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

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Summary
Heat stress sufficient to cause cellular damage triggers the heat-shock response, the enhanced expression of a group of molecular chaperones called heat-shock proteins (hsp). We compared the heat-shock responses of four species of marine snails of the genus Tegula that occupy thermal niches differing in absolute temperature and range of temperature. We examined the effects of short-term heat stress and thermal acclimation on the synthesis of hsp of size classes 90, 77, 70 and 38 kDa by measuring incorporation of 35S-labeled methionine and cysteine into newly synthesized proteins in gill tissue. Temperatures at which enhanced synthesis of hsp first occurred (T_{on}), temperatures of maximal induction of hsp synthesis (T_{peak}) and temperatures at which hsp synthesis was heat-inactivated (T_{off}) were lowest in two low-intertidal to subtidal species from the temperate zone, T. brunnea and T. montereyi, intermediate in a mid- to low-intertidal species of the temperate zone, T. funebralis, and highest in a subtropical intertidal species from the Gulf of California, T. rugosa. Synthesis of hsp and other classes of protein by T. brunnea and T. montereyi was heat-inactivated at temperatures commonly encountered by T. funebralis during low tides on warm days. In turn, protein synthesis by T. funebralis was blocked at the upper temperatures of the habitat of T. rugosa. Acclimation of snails to 13 °C, 18 °C and 23 °C shifted T_{on} and T_{peak} for certain hps, but did not affect T_{off}. The heat-shock responses of field-acclimatized snails were generally reduced in comparison with those of laboratory-acclimated snails. Overall, despite the occurrence of acclimatory plasticity in their heat-shock responses, genetically fixed differences in hsp, T_{on}, T_{peak} and T_{off} appear to exist that reflect the separate evolutionary histories of these species and may play important roles in setting their thermal tolerance limits and, thereby, their biogeographic distribution patterns.

Key words: acclimation, biogeography, heat-shock protein, intertidal zone, protein synthesis, Tegula spp., thermotolerance.

Introduction
The heat-shock response has received an enormous amount of study, especially since the discovery that heat-shock proteins (hsp), which are also termed stress proteins, are a subset of the large family of molecular chaperones that function in all types of cell to ensure the proper folding and compartmentation of proteins (Feige et al., 1996; Gething, 1997; Bukau and Horwich, 1998). Because hsp prevent the aggregation of heat-damaged proteins and facilitate their renaturation following a heat shock, they are likely to play an important role in thermotolerance (Parsell and Lindquist, 1993, 1994). Despite the large literature related to hsp, surprisingly few studies have examined their expression under natural (field) temperature conditions or the variation in the heat-shock response between species, which may contribute to establishing differences in their thermal tolerance limits (for a review, see Feder and Hofmann, 1999).

Among the important and largely unanswered questions that need to be addressed are the following. What characteristics of the heat-shock response differ between species adapted to different thermal niches? How do such important characteristics as the onset temperature of enhanced synthesis of hsp (T_{on}), the temperature of maximal hsp expression (T_{peak}) and the upper thermal limit for synthesis of hsp (T_{off}) correspond to habitat temperatures? Which of these characteristics of the heat-shock response are genetically fixed, and which can be modified by acclimatization in the field or acclimation in the laboratory? How large a change in exposure temperature is required to elicit an acclimatory response? Over what range of temperatures can acclimation be induced? Do acclimatory responses differ between steno- and eurythermal species? Do the expression patterns of different size classes of hsp differ within a species? Does expression of different
classes of hsp differ among species? Answers to these questions may reveal how evolutionary variation in the heat-shock response adapts organisms to their thermal niches and contributes to biogeographic patterning.

To address the above questions, we have initiated studies of the heat-shock response in marine snails belonging to the genus Tegula. Because of their diverse latitudinal and vertical distribution patterns, congeners of Tegula occupy thermal niches that vary widely in absolute temperature and range of temperature (Riedman et al., 1981; Watanabe, 1984; Hellberg, 1998). In the present study, we examined four congeners from temperate and subtropical habitats. Tegula brunnea and T. montereyi are low-intertidal to subtidal cool-temperate species that seldom face exposure to air (emersion) and are unlikely to encounter temperatures in excess of approximately 20–25 °C. A mid- to low-intertidal-zone congener found at the same latitude, T. funebralis, encounters much higher peak temperatures, 33 °C or more, when emersed. A fourth species, T. rugosa, is endemic to the rocky intertidal zone of the Gulf of California, where it encounters air and water temperatures T. rugosa, is endemic to the rocky intertidal zone of the Gulf of California, where it encounters air and water temperatures, 33 °C or more, when emersed. A fourth species, T. rugosa, is endemic to the rocky intertidal zone of the Gulf of California, where it encounters air and water temperatures in the region of 40 °C. Thus, these four congeners would be expected to possess widely different thermal tolerances and, we hypothesized, adaptive differences in their heat-shock responses. Another advantage afforded by these congeners is that any differences observed in the heat-shock responses are likely to indicate adaptation due to selective pressures arising from different thermal environments during their recent separate evolutionary histories rather than merely reflecting phylogenetic distance (Hellberg, 1998).

Using field-acclimatized and laboratory-acclimated specimens of Tegula, we determined the effects of acute thermal exposure on rates of incorporation of 35S-labeled methionine and cysteine into proteins in gill tissue, which readily accumulates dissolved free amino acids (Wright, 1988). We quantified the amount of new synthesis of hsps belonging to four size classes, 90 kDa (hsp90), 77 kDa (hsp77), 70 kDa (hsp70) and 38 kDa (hsp38) relative to a non-heat-shocked control. Comparisons among the differently adapted, acclimatized and acclimated snails suggest that, despite there being acclimatory plasticity in their heat-shock responses, the four congeners have genetically fixed differences in these responses and in their upper thermal limits of protein synthesis that are of importance in determining their distinct vertical and latitudinal distribution patterns.

Materials and methods

Organisms, distribution patterns and collection sites

The vertical distributions of the three temperate-zone Tegula species used in this study are given in Fig. 1. Tegula funebralis (Adams) has the widest latitudinal range, from Vancouver Island, British Columbia, Canada (48°25′N), to central Baja California, Mexico (28°00′N) (Abbott and Haderlie, 1980; Hellberg, 1998). Tegula brunnea (Philippi) is found from Cape Arago, Oregon, USA (43°21′N), to the Channel Islands, California, USA (34°00′N). Tegula montereyi (Kiener) occurs from Sonoma County, California, USA (38°17′N), to the Channel Islands, USA (Abbott and Haderlie, 1980; Hellberg, 1998). The three temperate Tegula species were collected at Hopkins Marine Station (HMS) of Stanford University in Pacific Grove, California (36°36′N, 121°54′W). The mid-intertidal T. rugosa (Adams), which is endemic to the northern part of the Gulf of California (Hellberg, 1998), was collected in San Felipe, Baja California, Mexico (27°20′N, 106°00′W). Large adults were used exclusively in all experiments, and the sizes of specimens were similar in all four species.

Measurements of body temperatures in the field

We used gelatin-filled snail shells to record internal body temperatures under field conditions (100% gelatin, Nabisco Foods). During hardening of the gelatin, we inserted a thermistor (Yellow Springs Instruments, model 44006; accuracy ±0.2 °C) into the interior of the shell and subsequently covered the opening with silicone sealant. The extension of the thermistor was connected to a Stow Away XTI (Onset, Massachusetts, USA) temperature data logger.

Fig. 1. Vertical distribution ranges of three temperate snail species of the genus Tegula (T. funebralis, T. brunnea and T. montereyi) along the intertidal–subtidal transition at Hopkins Marine Life Refuge, Pacific Grove, California, USA (after Riedman et al., 1981; Watanabe, 1984). The subtropical T. rugosa has a vertical distribution range similar to that of T. funebralis.
Heat-shock proteins, thermotolerance and biogeography

Heat-shock protocol and tissue preparation

We dissected gill tissue under non-heat-shock-inducing conditions (13 °C sea water for temperate and 23 °C sea water for subtropical species) and immediately placed the tissue into plastic microcentrifuge tubes containing filtered (0.2 µm) sea water containing 10 mmol l⁻¹ glucose. Tubes were pre-equilibrated at 13 °C (23 °C for T. rugosa) before the start of the experiment. Gill tissues were aerated every 30 min after dissection. The tubes containing gill tissue were then placed into different water baths preheated to the desired incubation temperature.

Samples of gill tissues were incubated at various temperatures for 2.5 h (see Figs 5–8). After incubation, tissues were placed at 13 °C (23 °C for T. rugosa) for 15 min before 35S-labeled methionine/cysteine (NEN) was added to the tube. The duration of the thermal exposure was less than the full period of aerial exposure that snails in the mid-intertidal would typically experience during emersion. Thus, a 2.5 h exposure is conservative in terms of the severity of the heat stress that might occur under field conditions. Higher concentrations of labeled amino acids were used for T. brunnea and T. montereyi (12.21 MBq ml⁻¹; 30–45 mg wet mass) than for T. funebralis and T. rugosa (8.14 MBq ml⁻¹; 15–25 mg) to compensate for higher tissue mass and lower uptake rates in the former two species. We then incubated the tubes for 4 h at 13 °C (23 °C for T. rugosa), a period adequate to allow protein synthesis to occur. After this incubation period, we washed the gill tissue in ice-cold sea water, added 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] to the tubes, and then froze the tubes on dry ice. The samples were then stored at −70 °C. To prepare homogenates for autoradiography, the frozen samples were thawed in a dry bath for 5 min at 100 °C and then homogenized with a silicon 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. No proteolytic activity was detected during this homogenization procedure. To determine the amount of 35S-labeled amino acids incorporated into newly synthesized proteins, we pipetted samples (5 or 10 µl) of gill supernatant onto GF-C glass-fiber filters (Whatman) and allowed them to dry in air. To remove unincorporated 35S-labeled amino acids, we washed the filters once in ice-cold 10 % trichloroacetic acid for 10 min, followed by two 5-min washes with 5 % trichloroacetic acid at room temperature (approximately 20 °C), and finally rinsed the filters in 95% ethanol. The filters were dried at room temperature, and counts per minute of incorporated 35S-labeled amino acids were quantified in a scintillation counter.

Gel electrophoresis and fluorography

For most tissue samples, we loaded approximately

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500×10³ counts min⁻¹ onto each lane of 10 % SDS–polyacrylamide gels. The gels were run first at 25 mA for 80 min (stacking gel) and then at 30 mA for 150 min (resolving gel). We obtained significantly lower incorporation rates for gill tissue exposed to higher temperatures (33 °C for T. brunnea and T. montereyi, 36 °C, 38 °C and 39 °C for T. funebralis and 40 °C and 42 °C for T. rugosa) and therefore loaded only 100×10³ counts min⁻¹ from these samples onto the gels to avoid over-loading of gel wells. Gels were stained overnight with Coomassie R-250 in 10 % acetic acid and destained for 2 h in 10 % acetic acid and 30 % methanol. The gels were treated with EN³HANCE (NEN) for 1 h according to the manufacturer’s instructions, and then dried and exposed to pre-flashed film (Kodak X-OMAT) at −70 °C for 8 h (500×10³ counts min⁻¹) or 18 h (100×10³ counts min⁻¹). We corrected for the fact that 500×10³ counts min⁻¹ samples emitted 2.23 times more counts over 8 h than did samples with 100×10³ counts min⁻¹ over 18 h. All levels of hsp expression are given after this correction, i.e. after normalization to 500×10³ counts min⁻¹ at 8 h exposure time.

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

We scanned film images on a densitometer (Sharp JX-330) and analyzed the digitized images with image-analysis software (ImageMaster 1D, Version 2.01, Pharmacia) to quantify the amount of newly synthesized protein in each hsp size class. To determine T on, T peak and T off, we developed a quantification protocol that used the intensity of the relevant hsp mass band at 13 °C, a temperature at which we incubated gill tissue of all specimens from all acclimatization and acclimation regimes (with the exception of T. rugosa, for which we used the band at 23 °C), as the index for normalization. The intensities of this band at other temperatures were expressed relative to the intensity at 13 °C for all classes of hsp. We emphasize that all 35S-incorporation experiments on the three temperate species were performed at a common incubation temperature (13 °C). Thus, no temperature effects on rates of protein synthesis are present in data from temperate species.

Note that hsps are named according to molecular mass, but have not been further characterized with respect to the number of isoforms present or homology with hsps of other species.

Comparisons of hsp band intensities were performed using a one-sided Dunnett test after a one-way analysis of variance (ANOVA; SYSTAT Software; Systat, Inc.). For the ANOVA, data were log-transformed; incubation temperature was used as the independent variable and expression level of hsps as the dependent variable. We describe the first temperature at which band intensity was significantly higher (P ≤ 0.05) than the band intensity of the 13 °C control group (23 °C in case of T. rugosa) as the onset temperature (T on) of the synthesis of a particular hsp. To reduce clutter in Figs 5–8, which present the digitized data, we have not indicated significant differences on the figures, but instead discuss these in the text.

Results

Vertical distributions, field temperature measurements and thermal tolerances

The three temperate species of Tegula examined in this study have distinct vertical distributions along the intertidal-to-subtidal transition at HMS (Fig. 1). In situ body temperatures of Tegula congeners reflect the vertical distribution ranges of the species and the effects of the tidal cycle. Fig. 2 displays a continuous series of temperature measurements of gelatin-filled shells of Tegula funebralis (black solid line) and T. brunnea (red broken line) from different intertidal heights (0.51 m above mean low low water, MLLW, for T. funebralis and 0.08 m below MLLW for T. brunnea) at Hopkins Marine Life Refuge, Pacific Grove, California. Tidal patterns and day (white) and night (gray) cycles are given for comparison in the upper graph. Seawater temperature measurements at 16 m depth are shown by the dotted line with open circles.
The rate of temperature increase was 1 °C every 12 min (N=20 for each data point).

Fig. 3. Percentage survival of field-collected specimens (mid-August 1996) of three species of the genus Tegula (T. funebralis, T. brunnea and T. montereyi) after exposure to increases in temperature. The rate of temperature increase was 1 °C every 12 min (N=20 for each data point).

measurements of gelatin-filled shells of T. funebralis and T. brunnea obtained at intertidal sites within the vertical distribution range of each species. The vertical heights that were chosen (Fig. 2) represent upper thermal extremes for these two species at HMS. For T. brunnea, this height represents the extreme upper limit of its vertical distribution range. Tegula funebralis does not move during emersion and frequently hides in or near crevices at its highest sites of occurrence. Thus, it experiences longer exposures to solar radiation and attains higher body temperatures in the center of the mid-intertidal zone (L. Tomanek and G. N. Somero, unpublished observations).

Bole temperatures for both species varied with the daily tidal rhythm (Fig. 2), but maximal changes in body temperature during a tidal cycle were more than twice as great for T. funebralis (approximately 19 °C) as for T. brunnea (approximately 7 °C). The maximal body temperatures measured for T. funebralis (approximately 33 °C) are approximately 10 °C higher than those for T. brunnea (approximately 24 °C). Tegula funebralis frequently experienced temperatures between 27 °C and 33 °C (Fig. 2), but T. brunnea experienced temperatures as high as 21 °C only during three extreme midday low tides (11, 12 and 14 April; Fig. 2).

We also observed longer-term periodicity linked to the timing of low tides. For example, the series of high body temperatures observed during daytime low tides for T. funebralis during the last week of March (Fig. 2) was followed by an 8–10 day period of low peak body temperatures in early April, when low tides occurred between the early evening and early morning hours. In general, we assume that the temperatures determined or estimated for T. brunnea approximate those that T. montereyi experiences, because both species remain immersed most of the time. Although we do not have field body temperature measurements for T. rugosa, summer water temperatures at the collection sites near San Felipe, Baja California, reach at least 36 °C (Dietz and Somero, 1992), and snails exposed during daytime low tides are apt to be even warmer.

To determine whether the differences in vertical distribution and field body temperature of the three temperate-zone congeners were associated with differences in heat tolerance, we assessed the survival of field-acclimatized T. funebralis, T. brunnea and T. montereyi exposed to an increase in seawater temperature of 1 °C every 12 min (Fig. 3). Under this heating regimen, the temperature of 50% mortality (LT50) was 42.5 °C for T. funebralis and 36.0 °C for T. brunnea and T. montereyi.

Effect of acclimation

Interspecific differences

The autoradiographs in Fig. 4 show the patterns of protein synthesis, at a common incubation temperature of 13 °C, following exposure of isolated gills from 13 °C- and 23 °C-acclimated T. funebralis and T. brunnea to several temperatures. We used densitometric analysis of such autoradiographs to generate Figs 5–8, which show the relative levels of synthesis of hsp70, hsp38, hsp90 and hsp77, respectively. Data are shown for 23 °C-acclimated individuals of all four species, as well as for the 13 °C- and 18 °C-acclimated and summer field-acclimatized specimens of the three temperate-zone species.

Three characteristic interspecific differences were observed in most comparisons. First, T_on varied positively with habitat (adaptation) temperature. For example, comparisons of 13 °C-acclimated T. brunnea and T. funebralis (Figs 4, 5) show that T_on of hsp70 occurred at 24 °C in T. brunnea and at 27 °C in T. funebralis. For hsp90, comparisons of the four species (23 °C acclimation groups) show a T_on of 24 °C for T. brunnea and T. montereyi, of 27 °C for T. funebralis and of 30 °C for T. rugosa (Fig. 7).

Second, T_peak showed a positive trend with habitat temperature for hsp70 for all species over all acclimation temperatures (Fig. 5). This trend also was found for hsp38 (Fig. 6) and hsp77 (Fig. 8), but not consistently for the expression of hsp90 (Fig. 7). Third, among all classes of hsp, a positive trend was found between adaptation temperature and T_off. This trend was also evident for the thermal limits of protein synthesis per se (Figs 5–9). The values for T_off were near the upper limits of the thermal tolerance ranges for T. funebralis and T. brunnea (Figs 9, 10). This is shown by the organismal heat tolerance data in Fig. 10, which were obtained using an incubation temperature regimen similar to that employed in the protein synthesis experiments. In addition, the difference between temperate intertidal and subtidal species in T_off (39 °C versus 33 °C) was similar to the interspecific differences in survival temperature, 6.5 °C, obtained using the protocol shown in Fig. 3. Furthermore, in T. brunnea and T.
Acclimation temperature 13 °C

Acclimation temperature 13 °C

Incubation temperature

Acclimation temperature 23 °C

Incubation temperature

Acclimation temperature 23 °C

Incubation temperature

Fig. 4. Autoradiographs showing the effects of incubation temperature on incorporation of 35S-labeled methionine/cysteine into proteins of gill tissue of *Tegula funebralis* and *T. brunnea* acclimated to either 13 or 23 °C. For each incubation temperature, duplicates of a gill sample from a single snail were loaded on a 10% SDS–polyacrylamide gel. 14C molecular mass markers are shown in the far left lane of each autoradiograph. All lanes were loaded with 500×10^3 counts min⁻¹ and exposed for 8 h to pre-flashed X-ray film.

For *T. brunnea* and *T. montereyi*, an increase in acclimation temperature from 13 to 23 °C led to an increase in *T*ₚₑ𝐚ᵏ of hsp70 from 24 to 27 °C (Figs 4, 5). The temperature of maximal induction (*T*ₚᵉᵃᵏ) of hsp70 in these two species shifted from 27 to 30 °C as acclimation temperature rose from 13 to 18 °C (Fig. 5). No additional increase in *T*ₚᵉᵃᵏ was seen following acclimation to 23 °C. *T. funebralis* showed no acclimation-induced changes in *T*ₚᵉᵃᵏ or *T*ₒᵣᵣ for hsp70 (Figs 4, 5). However, *T. brunnea* and *T. montereyi* exhibited increased synthesis of hsp70 at temperatures above 30 °C in all acclimation groups. For hsp90, *T. funebralis* exhibited a shift in *T*ₒᵣᵣ and a consistent attenuation of the maximal level of hsp90 synthesis with an increase in acclimation temperature from 13 to 18 °C, but *T. brunnea* and *T. montereyi* did not show these effects (Fig. 7).

*y. montereyi*, protein synthesis was blocked at temperatures (30–33 °C) that were frequently experienced by *T. funebralis* during the spring (Fig. 2). In turn, protein synthesis in *T. funebralis* ceased at temperatures below the maximal body temperatures likely to be encountered by the subtropical intertidal species *T. rugosa*.

Differences among the four species were also noted in the relative intensities of induction of the different size classes of hsp. For example, for hsp90, comparisons among the 23 °C-acclimated snails showed that *T. brunnea* and *T. montereyi* increased synthesis most, and *T. rugosa* increased synthesis least (Fig. 7). In contrast, the increase in synthesis of hsp38 was greatest in *T. rugosa* and *T. funebralis* and smallest in *T. brunnea* and *T. montereyi* (Fig. 6). For hsp77, relative synthesis was greatest in *T. funebralis* (Fig. 8).

Specific expression patterns of heat-shock proteins

To examine the phenotypic plasticity of the heat-shock response, we acclimated specimens of the three temperate-zone species to 13 °C, 18 °C and 23 °C under constant submersion. These temperatures lie within the range routinely encountered by *T. funebralis*, whereas 23 °C is near the upper limit of the temperature ranges of the lower-occurring species *T. brunnea* and *T. montereyi*.
Heat-shock proteins, thermotolerance and biogeography

Fig. 5. Relative induction (compared with the 13 °C control group) of hsp70 in the three temperate
Tegula congeners T. funebralis, T. brunnea and T. montereyi after laboratory acclimation at 13 °C, 18 °C and 23 °C for 30–34 days and field acclimatization (July 1997). Data are also given for 23 °C-acclimated T. rugosa (23 °C control group). T_on indicates the onset temperature, T_peak the temperature of maximal induction and T_off the cessation temperature of hsp synthesis. Values are means ±1 S.E.M. N=5 for all data points except for 13 °C-acclimated T. funebralis at 36 °C (N=4), for 18 °C-acclimated T. montereyi at 13 °C (N=4), 21 °C (N=4), 24 °C (N=4), 27 °C (N=4) and 30 °C (N=4), for 23 °C-acclimated T. funebralis at 33 °C (N=4) and for field-acclimatized T. funebralis at 27 °C and T. montereyi at 13 °C and 30 °C (N=4).

Fig. 6. Relative induction (compared with the 13 °C control group) of hsp38 in the three temperate Tegula congeners T. funebralis, T. brunnea and T. montereyi after laboratory acclimation at 13 °C, 18 °C and 23 °C for 30–34 days and field acclimatization (July 1997). Data are also given for 23 °C-acclimated T. rugosa (23 °C control group). Values are means ±1 S.E.M. N=5 for all data points, except for 13 °C-acclimated T. funebralis at 36 °C (N=4), for 18 °C-acclimated T. montereyi at 13 °C (N=4), 21 °C (N=4), 24 °C (N=4), 27 °C (N=4) and 30 °C (N=4), for 23 °C-acclimated T. funebralis at 33 °C (N=4) and for field-acclimatized T. funebralis at 27 °C (N=4) and T. montereyi at 13 °C (N=4) and 30 °C (N=4).

An increase in acclimation temperature from 18 to 23 °C did not change hsp expression patterns in any species. For hsp77, the responses of T. brunnea and T. montereyi were little affected by acclimation, whereas T. funebralis showed a large increase in intensity of response at the two higher acclimation temperatures (Fig. 8).

In general, acclimatory changes in T_on and T_peak occurred with a shift in acclimation temperature from 13 to 18 °C, but only minor additional changes were elicited by a further increase in acclimation temperature to 23 °C. Unlike T_on and T_peak, T_off showed no response to acclimation (Figs 5–9).

At higher heat-shock-inducing temperatures, synthesis of proteins other than hsps was typically much reduced, especially in 13 °C-acclimated snails (Figs 4, 9). However, in all three temperate-zone species, increasing acclimation temperatures restored to some degree the synthesis of non-hsps at higher temperatures, e.g. at 30 °C in T. brunnea and T. montereyi (Fig. 9). Thus, acclimation to higher temperatures allowed synthesis of non-hsps to occur at higher levels at the maximal temperatures at which synthesis was possible, even though T_off values were not altered by acclimation. Synthesis of hsps was also increased at the highest temperatures at which synthesis was possible. In some cases, the maximal levels of hsp synthesis occurred at higher temperatures (Figs 4–9).

Variation was also observed among size classes of hsps...
Field-acclimatized specimens versus laboratory-acclimated specimens

As shown in Figs 5–8, the heat-shock responses of field-acclimatized snails both resembled and differed from those of the laboratory-acclimated conspecifics. The $T_{off}$ values were the same in the field-acclimatized and laboratory-acclimated snails, which is further evidence for a genetically fixed upper thermal limit to protein synthesis in these species.

The most striking difference was found for hsp70. For all three temperate species, field-acclimatized specimens appeared to induce hsp70 only approximately one-quarter to one-third as strongly as snails from the 13°C and 18°C acclimation treatments (Fig. 5). However, this apparently lower intensity of induction in field-acclimatized snails is, in large measure, a consequence of the normalization procedure (see Materials and methods). Absolute levels of hsp70 synthesis were in fact higher in field-acclimatized specimens at all treatment temperatures (data not shown). This observation indicates that normalization to a common treatment temperature (13°C in the present experiment) provides a means for evaluating how the heat-shock response varies among treatment temperatures, but
Heat-shock proteins, thermotolerance and biogeography

Acclimation temperature

T. brunnea

13°C 30 30 33 33 °C 30 30 33 33 °C

23°C

Incubation temperature

T. montereyi

13°C 30 30 33 33 °C 30 30 33 33 °C

23°C

Fig. 9. Expression of newly synthesized proteins in specimens of Tegula congeners acclimated to different temperatures (13 °C and 23 °C). Arrows indicate the major hsps of size classes 90, 77, 70 and 38 kDa. Each lane of a 10% SDS–polyacrylamide gel was loaded with 100×10^3 counts min⁻¹. Pre-flashed X-ray film was exposed to the gel for 18 h for all temperature incubations except for the 30 °C lanes in T. brunnea and T. montereyi, which were loaded with 500×10^3 counts min⁻¹ and exposed for 8 h to pre-flashed film. 14C molecular mass markers are shown in the far left lane of the autoradiographs for T. brunnea, T. montereyi and T. funebralis.

it does not provide an index of the absolute level of synthesis that occurs in different acclimation or acclimatization groups (see Discussion).

Discussion

Biogeographic and ecological implications of interspecific variation in the heat-shock responses of Tegula congeners

Comparisons of heat-shock responses of four congeners of Tegula suggest that, despite some acclimatory plasticity in this response, genetically fixed differences exist between species in T_on, T_peak and T_off (Fig. 5) as well as in the upper thermal limits of protein synthesis in general. In the light of the thermal features of the distribution ranges of these species, each of these interspecific differences has implications for biogeographic patterning and for the in situ function of the heat-shock response.

First, the frequency with which the heat-shock response is induced in situ is likely to vary considerably between the four species. In their natural habitats, T. brunnea and T. montereyi are much less likely to experience temperatures that elicit enhanced expression of hsps than are T. funebralis and T. rugosa. For example, during the 26 day period of midday low tides between late March and mid-April, 1996 (Fig. 2), on at least 11 days body temperatures of T. funebralis may have exceeded 27 °C, a temperature above or at the T_on of certain hsps, e.g. hsp70 and hsp90 (in 13 °C-acclimated specimens, Figs 5, 7). During this same 26 day period, on only a single day did the body temperature of T. brunnea reach a value as high as 24 °C, which is the T_on for hsp70 and hsp90 synthesis in 13 °C-acclimated specimens. Since water temperatures in the shallow intertidal habitat of T. rugosa rise to at least 36 °C in summer (Dietz and Somero, 1992), this intertidal snail must also commonly activate the heat-shock response in situ.

The heat-shock response is energetically costly (Sanchez et al., 1992; Heckathorn et al., 1996), so interspecific differences in the frequency with which it is induced could be associated with significant differences among these species in how temperature affects their energy budgets. Energy is required for the synthesis of hsps, and the chaperoning activity of most hsps requires hydrolysis of ATP. Over-expression of hsps can significantly decrease fitness (Feder et al., 1992; Krebs and Loeschcke, 1994), possibly as a consequence of the energy costs associated with the heat-shock response and the preferential synthesis of hsps, at the expense of synthesis of other types of proteins, at elevated temperatures (Figs 4, 9).
Therefore, the species of *Tegula* that inhabit the mid-intertidal zone may face substantially higher energy costs related to synthesis of hsps and more frequent interruption of synthesis of non-hsp proteins than their lower-occurring congeners. The costs entailed in activating the heat-shock response may contribute to the upper limits of vertical distribution of intertidal invertebrates. A correlation between the induction of hsps at habitat temperatures and the variability of the thermal habitat has also been reported in a comparison of several species of *Hydra*, a freshwater cnidarian (Bosch et al., 1988, 1991; Gellner et al., 1992).

A second major difference found among the four congeners of *Tegula* is the upper thermal limit for the synthesis of hsps and other proteins. Protein synthesis of the two temperate species occurring lowest in the intertidal zone, *T. brunnea* and *T. montereyi*, was heat-inactivated at temperatures at which the synthesis of some hsps, for instance hsp70 (Fig. 5), is maximal in this species. The thermal sensitivity of protein synthesis by *T. brunnea* and *T. montereyi* may prevent these two species from occurring in the mid-intertidal region inhabited by *T. funebralis*. Thermotolerance measurements (Fig. 10) certainly suggest that *T. brunnea* would not survive exposures between 30 and 33 °C (over 2.5 h) that we frequently observed for *T. funebralis*. In turn, protein synthesis by *T. funebralis* ceased by 39 °C, a temperature at which hsp synthesis was maximal in the most warm-adapted species, *T. rugosa*. The observation that thermotolerance of protein synthesis could not be modified through acclimation suggests a fixed genetic basis for these differences (Figs 5–9).

Further evidence that the heat sensitivity of protein synthesis may play a role in establishing thermal tolerance limits and, thereby, contribute to biogeographic patterning along latitudinal and vertical gradients, is found in other studies of ectothermic animals with different distribution patterns. For example, a study of limpets of the genus *Collisella* demonstrated a 2°C difference in \( T_{\text{off}} \) between mid- and high-intertidal species (Sanders et al., 1991). Comparisons of a heat-adapted ant (genus *Cataglyphis*) from the Sahara with an ant living in temperate conditions (genus *Formica*) revealed a 6°C higher \( T_{\text{off}} \) in the former species (Gehring and Wehner, 1995). Protein synthesis in a more northern-occurring congener of *Mytilus*, *M. trossulus*, is heat-inactivated at temperatures near 30°C, whereas synthesis in the more southern-occurring species, *M. galloprovincialis*, continues at this temperature (Hofmann and Somero, 1996). These observations, taken in conjunction with the differences observed among *Tegula* congeners, provide evidence that differences in \( T_{\text{off}} \) may play an important role in establishing the biogeographic distributions of ectotherms.

**Plasticity in the heat-shock response: laboratory acclimation and field acclimatization**

We noted two types of plasticity in the heat-shock responses of differently acclimated and acclimatized conspecifics. First, we observed variation in the thermal responses of a given size class of hsp in differently acclimated or acclimatized individuals, which has also been observed in other species, including eurythermal goby fishes (*Gillichthys* spp.; Dietz and Somero, 1992) and intertidal mussels (*Mytilus* spp.; Roberts et al., 1997). Second, we found differences in response among size classes of hsp within a particular treatment group. The bases of the differences in relative amounts of synthesis and induction temperatures are undoubtedly complex, and the differences are probably based on the different roles played by the various classes of hsp (Parsell et al., 1993). For instance, although hsp90 is synthesized during heat stress, it does not seem to prevent heat inactivation of proteins under *in vivo* conditions (Nathan et al., 1997). *In vivo*, its contribution to the heat-shock response seems to be through an involvement in several signal-transducing pathways (Nathan et al., 1997; Pratt, 1998). Thus, more needs to be learned about the specific functions of the different classes of hsp in the heat-shock response before we can fully understand the importance of the inter- and intraspecific differences observed in this and other studies.

Although considerable acclimation-induced plasticity in the heat-shock response was noted, it did not usually occur across the full range of acclimation temperatures. Acclimatory effects occurred as acclimation temperature increased from 13 to 18 °C, but a further increase in acclimation temperature from 18 to 23 °C led to no major additional changes. The finding that neither *T. brunnea* nor *T. montereyi* showed additional acclimation effects when the temperature was raised from 18 to 23 °C may reflect the infrequency with which these two species encounter seawater temperatures above 23 °C (Fig. 2). However, *T. funebralis*, which frequently encounters seawater temperatures above 23 °C at its southern distribution limit, also

![Fig. 10. Survival of 13°C-acclimated (5–7 weeks) *Tegula funebralis* and *T. brunnea* after exposure for 2.5 h at different temperatures (\( N=20 \) for each time point). Survival was assessed within 30 min of exposure by examining the withdrawal response of the foot.](image-url)
Heat-shock proteins, thermotolerance and biogeography

showed no additional acclimatory changes when temperature was increased from 18 to 23 °C. Thus, for T. funebralis, the range of temperatures over which acclimation effects were observed was narrower than the range of body temperatures the organisms encounter in their habitats. Studies of acclimatory effects on mitochondrial function in congeneres of abalone (genus Haliotis) also found that the range of temperatures over which acclimatory changes occurred was narrower than the range of habitat temperatures (Dahlhoff and Somero, 1993).

Despite the variation in $T_{on}$ of hsp synthesis due to acclimatory history, the fact that interspecific differences in $T_{on}$ were retained whatever the temperature of acclimation suggests that fixed genetic differences are important in setting the temperatures of induction of hsp synthesis. These genetically based differences could be due to gene regulatory factors that establish the set points for induction of hsp synthesis (Craig and Gross, 1991; Morimoto, 1998; Morimoto and Santoro, 1998) or to interspecific variations in the thermal stability of cellular proteins (Somero, 1995).

One additional effect of acclimation merits emphasis, the increased ability of gills from the most warm-acclimated specimens to synthesize proteins at high temperatures (Fig. 9). The synthesis of hsps during heat shock blocks the synthesis of non-hsps in some organisms because of the preferential translation of hsp70 mRNA (Lindquist, 1980, 1981, 1993; Storti et al., 1980). A mild heat shock that induces hsp synthesis can prevent the inhibition of non-hsp synthesis during subsequent exposures to heat, indicating that pretreatment may affect the thermotolerance of translation (Petersen and Mitchell, 1981) by stabilizing translational initiation and/or chain elongation during heat shock (Beck and De Maio, 1994). Our observations therefore indicate that acclimation to higher temperatures, as well as a previous acute sub-lethal heat shock (Petersen and Mitchell, 1981), can lead to an increased ability to synthesize proteins at high temperatures. The mechanisms that allow protein synthesis to occur at higher rates near the maximal temperatures at which protein synthesis is possible appear to have no effect on $T_{off}$ itself, which was unchanged by acclimation.

The influence of experimental design on the observed properties of the heat-shock response

Several results of this study illustrate how experimental design can influence the observed properties of the heat-shock response. First, the differences that were noted between summer field-acclimatized snails and laboratory-acclimated conspecifics provide a caveat about extrapolation from laboratory acclimation studies to the responses occurring in the field. Roberts et al. (1997), in a study of field-acclimatized and laboratory-acclimated mussels (Mytilus californianus), also found a greatly attenuated hsp70 response in summer-acclimatized mussels. These observed differences between field- and laboratory-based studies suggest that the complex set of abiotic factors other than temperature that are present in field settings, such as nutritional status, desiccation stress, oxygen availability and ultraviolet radiation, may alter the heat-shock response in ways that have not been simulated in laboratory acclimation studies.

Second, and following from the preceding observation, the normalization procedure used to gauge the amounts of newly synthesized hsps can affect the conclusions. If constitutive synthesis of a particular class of hsp is high, as in the case of field-acclimatized snails incubated at 13 °C, then the intensity of induction of hsps at hsp-inducing temperatures may be underestimated.

In conclusion, although several characteristics of the heat-shock responses in Tegula congeners responded to laboratory acclimation or field acclimatization, we interpret the consistent correlation between three key characteristics of the heat-shock response, $T_{on}$, $T_{peak}$ and $T_{off}$, and normal habitat temperatures as a manifestation of genetically based interspecific differences that may play important roles in setting the latitudinal and vertical distribution limits of these species. Furthermore, the findings that protein synthesis is heat-inactivated at temperatures only slightly above the highest body temperatures measured and that the costly heat-shock response is frequently induced in higher-occurring intertidal species suggest that these species may currently be living near the upper extremes of their thermal tolerance ranges. This may put such species at a higher risk from global warming.

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References


Dietz, T. J. and Somero, G. N. (1992). The threshold induction temperature of the 90-kDa heat shock protein is subject to


