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Interspecific- and acclimation-induced variation in levels of heat-shock proteins 70 (hsp70) and 90 (hsp90) and heat-shock transcription factor-1 (HSF1) in congeneric marine snails (genus *Tegula*): implications for regulation of *hsp* gene expression

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Summary

In our previous studies of heat-shock protein (hsp) expression in congeneric marine gastropods of the genus *Tegula*, we observed interspecific and acclimation-induced variation in the temperatures at which heat-shock gene expression is induced (T_{on}). To investigate the factors responsible for these inter- and intraspecific differences in T_{on}, we tested the predictions of the 'cellular thermometer' model for the transcriptional regulation of hsp expression. According to this model, hsps not active in chaperoning unfolded proteins bind to a transcription factor, heat-shock factor-1 (HSF1), thereby reducing the levels of free HSF1 that are available to bind to the heat-shock element, a regulatory element upstream of *hsp* genes. Under stress, hsps bind to denatured proteins, releasing HSF1, which can now activate *hsp* gene transcription. Thus, elevated levels of heat-shock proteins of the 40, 70 and 90 kDa families (hsp 40, hsp70 and hsp90, respectively) would be predicted to elevate T_{on}. Conversely, elevated levels of HSF1 would be predicted to decrease T_{on}. Following laboratory acclimation to 13, 18 and 23 °C, we used solid-phase immunochemistry (western analysis) to quantify endogenous levels of two hsp70 isoforms (hsp74 and hsp72), hsp90 and HSF1 in the low- to mid-intertidal species *Tegula funebralis* and in two subtidal to low-intertidal congeners, *T. brunnea* and *T. montereyi*. We found higher endogenous levels of hsp72 (a strongly heat-induced isoform) at 13 and 18 °C in *T. funebralis* in comparison with *T. brunnea* and *T. montereyi*. However, *T. funebralis* also had higher levels of HSF1 than its congeners. The higher levels of HSF1 in *T. funebralis* cannot, within the framework of the cellular thermometer model, account for the higher T_{on} observed for this species, although they may explain why *T. funebralis* is able to induce the heat-shock response more rapidly than *T. brunnea*. However, the cellular thermometer model does appear to explain the cause of the increases in T_{on} that occurred during warm acclimation of the two subtidal species, in which warm acclimation was accompanied by increased levels of hsp72, hsp74 and hsp90, whereas levels of HSF1 remained stable. *T. funebralis*, which experiences greater heat stress than its subtidal congeners, consistently had higher ratios of hsp72 to hsp74 than its congeners, although the sum of levels of the two isoforms was similar for all three species except at the highest acclimation temperature (23 °C). The ratio of hsp72 to hsp74 may provide a more accurate estimate of environmental heat stress than the total concentrations of both hsp70 isoforms.

Key words: acclimation, heat-shock protein, HSF1, hsp70, hsp90, intertidal zone, *Tegula* spp., thermotolerance.

Introduction

In the face of thermal, chemical or physiological stresses that cause unfolding of proteins, cells preferentially express heat-shock (stress) proteins (hsps), a process called the heat-shock (or stress) response. As molecular chaperones, hsps protect proteins from denaturation and facilitate either their refolding into native conformations or, in the case of irreversibly damaged proteins, their removal from the cell through proteolysis (Bukau and Horwich, 1998; Frydman, 2001; Hartl, 1996; Morimoto, 1998; Parsell and Lindquist, 1993, 1994; Sherman and Goldberg, 1996). The heat-shock response is regulated in part by a transcription factor, heat-shock factor-1 (HSF1), which specifically stimulates the transcription of hsp-encoding genes (Fig. 1) (Voellmy, 1996; Wu, 1995).

Regulation of HSF1 activity is complex and is determined in part by intrinsic properties of the molecule, notably by heat-induced changes in its conformation (Farkas et al., 1998; Goodson and Sarge, 1995; Larson et al., 1995; Zhong et al., 1998), which enable inactive monomers to assemble into trimers that can bind to the heat-shock element (HSE), a gene

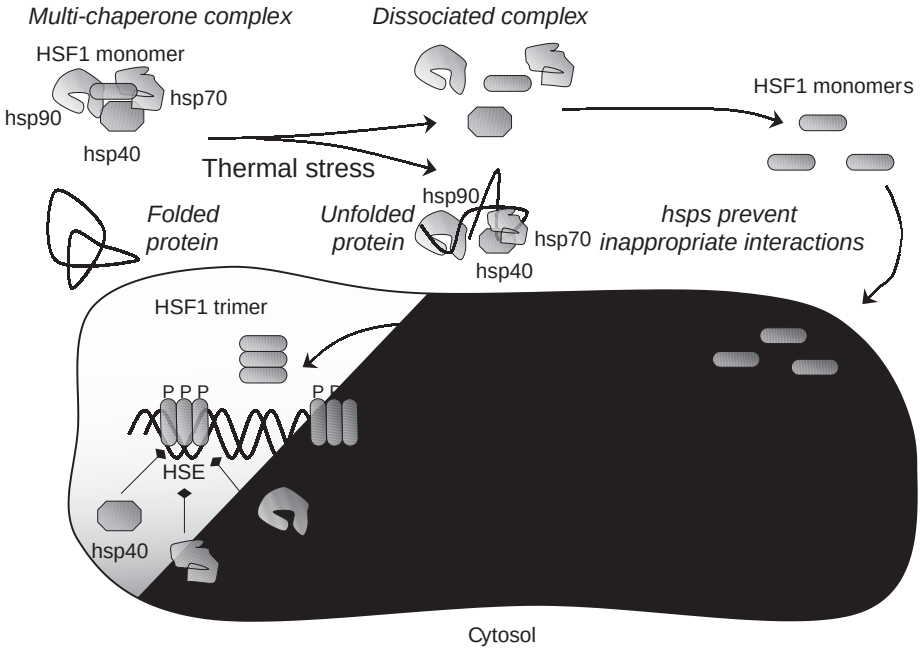


Fig. 1. The regulatory model of transcriptional activation leading to the *de novo* synthesis of heat-shock proteins (hsps). Under non-stressful conditions, heat-shock factor-1 (HSF1) monomers are associated with a chaperone complex that consists at least of hsp70, hsp90 and hsp40 (for references, see text). During thermal stress, the chaperones dissociate from the complex and bind to unfolded proteins. Dissociation of the complex thus frees HSF1 monomers, which are then able to move into the nucleus and bind to the heat-shock element (HSE). HSF1 trimers bound to the HSE become hyper-phosphorylated (P) before they are transcriptionally competent. As hsp levels increase, their binding to HSF1 triggers its dissociation from the HSE, leading to a decrease in *hsp* gene transcription (diamond-shaped ends indicate this inhibitory effect).

regulatory element found upstream of most hsp-encoding genes (Bienz and Pelham, 1987; Fernandes et al., 1994). These studies suggested that HSF1 itself 'senses' thermal stress and may be an important type of 'cellular thermometer'. However, several studies have shown that the temperature at which HSF1 is activated is not fixed (Abravaya et al., 1991; Clos et al., 1993), which implies that extrinsic factors also play important roles in regulating the activity of HSF1. Among these factors are hsps of the 40, 70 and 90 kDa size classes (hsp40, hsp70 and hsp90, respectively), which interact directly with HSF1 in a multi-protein complex under *in vivo* conditions (Fig. 1) (Abravaya et al., 1992; Ali et al., 1998; Baler et al., 1992, 1996; Bharadwaj et al., 1999; Duina et al., 1998; Marchler and Wu, 2001; Mosser et al., 1993; Rabindran et al., 1994; Shi et al., 1998; Zou et al., 1998).

It has been proposed that these hsps keep HSF1 'locked' in the multi-protein complex under non-stressful conditions and thereby prevent it from binding to the heat-shock element and stimulating transcription of *hsp* genes. Upon environmental or physiological stresses that cause proteins to unfold, this complex dissociates because hsps begin to bind preferentially to unfolded proteins rather than to HSF1 (Parsell and Lindquist, 1993, 1994). Free HSF1 monomers are then able to move to the nucleus (Mercier et al., 1999; Westwood et al., 1991), trimerize and bind to the HSE. HSF1 trimers that are transcriptionally competent when bound to the HSE are hyperphosphorylated (Holmberg et al., 2001) (and see references therein). To render HSF1 transcriptionally active, an additional unknown structural transition is required (Shi et al., 1998; Zuo et al., 1995). This cascade of events is, in general, reversed during the attenuation of the heat-shock response, but additional proteins play a regulatory role (Satyal et al., 1998).

In this regulatory scheme, which is termed the 'cellular thermometer' model of hsp expression, hsps play an autoregulatory role in governing their own synthesis by determining the levels of free HSF1 that exist in the cell (Craig and Gross, 1991; DiDomenico et al., 1982; Lindquist, 1993; Morimoto, 1998). Thus, under conditions at which endogenous levels of hsps increase, for example, in response to acclimation to increasing temperature, the formation of the HSF1–hsp70–hsp90–hsp40 complex is favored, leading to a higher T_{on} (the temperature at which heat-shock gene expression is induced) for synthesis of hsps. Although HSF1 transcription is not induced by heat stress and HSF1's activity is regulated post-translationally (Rabindran et al., 1991; Sarge et al., 1993), the cellular thermometer model predicts that elevated concentrations of HSF1 would, other things such as hsp70 and hsp90 levels being equal, favor lower T_{on} values for *hsp* gene transcription because of the presence of large amounts of free HSF1 for binding to the HSE.

The experiments described below were conducted to test these and other predictions of the cellular thermometer model in the context of the observed interspecific variation in T_{on} and in the kinetics of induction of hsps in gastropods of the genus *Tegula* (Tomanek and Somero, 1999, 2000). Congeners of the turban snail *Tegula* from temperate regions inhabit widely differing thermal environments, including cool subtidal habitats (e.g. *T. brunnea* and *T. montereyi*) and warmer and thermally variable intertidal habitats (e.g. *T. funebralis*). These three congeners have been shown to differ in (i) heat tolerance, (ii) T_{on} for the synthesis of hsp38, hsp70 and hsp90, (iii) temperatures at which synthesis of hsps is maximal (T_{peak}), (iv) upper thermal limits for hsp synthesis (T_{off}), (v) rates of hsp induction and (vi) the duration of hsp synthesis following heat shock (Tomanek and Somero, 1999, 2000). Thus, these closely related species (Hellberg, 1998) provide an excellent study system for elucidating interspecific variation in the regulatory mechanisms that govern the expression of hsps. In addition,

acclimatory changes in the heat-shock responses of these species occur: T_{on} and T_{peak} shift upwards with warmer acclimation temperatures (Tomanek and Somero, 1999). Thus, the applicability of the cellular thermometer model can be tested in the context of acclimation-induced variation within a species as well as in the analysis of interspecific variation.

To test the predictions of the cellular thermometer model, we applied solid-phase immunochemical methods (western analysis) to quantify endogenous levels of hsp70, hsp90 and HSF1 in field-acclimatized and laboratory-acclimated (13, 18 and 23 °C) *Tegula*. Because *T. funebralis* encounters greater heat stress in its habitat and grows significantly more slowly than *T. brunnea* and *T. montereyi* (Frank, 1965; Paine, 1969), we also analyzed the relative levels of hsp70 isoforms that appear to be associated either with normal protein synthesis or with repair of thermal damage to proteins. Our results indicate that the cellular thermometer model can account for intraspecific but not interspecific variation in T_{on} and that the relative levels of expression of different isoforms of hsp70 may provide a better measure of heat stress than the total expression of all hsp70 isoforms.

Materials and methods

Organisms, distribution patterns and collection sites

The three *Tegula* congeners used in this study differ in their biogeographic patterns and vertical distributions as follows: *Tegula brunnea* and *T. montereyi* inhabit the subtidal to low-intertidal zones of the eastern Pacific Ocean from Cape Arago, Oregon, USA (43°25′N), to the Channel Islands, California, USA (34°0′N), and from Sonoma County, California (38°17′N), to the Channel Islands, respectively (Abbott and Haderlie, 1980; Riedman et al., 1981; Watanabe, 1984). *Tegula funebralis* is found in the low- to mid-intertidal zone and has a wider latitudinal distribution range, from Vancouver Island, British Columbia, Canada (48°25′N), to central Baja California, Mexico (28°0′N) (Abbott and Haderlie, 1980; Riedman et al., 1981).

Specimens of all species were collected at Hopkins Marine Station of Stanford University in Pacific Grove, California, USA (36°36′N, 121°54′W). Large adults were used exclusively in all experiments, and the sizes of specimens were similar among all three species.

Thermal acclimation and experimental design

Specimens of all species were collected for acclimation in mid-July 1997 and either dissected immediately (field-acclimatized control group) or kept in temperature-controlled (13, 18 or 23 °C) circulating seawater aquaria for 30–34 days. Acclimation temperatures were chosen to be within the range of sea surface temperatures found within the latitudinal distribution range of the species, except for 23 °C, which is above the commonly experienced sea surface temperatures of *T. brunnea* and *T. montereyi*.

Specimens were kept constantly immersed and fed regularly with freshly collected giant kelp (*Macrocystis pyrifera*).

Tissue preparation

Gill tissue was dissected under conditions that do not induce heat shock (13 °C) and immediately placed in 200 µl (*T. funebralis*, 15.0–25.0 mg wet mass) or 300 µl (*T. brunnea* and *T. montereyi*, 30.0–45.0 mg wet mass) of homogenization buffer [32 mmol l^{-1} Tris-HCl, pH 7.5 at 4 °C, 2 % (w/v) SDS, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} Pefabloc (Boehringer Mannheim), 10 µg ml^{-1} pepstatin and 10 µg ml^{-1} leupeptin]. Samples were stored at –70 °C. To prepare homogenates for immunoblotting, the frozen samples were thawed in a dry bath for 5 min at 100 °C and then homogenized with a silicone pestle. Homogenates were incubated at 100 °C for 5 min, homogenized a second time, and then centrifuged at 15 800 ***g*** for 15 min. The supernatant was removed and stored at –70 °C. Protein concentrations were determined using the Micro-BCA assay (Pierce) according to the manufacturer's instructions.

Gel electrophoresis and immunodetection protocol

Proteins were separated electrophoretically according to size on a mini-gel apparatus (BioRad) for 45–50 min at 200 V (5 µg of protein per lane for hsp70 and 25 µg of protein per lane for hsp90 and HSF1). Subsequently, proteins were transferred for either 75 min (hsp70 and hsp90) or 90 min (HSF1) at 80 V (BioRad Protean apparatus) onto nitrocellulose membranes (Nitrobind, Schleicher and Schuell) soaked for at least 2 h in transfer buffer [25 mmol l^{-1} Tris-base, 0.193 mol l^{-1} glycine, 20 % (v/v) methanol, pH 8.3 at 20 °C]. Membranes were dried overnight on tissue paper.

Hsp70/hsp90 western protocol

Nitrocellulose membranes were blocked with blocking buffer [25 mmol l^{-1} Tris-Cl, pH 7.5 at 20 °C, 150 mmol l^{-1} NaCl, 0.1 % (v/v) Tween, 0.02 % (w/v) Thimerosol, 5 % (w/v) non-fat dried milk] for 1 h, subsequently washed twice for 5 min with Tris-buffered saline (TBS; 25 mmol l^{-1} Tris-Cl, pH 7.5 at 20 °C, 150 mmol l^{-1} NaCl) and then incubated with a solution of a monoclonal rat antibody (IgG) against hsp70 (clone 7.10; Affinity BioReagent, MA3-001; 1:2500 dilution of hsp70 antibody in buffer A) [BA: TBS, 2.5 % (w/v) bovine serum albumin, 0.02 % (w/v) Thimerosol] for 1 h or, in the case of hsp90, with a solution of a monoclonal rat antibody (IgG_{2a}) against hsp90 (StressGen, SPA-835; 1:500 dilution of hsp90 antibody in BA) for 90 min. This was followed by a 5 min wash with TBS, two 5 min washes with TBS containing 0.1 % (v/v) Tween and a final 5 min wash with TBS. Subsequently, the membrane was incubated for 30 min with a rabbit anti-rat bridging antibody (IgG) solution (1:2000 dilution in BA; Vector, AI-4000) followed by four 5 min washes with TBS, TBS containing 0.1 % Tween (twice) and TBS again. Finally, we incubated membranes with a horseradish-peroxidase Protein A solution (1:5000 dilution in BA; BioRad) for 30 min. The incubation was followed by a 5 min wash with TBS, three 10 min washes with TBS containing 0.1 % Tween and a final 5 min wash with TBS. Membranes were overlaid with a solution of enhanced

chemiluminescent (ECL) reagent (Amersham) according to the manufacturer's instructions for 1 min. Under dark-room conditions, we exposed membranes onto pre-flashed Hyperfilm (Amersham) for 5, 10 (hsp70) and 45 s (hsp90 and HSF1) 10, 30 and 50 min after ECL treatment to obtain various exposures within the linear range of detection. All samples were run at least twice. Results for hsp70 were also confirmed with an iodinated tertiary antibody (^{125}I-protein A, data not shown).

HSF1 western protocol

Nitrocellulose membranes were blocked with blocking buffer (see above) for 1 h and subsequently incubated with a polyclonal rabbit HSF1 antibody prepared against recombinant human HSF1 (Affinity BioReagents, PA3-017; 1:500 dilution in BA) for 1 h. Membranes were then washed for 3 min with TBS, TBS containing 0.1 % Tween and TBS and subsequently incubated with a goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (StressGen, SAB-300) for 30 min. After repeating the three washing steps, we detected bands by treating the blot with ECL according to the manufacturer's instructions. Results obtained with this human HSF1 antibody were confirmed using another antibody also prepared against human HSF1 in rabbit (StressGen, SPA-901).

Image analysis, quantification of expression of heat-shock proteins and statistical analyses

Film images were scanned on a densitometer (Sharp JX-330), and the digitized images were analyzed with image-analysis software (ImageMaster 1D, Version 2.01, Pharmacia) to quantify the band intensities of two hsp70 isoforms, one with a molecular mass of approximately 72 kDa (hsp72), the other of approximately 74 kDa (hsp74), hsp90 and HSF1. We express band intensities relative to a known amount of a bovine heat-shock cognate 70 (80 ng; StressGen, SPP-750), a bovine hsp90 (120 ng; StressGen, SPP 780) and a human HSF1 standard of 80 kDa (20 ng; StressGen, SPA-900WB) to account for variation among western blots (Fig. 2).

Differences in endogenous levels of hsp70 isoforms (see Figs 3, 4), their levels relative to each other (see Fig. 5) and their total sum (see Fig. 6), hsp90 (see Fig. 7) and HSF1 (see Fig. 8) were tested among species within treatments (four comparisons of *T. funebralis* relative to *T. brunnea* and *T. montereyi*) and among treatments within species (three comparisons of the 23 °C treatment relative to all other treatments). The resulting seven comparisons were of particular interest to us because of the differences in induction temperatures observed previously (Tomanek and Somero, 1999). We used the least significant difference (LSD)-test for a *post-hoc* pairwise comparison (SYSTAT software, Systat, Inc.) after a two-way analysis of variance (ANOVA). For the two-way ANOVA, species and treatment were used as independent categorical variables, and endogenous levels of hsps and HSF1 were used as the dependent variable. LSD was used because we were specifically interested in seven planned comparisons. We accounted for the fact that these comparisons are not independent by multiplying the *P*-value we obtained by the number of comparisons made (in this case seven). Differences are described as statistically significant when the resulting *P*-level was $\leqslant 0.05$.

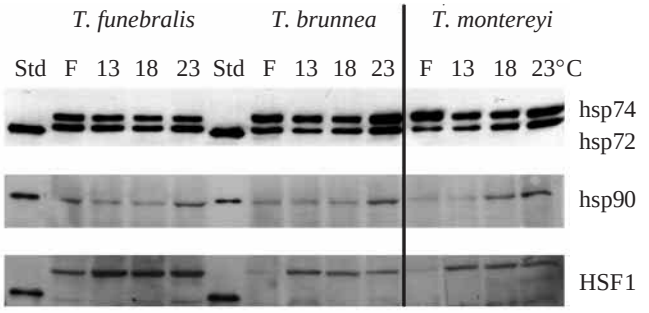


Fig. 2. Western blots of hsp70 isoforms, hsp90 and heat-shock factor-1 (HSF1) in a field-acclimatized (F) group of three *Tegula* congeners and after 30–34 days of laboratory-acclimation to 13, 18 or 23 °C. Lanes contain equal amounts of protein (5 µg for hsp70 blots and 25 µg for hsp90 and HSF1 blots). A known amount of an hsp70, hsp90 or HSF1 standard (Std; see text) was loaded on each gel.

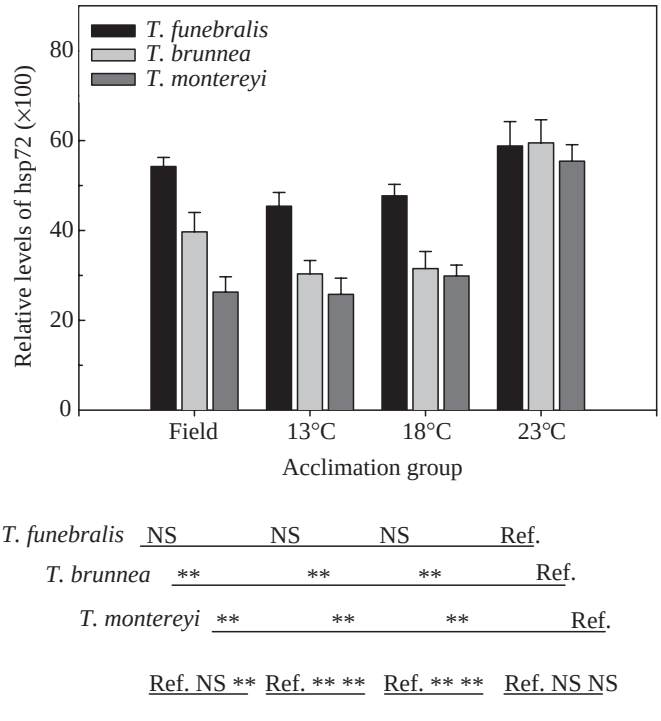


Fig. 3. Endogenous levels of hsp72 in three *Tegula* congeners field-acclimatized (July 1997) and laboratory-acclimated to 13, 18 or 23 °C for 30–34 days. Long lines indicate pairwise comparisons within species among treatments; short lines indicate comparisons within treatments among species. Ref. refers to the group with which all other groups along a line are compared. ***P*<0.05; NS, not significant. Values are means +1 S.E.M. (*N*=5 for all data points except for *T. brunnea* at 18 °C, for which *N*=4).

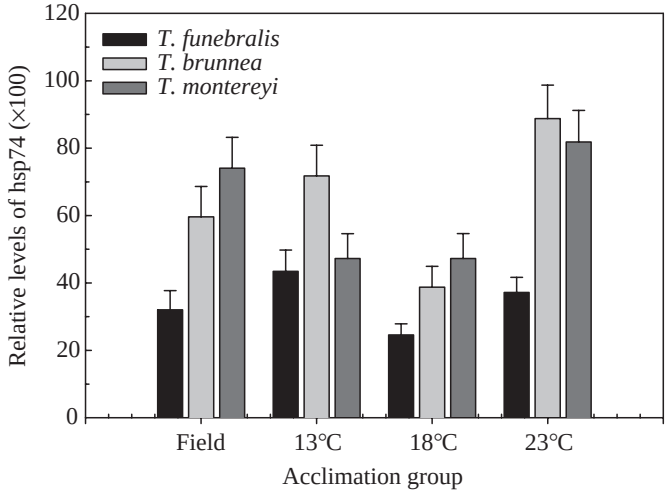
Results

Endogenous levels of hsp70 isoforms and hsp90

The hsp70 antibody we used for western analysis detected one low-molecular-mass (hsp72) and one high-molecular-mass (hsp74) isoform (Fig. 2). Endogenous levels of hsp72 were higher in *T. funebralis* than in *T. brunnea* and *T. montereyi* in field-acclimatized specimens and following acclimation to 13 and 18 °C (Fig. 3). No interspecific differences in levels of hsp72 were found at 23 °C. In *T. funebralis*, no significant differences in levels of hsp72 were observed among the four treatments. In contrast, hsp72 levels increased with increasing acclimation temperature (23 °C in comparison with 18 and 13 °C) in both subtidal *Tegula* congeners.

Endogenous levels of hsp74 were invariably higher in *T. brunnea* and *T. montereyi* than in *T. funebralis*, although these differences were not always significant (Fig. 4). Levels of hsp74 increased following acclimation to 23 °C, in comparison with 18 °C, in both subtidal congeners but, as for hsp72, *T. funebralis* showed no significant differences among the four treatments.

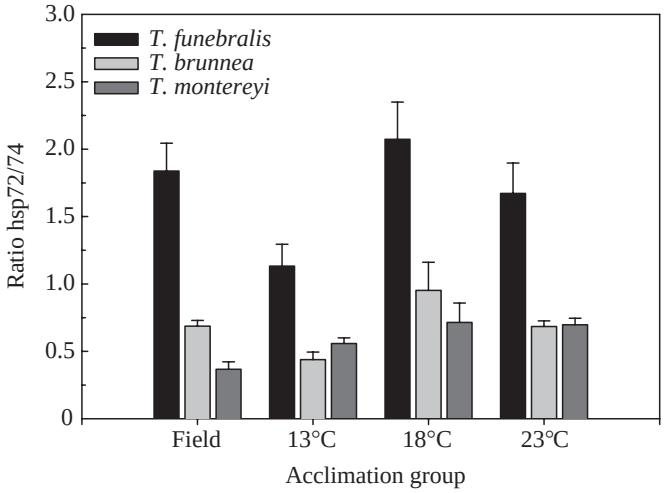
To determine whether the three congeners differed in the relative levels of the weakly (hsp74) and strongly (hsp72) heat-induced hsp70 isoforms, we calculated the ratio of hsp72 to hsp74 levels in the four treatment groups (Fig. 5). *T. funebralis* had, under all four treatments, an approximately twofold higher ratio of hsp72 to hsp74 relative to the two subtidal congeners. Within a species, the ratio of hsp72 to hsp74 did not vary among treatment groups.



T. funebralis NS NS NS Ref.
T. brunnea NS NS ** Ref.
T. montereyi NS ** ** Ref.

Ref. NS ** Ref. NS NS Ref. NS NS Ref. ** **

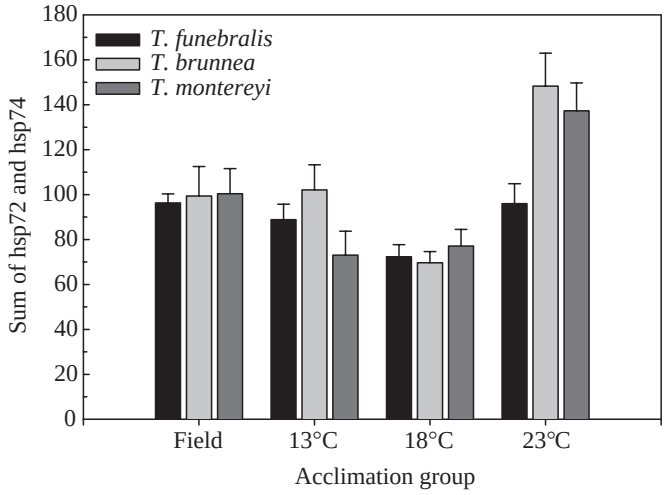
Fig. 4. Endogenous levels of hsp74 in three *Tegula* congeners field-acclimatized (July 1997) and laboratory-acclimated to 13, 18 or 23 °C for 30–34 days. Values are means +1 S.E.M. (*N*=5 for all data points). For further details, see Fig. 3.



T. funebralis NS NS NS Ref.
T. brunnea NS NS NS Ref.
T. montereyi NS NS NS Ref.

Ref. ** ** Ref. ** NS Ref. ** ** Ref. ** **

Fig. 5. Ratios of hsp72 to hsp74 levels in three *Tegula* congeners field-acclimatized (July 1997) and laboratory-acclimated to 13, 18 or 23 °C for 30–34 days. Values are means +1 S.E.M. (*N*=5 for all data points except for *T. brunnea* at 18 °C, for which *N*=4). For further details, see Fig. 3.



T. funebralis NS NS NS Ref.
T. brunnea ** ** ** Ref.
T. montereyi NS ** ** Ref.

Ref. NS NS Ref. NS NS Ref. NS NS Ref. ** **

Fig. 6. Sum of hsp72 and hsp74 levels in three *Tegula* congeners field-acclimatized (July 1997) and laboratory-acclimated to 13, 18 or 23 °C for 30–34 days. Values are means +1 S.E.M. (*N*=5 for all data points except for *T. brunnea* at 18 °C, for which *N*=4). For further details, see Fig. 3.

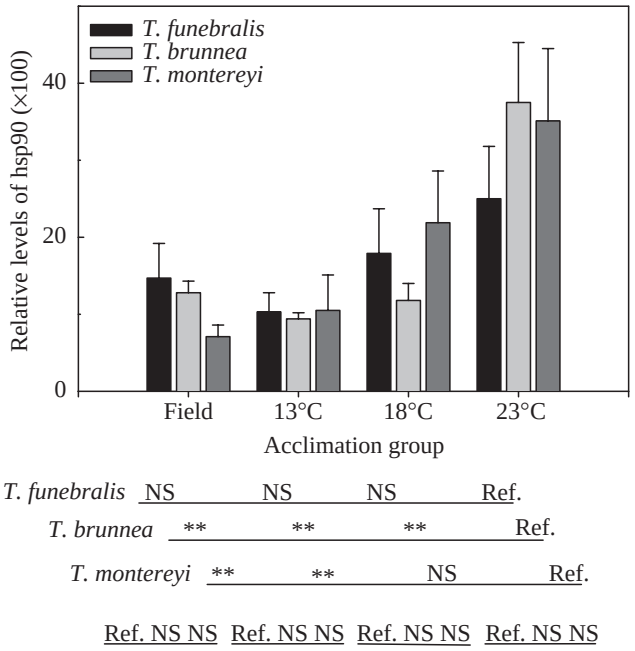


Fig. 7. Endogenous levels of hsp90 in three *Tegula* congeners field-acclimatized (July 1997) and laboratory-acclimated to 13, 18 or 23 °C for 30–34 days. Values are means +1 S.E.M. (*N*=5 for all data points). For further details, see Fig. 3.

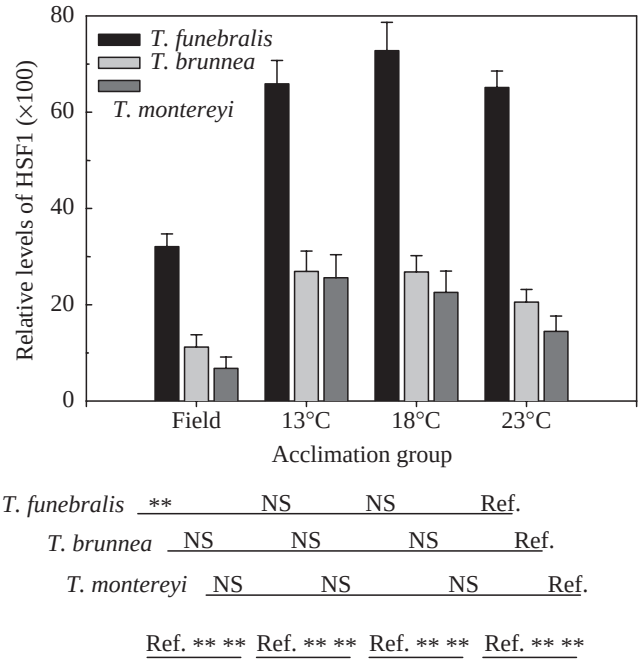


Fig. 8. Endogenous levels of heat-shock transcription factor-1 (HSF1) in three *Tegula* congeners field-acclimatized (July 1997) and laboratory-acclimated to 13, 18 or 23 °C for 30–34 days. Values are means +1 S.E.M. *N*=5 for all data points except for *T. funebralis* under field conditions (*N*=4), at 13 °C (*N*=3) and 18 °C (*N*=4) and for *T. montereyi* at 18 °C (*N*=4) and 23 °C (*N*=4). For further details, see Fig. 3.

Hsp70 isoforms may act together in repressing the induction of hsp synthesis, i.e. the inhibition of HSF1 may be the summed effect of all hsp70 isoforms in the cell. We therefore calculated the total levels of hsp72 plus hsp74 in the experimental groups (Fig. 6). The total levels of the two isoforms were similar in all three congeners for any treatment group. Acclimation had no effect on the total amount of hsp70 isoforms in *T. funebralis*, but acclimation to 23 °C led to significantly higher total hsp70 levels in the two subtidal species.

Endogenous levels of hsp90 did not differ interspecifically under any comparison (P=0.905 for species effect, ANOVA), but generally increased in all species with increasing acclimation temperature, significantly so in the two subtidal species (Figs 2, 7).

Endogenous levels of HSF1

To determine whether the inter- and intraspecific variation in T_{on} observed in our previous study was related to different endogenous levels of HSF1, we used an antibody against human HSF1 to quantify HSF1 levels in differently acclimated congeners of *Tegula* (Figs 2, 8). Note that human HSF1 (80 kDa) has a lower molecular mass than *Tegula* HSF1 (approximately 100 kDa) (Fig. 2). This is not unusual considering how widely the molecular mass of HSF1 varies among species (yeast, 150 kDa; *Drosophila melanogaster*, 110 kDa; human, 80 kDa) (Wu, 1995).

Endogenous levels of HSF1 were significantly higher in *T. funebralis* than in *T. brunnea* and *T. montereyi* in field-acclimatized and laboratory-acclimated specimens (Fig. 8). Within a species, HSF1 levels changed little with increasing acclimation temperature in most cases. However, levels of HSF1 were always lowest in the field-acclimatized group (P<0.05 for all *post-hoc* comparisons between the field-acclimatized and all other acclimation groups, except for 23 °C acclimation in the two subtidal congeners; statistical results not shown). Thus, laboratory acclimation had an effect on endogenous levels of HSF1 even at a temperature (13 °C) that represents a typical sea surface temperature at the collection site.

Discussion

Regulation of interspecific variation in the heat-shock responses of Tegula *congeners*

Endogenous levels of hsps and HSF1 were measured to elucidate the mechanisms underlying the inter- and intraspecific variation in *hsp* gene expression discovered in our earlier studies of hsp synthesis in congeners of *Tegula* (Tomanek and Somero, 1999, 2000). Specifically, we tested predictions based on the cellular thermometer hypothesis, which emphasizes the central regulatory role played by a multi-protein complex comprising HSF1 and hsps in regulating *hsp* gene transcription (see Introduction). Following acclimation to 13 and 18 °C under constant submersion, we found that the heat-tolerant, low- to mid-intertidal species *T. funebralis* induced *de novo* synthesis of hsp70, the major hsp in *Tegula*,

at 27 °C, whereas the heat-sensitive subtidal to low-intertidal *T. brunnea* and *T. montereyi* induced hsp synthesis at 24 °C (Tomanek and Somero, 1999). All three species induced hsp synthesis at 27 °C after acclimation to 23 °C, a temperature close to the upper end of the sea surface temperatures found within the distribution range of the two subtidal congeners (Tomanek and Somero, 1999). Our prediction, based on the cellular thermometer model, was that higher endogenous levels of two repressors of the heat-shock response, hsp70 and hsp90, would be found in *T. funebralis* following acclimation to 13 and 18 °C, but not to 23 °C. This initial hypothesis assumed that the levels of HSF1 would be the same for all three species.

Levels of hsp72, but not of hsp74 or hsp90, agreed with this prediction (Figs 3, 4, 7). Thus, endogenous levels of hsp72 appear to provide a potential basis for the interspecific differences in T_{on} found in specimens acclimated to 13 and 18 °C. However, two other observations call into question the role of hsp70 isoforms in accounting for these interspecific differences in T_{on}. First, consistently higher levels of HSF1 were present in *T. funebralis* than in *T. brunnea* and *T. montereyi* (Fig. 8). Second, total levels of hsp72 plus hsp74 did not differ among species in the specimens acclimated to 13 and 18 °C. According to the cellular thermometer model, the higher levels of HSF1 in *T. funebralis*, *vis à vis* the equal levels of total hsp70 proteins in the three species, would favor a lower rather than a higher T_{on} in this species. Thus, interspecific differences in the T_{on} of hsp synthesis cannot be explained by a cellular thermometer model, which has as its central mechanism the inhibition of HSF1 activity by hsp70 and hsp90.

The higher levels of HSF1 present in *T. funebralis* may, however, account for another difference observed between this species and *T. brunnea*: a more rapid induction of hsp synthesis by *T. funebralis* following severe heat stress (Tomanek and Somero, 2000). Higher endogenous levels of HSF1 could facilitate a faster conversion of inactive HSF1 monomers to active trimers and increased binding of HSF1 to the HSE following heat-shock. When HSF1 was overexpressed in *Escherichia coli*, the increased levels of HSF1 led to constitutive binding of HSF1 trimers to the HSE (Clos et al., 1990; Rabindran et al., 1991; Sarge et al., 1993). Whether warm-adapted species such as *T. funebralis* generally contain higher levels of HSF1 than more cold-adapted species remains to be determined. In only a few cases have comparisons of this type been made. In fact, Zatsepina et al. (2000) reported that two heat-tolerant desert lizard species had lower levels of HSF1 than a distantly related more heat-sensitive species. On the basis of current understanding of the mechanisms that regulate *hsp* gene expression, it appears likely that the total concentration of HSF1 in the cell is an important, but only partial, determinant of T_{on} and the rate of *hsp* gene expression. As suggested by the model in Fig. 1, conformational changes and the phosphorylation state of HSF1 also contribute to regulation of *hsp* genes (Shi et al., 1998; Zuo et al., 1995). In a recent study of *hsp* gene expression in *Mytilus* spp., Buckley et al. (2001) showed that binding of HSF1 to the HSE occurred at temperatures several degrees below T_{on}. This observation suggests that additional steps that occur subsequent to the binding of HSF1 to the HSE may be of importance in transcriptional regulation of *hsp* genes. Nonetheless, higher levels of HSF1 present in *T. funebralis* are likely to poise the hsp70 system towards rapid expression.

Regulation of acclimation-induced intraspecific variation in the heat-shock responses of Tegula *congeners*

Acclimation to a temperature (23 °C) close to the habitat upper thermal extreme for the subtidal species induced a shift in T_{on} in *T. brunnea* and *T. montereyi* (from 24 to 27 °C), but not in *T. funebralis* (27 °C) (Tomanek and Somero, 1999). Corresponding increases in hsp72, hsp74, the sum of both hsp70 isoforms and hsp90 (Figs 3, 4, 6, 7) levels in both subtidal species, but not in *T. funebralis*, following acclimation to 23 °C suggest that the 3 °C increase in T_{on} could be due to elevated levels of HSF1-binding hsps. This conclusion is supported by the observation that HSF1 levels were the same under all acclimation conditions within a species (Fig. 8). Thus, it seems that the cellular thermometer model can explain acclimatory changes in T_{on}. Similar intraspecific changes in induction temperatures and endogenous levels of hsp90 or hsp70 have been observed for summer- and winter-acclimatized gobies of the genus *Gillichthys* (Dietz and Somero, 1992) and for intertidal mussels of the genus *Mytilus* (Buckley et al., 2001; Roberts et al., 1997), respectively.

Heat stress and growth in Tegula *congeners: using hsp70 isoforms as indicators of physiological state*

Multiple isoforms of hsp70 are present in most species, and these isoforms generally differ in the extent to which they are induced by heat stress (Parsell and Lindquist, 1993, 1994). It is common for a constitutively expressed hsp70 cognate (hsc70) to be present in cells under all conditions, where it functions as a chaperone during normal protein synthesis. In contrast, some hsp70 isoforms may be absent or present only at low levels until the cell encounters heat stress. This type of variation in hsp70 expression has been observed in marine mussels of the genus *Mytilus*.

In the bay mussel *Mytilus trossulus*, levels of a high-molecular-mass isoform of hsp70 (hsp76) exhibited little variation with exposure temperature, whereas levels of a small hsp70 (hsp68) varied several-fold as a function of habitat temperature conditions (Hofmann and Somero, 1995). In some specimens from a cold-acclimatized population, only the larger isoform could be detected, indicating that the low-molecular-mass isoform is not constitutively expressed. Similar observations were made in the ribbed mussel *Mytilus californianus*: the low-molecular-mass hsp70 isoform was more strongly induced by high temperatures than the larger isoform (Roberts et al., 1997), and subtidal specimens acclimatized to low temperatures sometimes lacked the smaller isoform altogether (M. Lopuch and G. N. Somero, unpublished observations).

In the three species of *Tegula*, two hsp70 isoforms, hsp72 and hsp74, were detected in all specimens examined, but hsp72 exhibited stronger induction during heat stress (Figs 2–4). As in the case of the congeners of *Mytilus*, the larger of the two isoforms may be a constitutively expressed chaperone that is present at all times to assist in normal protein synthesis. Hsp72 may be a stress-induced isoform whose synthesis is strongly upregulated during heat stress. These differences in heat-inducibility between hsp70 isoforms in *Mytilus* and *Tegula* suggest that attempts to use endogenous levels of hsp70 as an index of environmental thermal stress may require separate analyses to be made of each isoform. Although hsp74 levels may be elevated under extreme heat stress (e.g. at 23 °C in the subtidal species, Fig. 4), its endogenous levels under non-stressful conditions may reflect the capacity of the tissue for normal protein synthesis. In contrast, levels of the strongly heat-inducible low-molecular-mass isoforms may be a valid index of heat stress (Hofmann and Somero, 1995; Roberts et al., 1997) (present study). Total levels of low- and high-molecular-mass hsp70 isoforms may obscure important relationships related to physiological state, including propensity for growth.

The interspecific differences observed in the field-acclimatized snails, as well as in the 13 and 18 °C acclimation groups support the latter possibility (Figs 3–5). In *T. funebralis*, levels of hsp72 are generally higher, and those of hsp74 lower, than in the subtidal species. However, the total amounts of hsp72 plus hsp74 do not differ among species. On the basis of the proposed different roles of the two hsp70 isoforms, it appears that a different balance between normal protein synthetic activity (indexed by levels of hsp74) and stress-induced repair of unfolded proteins (indexed by levels of hsp72) is present in the three species. In *T. funebralis*, which experiences greater heat stress than the two subtidal species (Tomanek and Somero, 1999), there may be a greater expenditure of energy for repair of heat damage to proteins, resulting in a lesser channeling of energy into normal protein synthesis.

This hypothesized interspecific difference in allocation of energy between repair and protein synthesis, based on the ratio of levels of hsp72 to hsp74, is consistent with observed differences among the congeners in growth rates: *T. brunnea* and *T. montereyi* grow approximately 3–6 times faster than *T. funebralis* under field conditions (Frank, 1965; Paine, 1969; Watanabe, 1982). A recent study on the intertidal predatory whelk *Nucella ostrina* also illustrated how heat stress and concomitant higher levels of hsp70 can affect metabolic activity and possibly growth rates (Dahlhoff et al., 2001).

In conclusion, using levels of molecular chaperones to gain insights into the physiological state of specimens requires that the distinct roles of these proteins in normal protein synthesis and in repair of heat-induced damage to proteins be taken into consideration. Total levels of all hsp70 isoforms may fail to provide a valid estimate of either protein synthetic capacity (growth potential) or level of heat stress.

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References

Abbott, D. P. and Haderlie, E. C. (1980). Prosobranchia: Marine snails. In *Intertidal Invertebrates of California* (ed. R. H. Morris, D. P. Abbott and E. C. Haderlie), pp. 230–307. Stanford: Stanford University Press.

Abravaya, K., Myers, M. P., Murphy, S. P. and Morimoto, R. I. (1992). The human heat shock protein hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression. *Genes Dev.* **6**, 1153–1164.

Abravaya, K., Phillips, B. and Morimoto, R. I. (1991). Attenuation of the heat shock response in HeLa cells is mediated by the release of bound heat shock transcription factor and is modulated by changes in growth and in heat shock temperatures. *Genes Dev.* **5**, 2117–2127.

Ali, A., Bharadwaj, S., O'Caroll, R. and Ovsenek, N. (1998). Hsp90 interacts with and regulates the activity of heat shock factor 1 in *Xenopus* oocytes. *Mol. Cell. Biol.* **18**, 4949–4960.

Baler, R., Welch, W. J. and Voellmy, R. (1992). Heat shock gene regulation by nascent polypeptides and denatured proteins: HSP70 as a potential autoregulatory factor. *J. Cell Biol.* **117**, 1151–1159.

Baler, R., Zou, J. and Voellmy, R. (1996). Evidence for a role of Hsp70 in the regulation of the heat shock response in mammalian cells. *Cell Stress Chaperones* **1**, 33–39.

Bharadwaj, S., Ali, A. and Ovsenek, N. (1999). Multiple components of the HSP90 chaperone complex function in regulation of heat shock factor 1 *in vivo*. *Mol. Cell. Biol.* **19**, 8033–8041.

Bienz, M. and Pelham, H. R. B. (1987). Mechanisms of heat-shock gene activation in higher eukaryotes. *Adv. Genet.* **24**, 31–72.

Buckley, B. A., Owen, M.-E. and Hofmann, G. E. (2001). Adjusting the thermostat: the threshold induction temperature for the heat-shock response in intertidal mussels (genus *Mytilus*) changes as a function of thermal history. *J. Exp. Biol.* **204**, 3571–3579.

Bukau, B. and Horwich, A. L. (1998). The hsp70 and hsp60 chaperone machines. *Cell* **92**, 351–366.

Clos, J., Rabindran, S., Wisniewski, J. A. N. and Wu, C. (1993). Induction temperature of human heat shock factor is reprogrammed in a *Drosophila* cell environment. *Nature* **364**, 252–255.

Clos, J., Westwood, J. T., Becker, P. B., Wilson, S., Lambert, K. and Wu, C. (1990). Molecular cloning and expression of a hexameric *Drosophila* heat shock factor subject to negative regulation. *Cell* **63**, 1085–1097.

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

Dahlhoff, E. P., Buckley, B. B. and Menge, B. A. (2001). Physiology of the rocky intertidal predator *Nucella ostrina* along an environmental stress gradient. *Ecology* **82**, 2816–2829.

DiDomenico, B. J., Bugaisky, G. E. and Lindquist, S. (1982). The heat shock response is self-regulated at both the transcriptional and posttranscriptional levels. *Cell* **31**, 593–603.

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

Duina, A. A., Kalton, H. M. and Gaber, R. F. (1998). Requirement for Hsp90 and a CyP-40-type cyclophilin in negative regulation of the heat shock response. *J. Biol. Chem.* **273**, 18974–18978.

Farkas, T., Kutskova, Y. A. and Zimarino, V. (1998). Intramolecular repression of mouse heat shock factor 1. *Mol. Cell. Biol.* **18**, 906–918.

Fernandes, M., O'Brien, T. and Lis, J. T. (1994). Structure and regulation of heat shock gene promoters. In *The Biology of Heat Shock Proteins and Molecular Chaperones* (ed. R. I. Morimoto, A. Tissieres and C. Georgopoulos), pp. 375–393. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Frank, P. W. (1965). Shell growth in a natural population of the turban snail, *Tegula funebralis*. *Growth* **29**, 395–403.

Frydman, J. (2001). Folding of newly translated proteins *in vivo*: The role of molecular chaperones. *Annu. Rev. Biochem.* **70**, 603–649.

Goodson, M. L. and Sarge, K. D. (1995). Heat-inducible DNA binding of purified heat shock transcription factor 1. *J. Biol. Chem.* **270**, 2447–2450.

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

Hellberg, M. E. (1998). Sympatric sea shells along the sea's shore: the geography of speciation in the marine gastropod *Tegula*. *Evolution* **52**, 1311–1324.

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.

Holmberg, C. I., Hietakangas, V., Mikhailov, A., Rantanen, J. O., Kallio, M., Meinander, A., Hellman, J., Morrice, N., MacKintosh, C., Morimoto, R. I., Eriksson, B. E. and Sistonen, L. (2001). Phosphorylation of serine 230 promotes inducible transcriptional activity of heat shock factor 1. *EMBO J.* **14**, 3800–3810.

Larson, J. S., Schuetz, T. J. and Kingston, R. E. (1995). *In vitro* activation of purified human heat shock factor by heat. *Biochemistry* **34**, 1902–1911.

Lindquist, S. (1993). Autoregulation of the heat shock response. In *Translational Regulation of Gene Expression 2* (ed. J. Ilan), pp. 279–320. New York: Plenum Press.

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

Mercier, P. A., Winegarden, N. A. and Westwood, J. T. (1999). Human heat shock factor 1 is predominantly a nuclear protein before and after heat stress. *J. Cell Sci.* **112**, 2765–2774.

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

Mosser, D. D., Duchaine, J. and Massie, B. (1993). The DNA-binding activity of the human heat shock transcription factor is regulated *in vivo* by hsp70. *Mol. Cell. Biol.* **13**, 5427–5438.

Paine, R. T. (1969). The *Pisaster–Tegula* interaction: prey patches, predator food preference and intertidal community structure. *Ecology* **50**, 950–961.

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

Parsell, D. A. and Lindquist, S. (1994). Heat shock proteins and stress tolerance. In *The Biology of Heat Shock Proteins and Molecular Chaperones*, vol. 26 (ed. R. I. Morimoto, A. Tissieres and C. Georgopoulos), pp. 457–494. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Rabindran, S. K., Giorgi, G., Clos, J. and Wu, C. (1991). Molecular cloning and expression of a human heat shock factor, HSF1. *Proc. Natl. Acad. Sci. USA* **88**, 6906–6910.

Rabindran, S. K., Wisniewski, J. A. N., Li, L., Li, G. C. and Wu, C. (1994). Interaction between heat shock factor and hsp70 is insufficient to suppress induction of DNA-binding activity *in vivo*. *Mol. Cell. Biol.* **14**, 6552–6560.

Riedman, M. L., Hines, A. H. and Pearse, J. S. (1981). Spatial segregation of four species of turban snails (genus *Tegula*) in central California. *Veliger* **24**, 97–102.

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., 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.

Sherman, M. Y. S. and Goldberg, A. L. (1996). Involvement of molecular chaperones in intracellular protein breakdown. In *Stress-Inducible Cellular Responses* (ed. U. Feige, R. I. Morimoto, I. Yahara and B. Polla), pp. 57–78. Basel: Birkhäuser Verlag.

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.

Tomanek, L. and Somero, G. N. (2000). Time course and magnitude of synthesis of heat-shock proteins in congeneric marine snails (genus *Tegula*) from different tidal heights. *Physiol. Biochem. Zool.* **73**, 249–256.

Voellmy, R. (1996). Sensing stress and responding to stress. In *Stress-Inducible Cellular Responses* (ed. U. Feige, R. I. Morimoto, I. Yahara and B. Polla), pp. 121–137. Basel: Birkhäuser Verlag.

Watanabe, J. M. (1982). Aspects of community organization in a kelp forest habitat: Factors influencing the bathymetric segregation of three species of herbivorous gastropods. PhD dissertation, University of California, Berkeley.

Watanabe, J. M. (1984). The influence of recruitment, competition and benthic predation on spatial distributions of three species of kelp forest gastropods (Trochidae: *Tegula*). *Ecology* **65**, 920–936.

Westwood, J. T., Clos, J. and Wu, C. (1991). Stress-induced oligomerization and chromosomal relocalization of heat-shock factor. *Nature* **353**, 822–827.

Wu, C. (1995). Heat shock transcription factors: Structure and regulation. *Annu. Rev. Cell Dev. Biol.* **8**, 441–469.

Zatsepina, O. G., Ulmasov, K. A., Beresten, S. F., Molodstov, 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.

Zhong, M. I. N., Orosz, A. and Wu, C. (1998). Direct sensing of heat and oxidation by *Drosophila* heat shock transcription factor. *Mol. Cell* **2**, 101–108.

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.

Zuo, J., Rungger, D. and Voellmy, R. (1995). Multiple layers of regulation of human heat shock transcription factor 1. *Mol. Cell. Biol.* **15**, 4319–4330.