig. 3). These data agree with measurements of daily body temperature ranges of more than 20 °C that have been recorded elsewhere in *Mytilus californianus* from the Pacific Northwest coast (Helmuth, 1999).

Endogenous levels of Hsp 70, HSF1 and ubiquitin conjugates

The upward shift in threshold induction temperature in field-acclimatized mussels was accompanied by an approximately
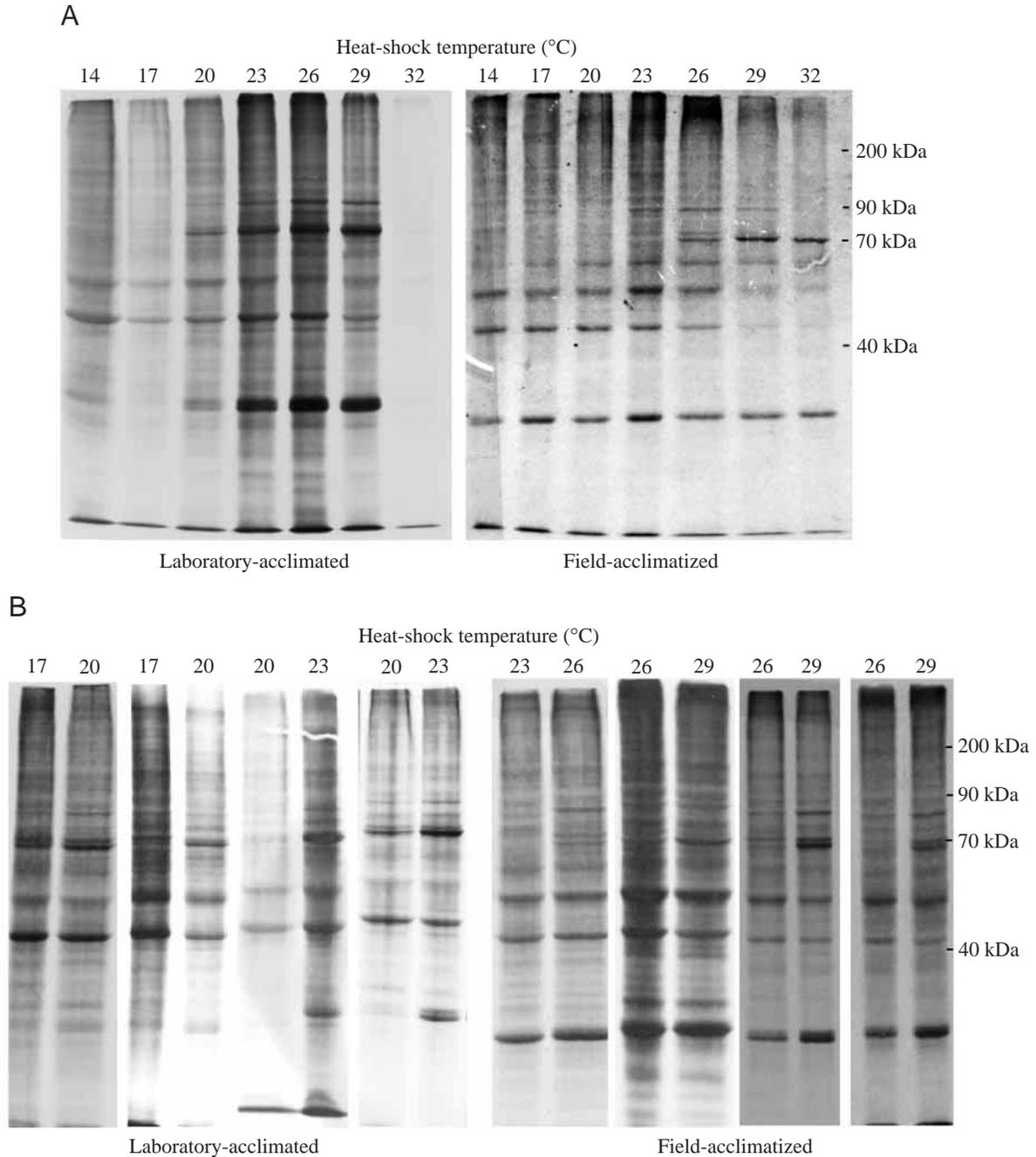
Fig. 2. Effects of thermal history on the induction of heat-shock protein (Hsp) synthesis in gill tissue from *Mytilus trossulus*. Gill sections from mussels acclimated to 10 °C in the laboratory or acclimatized in the field for 6 weeks were treated identically to those in Fig. 1 (see Fig. 1 legend for methods) with the exception of the specific heat-shock temperatures (listed above each lane). Protein synthesis patterns in the gill are depicted for an individual from each acclimation/acclimatization group (A) and for four additional mussels from each group (B). In A, protein synthesis patterns across the entire experimental thermal gradient are shown. The threshold induction temperature for laboratory-acclimated mussels was 20 °C and that for field-acclimatized mussels was 26 °C. In B, the first lane depicts the temperature immediately prior to induction and the second lane demonstrates Hsp induction threshold temperature for that particular individual. The locations of molecular mass markers (not shown) are given on the right.

sevenfold increase in the endogenous levels of the inducible isoform of Hsp70 in gill tissues over the levels measured in laboratory-acclimated individuals (Fig. 4A). However, levels of the constitutive isoform of Hsp70 (Hsc70 in Fig. 4A) and

those of HSF1 did not increase during field acclimatization and were not significantly different from those in the laboratory-acclimated group (Fig. 4A). Levels of ubiquitin-conjugated protein were four times higher in the tissues of the field-
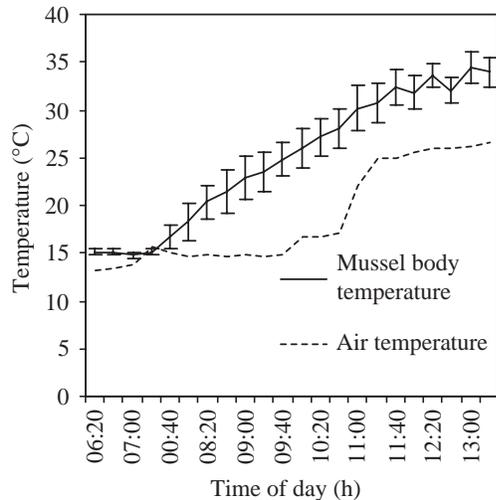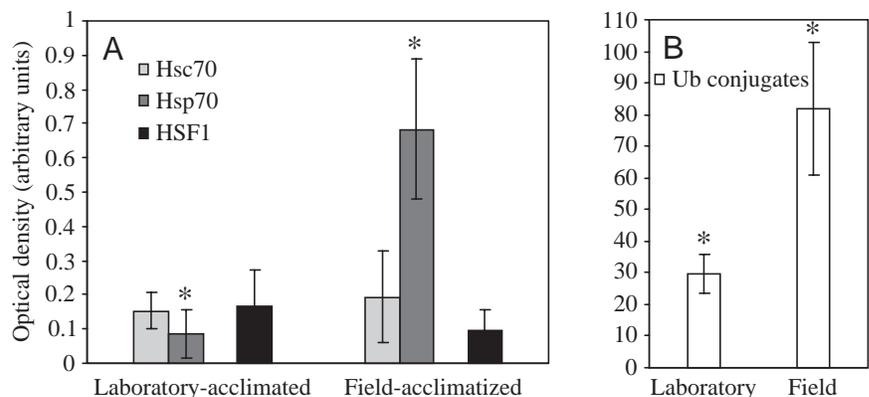
Fig. 3. Mussel body temperature (means ± S.D., N=5) and ambient air temperatures on 29 July 1996. Measurements were taken using a hand-held digital thermometer equipped with a thermistor during tidal emersion, when the mussels were exposed to direct solar irradiation. All body temperatures were taken sequentially on the same five mussels.

collected mussels than those acclimated in the laboratory (Fig. 4B).

Relationship between HSF1 DNA-binding activity and Hsp induction

In a separate experiment using *M. californianus*, we tested the relationship between the temperature of activation of HSF1 and the threshold induction temperature of Hsp synthesis (Fig. 5). Both assays were performed on gill tissue from the same individuals. There was some variability in the amount of constitutive binding of HSF1 to the HSE observed at the control temperature (13 °C) but, in all cases, a significant increase in activated HSF1 occurred in response to exposure to 17 °C (Fig. 6). However, there was no observable increase in Hsp levels in the gill of any of the individuals until 26 °C, as visualized by autoradiography (induction temperature is marked with an arrow in Fig. 5A).
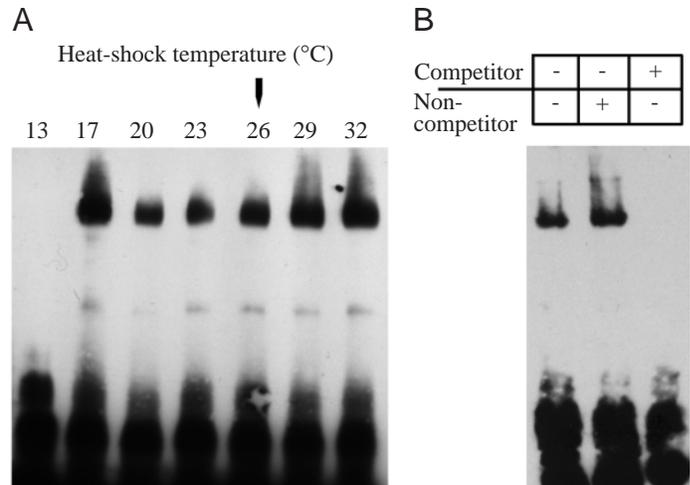
Discussion

We examined the plasticity of the heat-shock response, the highly regulated transcriptional activation of heat-shock genes in response to thermal stress, in intertidal mussels (genus *Mytilus*). Our larger goal was to begin to address the mechanisms that underlie the observed plasticity in the heat-shock response in natural populations of ectothermic organisms. We endeavored to build a connection between protein denaturation, the expression patterns of Hsp genes and the behavior of the Hsp transcriptional apparatus and to examine how these might vary in natural populations experiencing environmentally realistic temperatures.

Four salient findings in this study address the effects of laboratory acclimation and field acclimatization on the heat-shock response and its regulation in the intertidal mussel *Mytilus*. First, in all cases, the threshold temperature for Hsp induction displayed a great deal of plasticity and varied with the thermal history of *M. trossulus*. Second, endogenous levels of the inducible isoform of Hsp70 were approximately seven times higher in the field-collected group than in the laboratory-acclimated group, and this corresponded with a fourfold increase in the level of ubiquitin conjugates (i.e. irreversibly damaged proteins) in the field-acclimatized group. Third, endogenous levels of HSF1 and of the constitutive isoform of Hsp70 did not vary between the two groups. Finally, the activation temperature of HSF1 was not identical to that of the threshold temperature for Hsp synthesis in the congeneric *M. californianus*.

The temperature at which the first noticeable expression of Hsps occurred shifted from 20–23 °C to 26–29 °C in 6 weeks of acclimatization to warm and varying environmental temperatures (Fig. 2A,B). Furthermore, there was a strong induction of low-molecular-mass Hsps (sHsps) in the laboratory-acclimated individuals at 23 °C, whereas sHsps were constitutively expressed in the field-acclimatized mussels (Fig. 2A). sHsps (of which Hsp26 is the primary constituent) are thought to be involved in binding non-native, refoldable protein for subsequent refolding by other chaperones (Haslbeck et al., 1999). The regulation of sHsp activity involves a post-translational oligimerization and stress-responsive dissociation, so their expression patterns will not be discussed in the context of the HSF1-regulated mechanism of

Fig. 4. Relative levels of Hsp70, its constitutive form Hsc70 and HSF1 (A) and relative levels of ubiquitin-conjugated protein (B) in gill tissue from laboratory-acclimated and field-acclimatized *Mytilus trossulus* (means ± S.D., N=5). For western blotting, equivalent amounts of protein (10 μg) were electrophoresed in each lane. Scanning densitometry was performed on western blots developed with a standard enhanced chemiluminescent technique (see Materials and methods). *Values that are significantly different (P<0.005).

Fig. 5. Heat-shock protein induction and HSF1 activation profiles from *Mytilus californianus* (*N*=5). (A) HSF1 activation was visualized *via* an electrophoretic mobility shift assay, using a LightShift chemiluminescent EMSA kit (Pierce). Following a 1 h heat shock at the indicated temperature, homogenized gill tissue was incubated with approximately 15 pmol of biotinylated-DNA probe for 20 min at room temperature (approximately 20 °C) and then separated on 5 % non-denaturing polyacrylamide gels. The arrowhead marks the induction of Hsp synthesis, which occurred at 26 °C in all individuals. (B) A competitor/non-competitor assay in which the addition of excess non-biotinylated heat-shock element probe was used to outcompete the biotinylated probe for HSF1 binding activity. Unlabelled non-competitor sequence did not have the same effect, demonstrating the specificity of our probe.

interest in the present study. Their constitutive expression in warm-acclimatized mussels in the field may reflect the need to bind and 'hold' the large endogenous amount of denatured protein measured in these animals (see discussion of ubiquitin-conjugated protein below).

The finding that the induction set-point for Hsp synthesis changes with thermal history agrees with other studies in diverse organisms (for a review, see in Feder and Hofmann, 1999) including our own previous work on *Mytilus* (Hofmann and Somero, 1995; Hofmann and Somero, 1996). In general, organisms from warmer environments induce Hsps at a higher temperature than do closely related organisms from colder environments, whether that involves differences between congeners with different habitat distributions [e.g. in marine invertebrates (Tomanek and Somero, 1999); in insects (Huey and Bennett, 1990; Gehring and Werner, 1995)] or seasonal differences within a species (Dietz and Somero, 1992; Fader et al., 1994). In *Mytilus*, higher induction thresholds have been observed in *M. galloprovincialis*, a warm-water species, than in *M. trossulus*, which has a colder distribution in nature (Hofmann and Somero, 1995), and *M. californianus* has a demonstrably higher Hsp induction set-point in the summer than in the winter (Roberts et al., 1997). But what processes are responsible for this upward shift in threshold induction temperature?

Measurements of ubiquitin conjugation make it unlikely that the answer is that warm-acclimatized organisms simply sustain less total thermal denaturation of their protein pool (Fig. 4B). Ubiquitin is an abundant, low-molecular-mass protein that is crucial to the selective targeting of denatured proteins for proteolytic degradation (for a review, see Ciechanover, 1998). A thermally denatured protein that is not 'rescued' through chaperone-mediated stabilization and refolding is targeted for degradation by conjugation with a ubiquitin tag [see (Wickner et al., 1999)]. Therefore, the content of ubiquitin-conjugated proteins is a direct measure of the amount of thermal damage incurred by the cellular protein pool. In the present study, levels of ubiquitin conjugates were four times higher in gill tissue from field-acclimatized mussels than in gill tissue from

the laboratory-acclimated individuals. A similar result was found in seasonal comparisons of *M. trossulus* experiencing temperature variation in the field: levels of ubiquitin conjugates were higher in summer than in winter (Hofmann and Somero, 1995). It is apparent, therefore, that the process of warm-acclimatization in these organisms does not involve an increase in the inherent thermostability of the cellular protein pool (which would necessitate upregulation of a large number of more thermostable protein isoforms) and that significant temperature-related protein damage is occurring at elevated, but routinely experienced, environmental temperatures.

It is possible, however, that the gradual accumulation of Hsp70 during warm-acclimatization, such as that observed here and in *Mytilus* elsewhere (Hofmann and Somero, 1995; Roberts et al., 1997), might act as a buffer against subsequent heat stress, conferring increased thermotolerance. It is
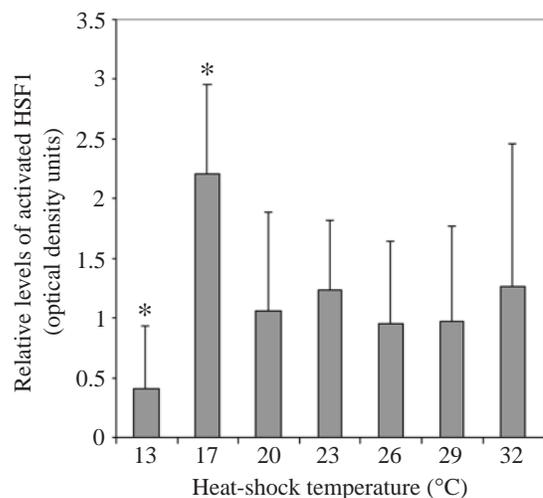
Fig. 6. Relative levels of activated HSF1 in *Mytilus californianus*. Densities of HSF1–HSE complex bands were calculated with Quantity One software and standardized to an internal standard (a single gill extract sample run on every gel). Values are means + S.D., *N*=5. *Values that are significantly different (*P*<0.005).
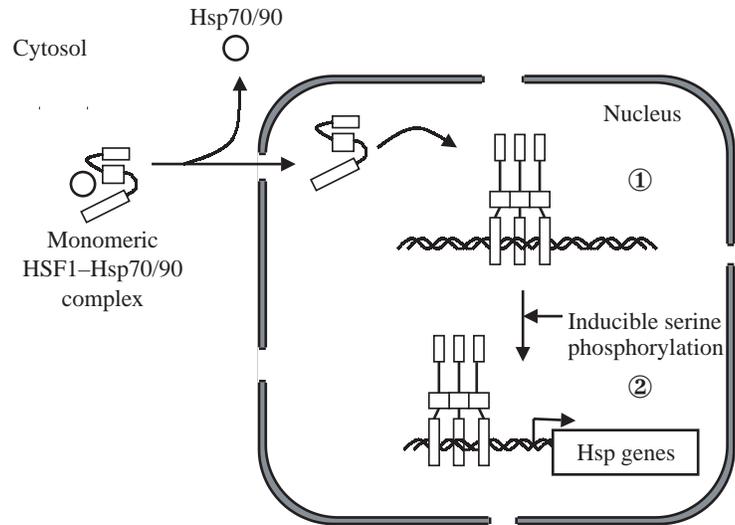
Fig. 7. Model of the regulation of HSF1 (after Morimoto, 1993). At non-stressful temperatures, HSF1 is present in the cytosol as an inactive, monomeric protein. Following heat shock, HSF1 localizes in the nucleus, trimerizes and acquires DNA-binding ability (step 1). Following inducible phosphorylation of serine residues, HSF1 transactivates Hsp genes (step 2). In *Mytilus*, it appears that the temperatures at which steps 1 and 2 occur are uncoupled, and that the temperature at which HSF1 binds the Hsp promoter is lower than that at which gene product appears in the cell.

important to note that it was levels of the stress-inducible isoform of Hsp70 that increased in intracellular concentration and not of the constitutive isoform (Fig. 4A), which is involved in non-stress-related chaperoning functions [see (Fink, 1999)]. This means that the build-up in standing stocks of intracellular Hsp70 is probably due to repeated induction events during acclimatization to gradually warming environmental temperatures rather than to a thermally triggered increase in overall protein synthesis rates (which could result in a similar increase in levels of the constitutive isoform of Hsp70).

The increased levels of endogenous Hsp70 could affect the threshold induction temperature in at least two ways. First, increased levels of free Hsp70 in the cell might be sufficient to handle mild temperature stress, and therefore the induction of Hsp genes and *de novo* synthesis of Hsps would not be necessary until a higher threshold temperature had been experienced. The second possibility is that rising endogenous Hsp70 levels might result in an upward shift in threshold induction temperature through a classic negative feedback loop, a hypothesized model for the regulation of Hsp genes (Craig and Gross, 1991; Morimoto, 1993; Wu, 1995) (Fig. 7). In this model, the activity of the heat-shock transcription factor (HSF1) responsible for stress-induced upregulation of all families of Hsp genes is controlled post-translationally through the complexing of inactive HSF1 monomers with molecular chaperones, particularly Hsp70 and Hsp90 (Shi et al., 1998; Zou et al., 1998). During heat stress, Hsp70 and Hsp90 would be recruited to chaperone the denaturing protein pool, releasing HSF1 and enabling this molecule to trimerize, localize in the nucleus and upregulate Hsp genes. In warm-acclimatized *M. trossulus*, it is possible that increased levels of Hsp70 (and potentially Hsp90, which was not measured here) maintain HSF1 in an inactive state for longer, and only when temperatures reach the new higher threshold temperature is HSF1 released in order to transactivate Hsp genes.

This hypothesis is contradicted, however, by our findings that HSF1 acquired DNA-binding ability in *M. californianus*

at a temperature 9 °C lower than that at which the gene product appears in the cell (Fig. 5A). That is, it appears that HSF1 is released by Hsp70 in response to relatively small increases in temperature, binds the HSE, as shown by positive EMSAs, and is then presumably kept inactive on the promoter until a higher temperature is reached. It should also be noted that, in *M. trossulus*, endogenous levels of HSF1 did not increase with warm-acclimatization and were similar to those in cold-acclimated animals (Fig. 4A), implying that a quantitative change in levels of HSF1 is not necessary to fine-tune the heat-shock response during acclimatization [as has been suggested by (Zatsepina et al., 2000)]. Rather, the controlling steps that underlie the adjustment in gene transactivation temperature are probably those that occur after HSF1 has bound the promoter. This might involve either protein–protein interactions with other transcriptional effector molecules, such as the heat-shock factor binding protein (HSBP1) (Satyal et al., 1998), and/or regulation *via* the MAPK signaling cascade, which is responsible for the phosphorylation of specific serine residues on HSF1 that precedes Hsp gene transactivation (Sarge et al., 1993; Xia and Voellmy, 1997). Alternatively, the controlling step could be translational, involving mRNA stability, translation rate or fidelity. The cellular 'thermostat' is likely to involve all these levels of regulation, and we suggest that integrative studies, such as the present one, that measure multiple aspects of the heat-shock response are required to elucidate not only the ecological importance of this response but also the transduction of environmental temperature signals to the machinery of gene expression.

References

Airaksinen, S., Rabergh, C. M. I., Sistonen, L. and Nikinmaa, M. (1998). Effects of heat shock and hypoxia on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) cells. *J. Exp. Biol.* **201**, 2543–2551.

Ciechanover, A. (1998). The ubiquitin–proteasome pathway: on protein death and cell life. *EMBO J.* **17**, 7151–7160.

Craig, E. A. and Gross, C. A. (1991). Is hsp70 the cellular thermometer? *Trends Biochem. Sci.* **16**, 135–140.

Dietz, T. J. (1994). Acclimation of the threshold induction temperatures for 70 kDa and 90 kDa heat shock proteins in the fish *Gillichthys mirabilis*. *J. Exp. Biol.* **188**, 333–338.

Dietz, T. J. and Somero, G. N. (1992). The threshold induction temperature of the 90 kDa heat shock protein is subject to acclimatization in the eurythermal goby fishes (genus *Gillichthys*). *Proc. Natl. Acad. Sci. USA* **89**, 3389–3393.

Fader, S. C., Yu, Z. and Spotila, J. R. (1994). Seasonal variation in heat shock proteins (hsp70) in stream fish under natural conditions. *J. Therm. Biol.* **19**, 335–341.

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

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

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

Fink, A. L. (1999). Chaperone-mediated protein folding. *Physiol. Rev.* **79**, 425–449.

Gehring, W. J. and Werner, R. (1995). Heat shock protein synthesis and thermotolerance in *Cataglyphis*, an ant from the Sahara desert. *Proc. Natl. Acad. Sci. USA* **92**, 2994–2998.

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

Haslbeck, M., Walke, S., Stromer, T., Ehrnsperger, M., White, H. E., Chen, S., Saibil, H. R. and Buchner, J. (1999). Hsp26: a temperature-regulated chaperone. *EMBO J.* **18**, 6744–6751.

Helmuth, B. (1999). Thermal biology of rocky intertidal mussels: quantifying body temperatures using climatological data. *Ecology* **80**, 15–34.

Hofmann, G. E. and Somero, G. N. (1995). Evidence for protein damage at environmental temperatures: seasonal changes in levels of ubiquitin conjugates and hsp70 in the intertidal mussel *Mytilus trossulus*. *J. Exp. Biol.* **198**, 1509–1518.

Hofmann, G. E. and Somero, G. N. (1996). Interspecific variation in thermal denaturation of proteins in the congeneric mussels *Mytilus trossulus* and *M. galloprovincialis*: evidence from the heat-shock response and protein ubiquitination. *Mar. Biol.* **126**, 65–75.

Huey, R. B. and Bennett, A. F. (1990). Physiological adjustments to fluctuating thermal environments: an ecological and evolutionary perspective. In *Stress Proteins in Biology and Medicine* (ed. R. I. Morimoto, A. Tissieres and C. Georgopoulos), pp. 37–59. Cold Spring Harbor, NY: Cold Spring Harbor Laboratories.

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

Lindquist, S. (1986). The heat-shock response. *Annu. Rev. Biochem.* **55**, 1151–1191.

Mogk, A., Tomoyasu, T., Goloubinoff, P., Ruediger, S., Roder, D., Langen, H. and Bukau, B. (1999). Identification of thermolabile *Escherichia coli* proteins: Prevention and reversion of aggregation by DnaK and ClpB. *EMBO J.* **18**, 6934–6949.

Morimoto, R. I. (1993). Cells in stress: Transcriptional activation of heat shock genes. *Science* **259**, 1409–1410.

Parsell, D. A. and Lindquist, S. (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* **27**, 437–439.

Pigliucci, M. (1996). How organisms respond to environmental changes: From phenotypes to molecules (and *vice versa*). *Trends Ecol. Evol.* **11**, 168–173.

Roberts, D. A., Hofmann, G. E. and Somero, G. N. (1997). Heat-shock protein expression in *Mytilus californianus*: acclimatization (seasonal and tidal-height comparisons) and acclimation effects. *Biol. Bull.* **192**, 309–320.

Sarge, K. D., Murphy, S. P. and Morimoto, R. I. (1993). Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity and nuclear localization and can occur in the absence of stress. *Mol. Cell. Biol.* **13**, 1392–1407.

Satyal, S. H., Chen, D. Y., Fox, S. G., Kramer, J. M. and Morimoto, R. I. (1998). Negative regulation of the heat shock transcriptional response by HSBP1. *Genes Dev.* **12**, 1962–1974.

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

Tomanek, L. and Somero, G. N. (1999). Evolutionary and acclimation-induced variation in the heat-shock responses of congeneric marine snails (genus *Tegula*) from different thermal habitats: implications for limits of thermotolerance and biogeography. *J. Exp. Biol.* **202**, 2925–2936.

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

Wu, C. (1995). Heat shock transcription factors: Structure and regulation. *Annu. Rev. Cell. Dev. Biol.* **11**, 441–469.

Xia, W. and Voellmy, R. (1997). Hyperphosphorylation of heat shock transcription factor 1 is correlated with transcriptional competence and slow dissociation of active factor trimers. *J. Biol. Chem.* **272**, 4094–4102.

Xiao, H. and Lis, J. T. (1988). Germline transformation used to define key features of heat-shock response elements. *Science* **239**, 1139–1142.

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

Zatsepina, O. G., Velikodvorskaia, V. V., Molodtsov, V. B., Garbuz, D., Lerman, D. N., Bettencourt, B. R., Feder, M. E. and Evgenev, M. B. (2001). A *Drosophila melanogaster* strain from sub-equatorial Africa has exceptional thermotolerance but decreased Hsp70 expression. *J. Exp. Biol.* **204**, 1869–1881.

Zou, J., Guo, Y., Guettouche, T., Smith, D. F. and Voellmy, R. (1998). Repression of heat shock transcription factor HSF1 activation by HSP90 (HSP90 complex) that forms a stress-sensitive complex with HSF1. *Cell* **94**, 471–480.