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Two-dimensional gel analysis of the heat-shock response in marine snails (genus *Tegula*): interspecific variation in protein expression and acclimation ability

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Summary

The degree to which temperature acclimation modifies the acute synthesis of the entire heat-shock protein (Hsp) complement is still unknown, but it may constitute an important mechanism for understanding the differences in acclimation ability among closely related ectothermic species that occupy widely varying thermal environments. In general, eurythermal (heat-tolerant) species modify physiological function in response to an increase in acclimation temperature to a greater extent than stenothermal (heat-sensitive) species. In the present work I used ^{35}S-labelled amino acids and two-dimensional gel electrophoresis to test this assumption for how acclimation affects acute Hsp expression (referred to as phenotypic plasticity) in two heat-sensitive, low-intertidal to subtidal zone turban snails, *Tegula brunnea* and *T. montereyi*, in comparison to a heat-tolerant, mid- to low-intertidal zone congener, *T. funebralis*. I was able (i) to detect the synthesis of over 30 proteins in gill tissue, primarily in the 70 kDa range, in response to an increase in temperature (13°C, 24°C, 27°C and 30°C), (ii) to assess the effect of acclimation (13°C *vs* 22°C) on acute Hsp synthesis, and (iii) to compare this effect among the three *Tegula* congeners. After increasing acclimation temperature from 13°C to 22°C, synthesis of the most highly expressed Hsps decreased more in *T. brunnea* and *T. montereyi* than in *T. funebralis*. Two highly expressed proteins of molecular mass 71 and 74 kDa, however, were also synthesized constitutively at 13°C and changed with increasing acclimation temperature in all three species. Although similar in phenotypic plasticity, *T. brunnea* and *T. montereyi* synthesized either a 76 or a 72 kDa cluster of proteins, respectively, and differed in how acclimation affected the acute synthesis of several 77 kDa proteins. Thus, in *Tegula*, the effect of acclimation on Hsp expression is (i) Hsp-specific, (ii) dependent on a protein's expression pattern (constitutive and inducible *vs* only inducible), (iii) and is actually limited in the more eurythermal mid- to low-intertidal congener. These results contradict the general assumption that greater heat tolerance correlates with an increased ability to modify physiological function in response to acclimation.

Key words: acclimation, heat-shock protein, molecular chaperone, phenotypic plasticity, *Tegula*, two-dimensional gel electrophoresis.

Introduction

Acclimation to higher temperatures leads to wide-ranging physiological changes in ectothermic organisms (Cossins and Bowler, 1987; Prosser, 1991; Withers, 1992). In general, animals from relatively constant thermal environments are less capable of modifying physiological function during acclimation than animals from environments that undergo more frequent and greater temperature changes. Such comparisons in phenotypic plasticity have been documented for animals that differ either in their biogeographical distribution pattern (e.g. temperate *vs* tropical), habitat preference (shallow *versus* deep waters) or seasonal temperature (e.g. summer *vs* winter) (Prosser, 1991). Expression patterns of heat-shock proteins (Hsps) have been shown to vary between species that differ in heat tolerance and within species due to short-term heat-hardening, longer term seasonal acclimatization or laboratory acclimation (Feder and Hofmann, 1999; Hoffmann et al., 2003). However, our knowledge of how species that differ in heat tolerance, e.g. closely related eurytherms *vs* stenotherms, vary in expression patterns of Hsps with changing acclimation temperature is still limited (Hoffmann et al., 2003; Tomanek and Somero, 1999, 2002; Tomanek, 2002).

As molecular chaperones, Hsps stabilize and refold reversibly denatured proteins and help degrade irreversibly denatured proteins (Feige et al., 1996; Frydman, 2001; Hartl and Hayer-Hartl, 2002; Young et al., 2004). Their role in conferring increased heat tolerance has been established. The onset temperature (T_{on}) of Hsp70 synthesis, the major heat-

induced protein in most organisms, correlates in general positively with a species' temperature range (Feder and Hofmann, 1999; Nakano and Iwama, 2002; Tomanek and Somero, 1999). T_{on} of Hsp expression, however, is not fixed and can shift to higher temperatures after laboratory acclimation (Tomanek and Somero, 1999) and seasonal acclimatization to increasing temperatures (Barua and Heckathorn, 2004; Buckley et al., 2001; Chapple et al., 1998; Dietz and Somero, 1992; Hamdoun et al., 2003; Hofmann and Somero, 1995; Roberts et al., 1997), an adjustment I refer to as the phenotypic plasticity of the heat-shock response (HSR). A shift in T_{on} in response to acclimation is equivalent to a decrease in Hsp synthesis, at least at the lower temperatures that normally elicit a stress response, and is in part due to Hsp70 itself, such that an increase in steady-state levels will shift the activation of its own transcription and synthesis towards higher temperatures (Morimoto, 1998; Tomanek and Somero, 2002; Voellmy, 2004). Interestingly, a heat-sensitive intertidal marine snail species of the genus *Tegula* shifts T_{on} of a Hsp70 band more after acclimation to higher temperatures than does a heat-tolerant congener (Tomanek and Somero, 1999). It is therefore possible that species that vary in heat tolerance activate the stress response at a common temperature following acclimation to warmer temperature. This suggests that species with the highest heat tolerance may have a relatively lower capacity to further adjust the stress response to warmer acclimation temperatures. If so, this limit to acclimation capacity of Hsp expression could explain the limited ability of more heat-tolerant organisms to increase tolerance to even greater heat stress (Cavicchi et al., 1995; Hoffmann et al., 2003; Stillman, 2003; Ushakov et al., 1977; Zatsepina et al., 2001).

However, it is not known if these results of a single Hsp band can be extrapolated to the entire complement of heat-induced proteins. Simply, previous acclimation studies have only used one-dimensional gel electrophoresis to analyze heat-induced protein bands that were close to a molecular mass class that is characteristic for a given Hsp family (Hofmann and Somero, 1996; Tomanek and Somero, 1999), despite the fact that the Hsp complement has been resolved since the early days of two-dimensional gel electrophoresis (2D-GE; Mirault et al., 1978).

In the present work, I address the effect of acclimation on the synthesis of the Hsp complement in three cool–temperate *Tegula* snail species that differ in their vertical distribution patterns along the subtidal to mid-intertidal axis in rocky shore habitats and differ in upper lethal temperatures by approximately 6.5°C (Tomanek and Somero, 1999). *T. funebralis*, which occupies the mid- to low-intertidal zone, experiences greater and more variable temperatures on a daily scale and is more heat-tolerant than the two low-intertidal to subtidal zone congeners *T. brunnea* and *T. montereyi* (Tomanek and Somero, 1999). Furthermore, *T. funebralis* differs from the two subtidal species in having (i) a higher T_{on} and a wider temperature range of Hsp synthesis, (ii) a faster rate to reach pre-stress levels of Hsp synthesis after a thermal exposure typical for the mid-intertidal zone, and (iii) smaller changes in inducible and steady-state levels of Hsp70 and generally higher levels of heat-shock transcription factor-1 (HSF-1) during acclimation to increasing temperatures (Tomanek, 2002; Tomanek and Somero, 1999, 2000, 2002). These interspecific differences enable *T. funebralis*, but not *T. brunnea*, to better cope with the thermal variation in the mid-intertidal zone (Tomanek and Sanford, 2003) and further support the hypothesis that greater heat tolerance may correlate with a limited capacity to modify the stress response.

In this study, I tested for the first time how acclimation affects the incorporation of ^{35}S-labeled methionine and cysteine into over 30 heat-induced proteins or their variants in gill tissue, by separating the Hsp complement using 2D-GE. My results show that the more heat-tolerant species displays a far lower capacity to adjust Hsp synthesis following acclimation to warmer temperatures than the two heat-sensitive species. These results contradict the general observation that animals from thermally more variable environments have a greater capacity to acclimate or modify physiological function in response to changing (here increasing) temperatures than animals from environments with moderate temperature variation. Furthermore, it has been proposed that the benefit of acclimation to increasing temperatures is to prepare the animal for the possibility that the environment will become warmer (Huey et al., 1999; Leroi et al., 1994). I discuss how the differences in phenotypic plasticity between the *Tegula* congeners support this interpretation in the context of the thermal characteristics of their respective vertical distribution ranges in the intertidal zone. Finally, the results also suggest that animals from thermally more-variable relative to less-variable environments may be more sensitive to future temperature increases.

Materials and methods

Animals and acclimation conditions

Large adult animals of all three *Tegula* congeners were collected at Hopkins Marine Station of Stanford University in Pacific Grove, California, USA (36°36′N, 121°54′W) at the beginning of the months of June and July 2003 and acclimated to 13°C and 22°C in circulating seawater aquaria for 21 and 20 days, respectively. Animals were kept submersed and fed with freshly collected kelp (*Macrocystis pyrifera*).

Temperature exposure, labeling protocol and sample preparation

After acclimation to 13°C and 22°C I dissected gill tissue under non-heat-shock-inducing conditions (13°C and 22°C, respectively). Tissues were placed into microcentrifuge tubes holding 300 µl of filtered (0.2 µm) seawater containing 10 mmol l^{-1} glucose. Tubes were pre-incubated to either 13°C or 24°C for 13°C- and 22°C-acclimated animals, respectively. Gill tissue was aerated every 20 min. At the start of the experiment tissues were incubated at 13°C, 24°C, 27°C and 30°C for 2.5 h (for further details, see Tomanek and Somero, 1999). Gill tissue was not directly transferred to 27°C and 30°C

but instead incubated first to 24°C (13°C-acclimated animals) and 27°C (for the 30°C incubation) for 5 min to simulate a more gradual temperature increase. After incubation at the experimental temperature they were transferred to a common temperature of 13°C for 15 min before being incubated with ^{35}S-labeled methionine/cysteine (6.78 MBq ml^{-1}; Perkin Elmer) for 4 h. At the end of the experiment tissues were washed in filtered seawater, frozen on dry ice and stored at –70°C. Tissues were first homogenized using a sample grinding kit containing an abrasive grinding resin in 200 µl of lysis buffer containing 8 mol l^{-1} urea, 4% CHAPS and 2% Pharmalyte 3-10 and subsequently precipitated using the 2-D clean-up kit (both kits were from Amersham Bioscience, Piscataway, NJ, USA). The precipitate was dissolved in 120 µl of rehydration buffer (8 mol l^{-1} urea, 2% CHAPS, 0.5% IPG buffer pH 4–7 (Amersham Bioscience), 0.002% Bromophenol Blue). Levels of ^{35}S-labelled amino acids incorporated into newly synthesized proteins were determined by precipitation with trichloroacetic acid (for details, see Tomanek and Somero, 1999).

Isoelectric focusing, 2D-GE and fluorography

Samples were added (5×10^5 cts min^{-1}) to the rehydration buffer (see above) during overnight rehydration of Immobiline DryStrip gels (7 cm long, pH 4–7; Amersham Bioscience). Rehydrated strips were run on a Multiphor II electrophoresis system (Amersham Bioscience) for 1 min at 200 V and for 2 h 50 min at 3500 V before being stored at –70°C.

For the second dimension, strips were equilibrated twice for 15 min in 10 ml of equilibration buffer (50 mmol l^{-1} Tris-HCl, pH 8.8, 6 mol l^{-1} urea, 30% glycerol, 2% SDS, 0.002% Bromophenol Blue); first with 100 mg dithiothreitol and second with 250 mg iodoacetamide. Strips were placed on ExcelGel 12–14% gradient gels (Amersham Bioscience) and run according to instructions. Afterwards gels were incubated in Amplify fluorographic reagent (Amersham Bioscience) for 30 min and subsequently dried overnight at 60°C (for the first 2 h) using a vacuum.

Image and statistical analyses

Gels were exposed to Hyperfilm (Amersham Bioscience) for 72, 48, 24, 12 and 6 h to obtain a range of exposure intensities, scanned on a densitometer (Sharp JX-330) and analyzed using Image Master 2D software (Amersham Bioscience). Specific spot volumes were normalized against total spot volume within a single gel and square root-transformed to normalize values for statistical analysis. Similar intensities were found for film that was exposed for 48 h after incubation at 13°C and 24°C, 24 h after 27°C incubation and 12 h after 30°C incubation. Expression values of a single Hsp of all species from all acclimation and incubation temperatures were fitted into a general linear model to estimate the average effect for each species, each acclimation and incubation temperature within a species, and an interaction effect between each combination of acclimation and incubation temperature (Software R, version 1.9.1). All treatments had an N-value of three (only exception: *T. funebralis* incubated to 24°C after acclimation to 22°C, N=1). The P-values ($\leqslant$0.05 and $\leqslant$0.10) that are reported in Table 1 are for the average effect of acclimation on Hsp expression within an incubation temperature for a particular species.

Results

Synthesis of heat-induced proteins

Most heat-induced proteins in *Tegula* had a molecular mass between 68 and 90 kDa; additional ones were found around 40

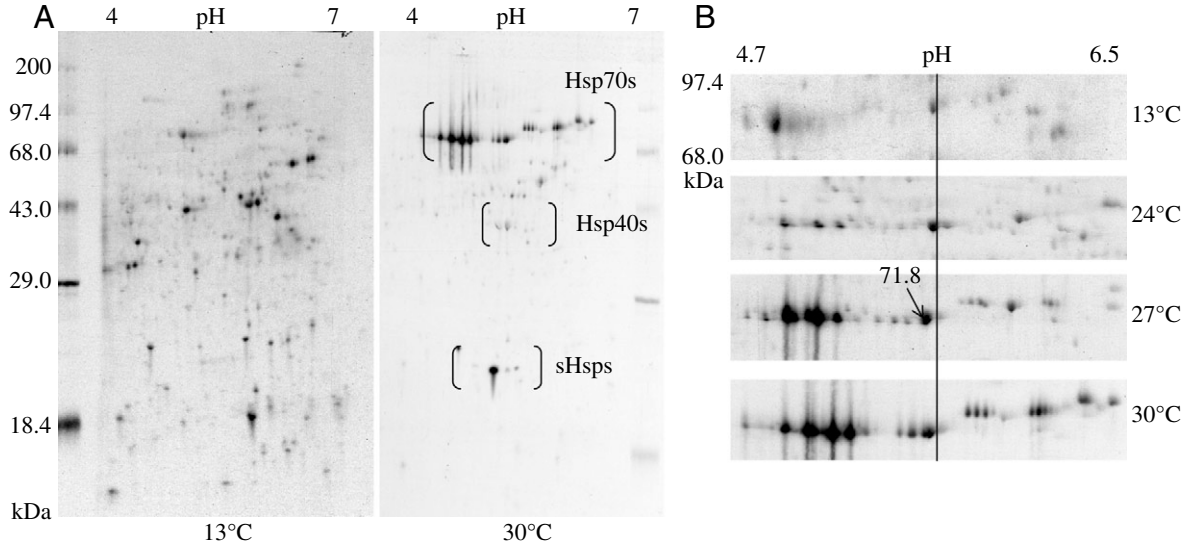

Fig. 1. (A) Autoradiographs of ^{35}S-labeled proteins following incubation to 13°C and 30°C (heat-shocked) for 2.5 h and 4 h of recovery (and labeling) at 13°C in gill tissues of 13°C-acclimated *T. funebralis*. Clusters of heat-induced proteins are further described in Fig. 2. (B) Heat-induced activation of synthesis of proteins between 70–90 kDa in 13°C-acclimated *T. funebralis*. The constitutively expressed protein spot Hsp71.8 is always found to the left of the vertical line. ^{14}C molecular mass markers are shown on the side of the gels (A).

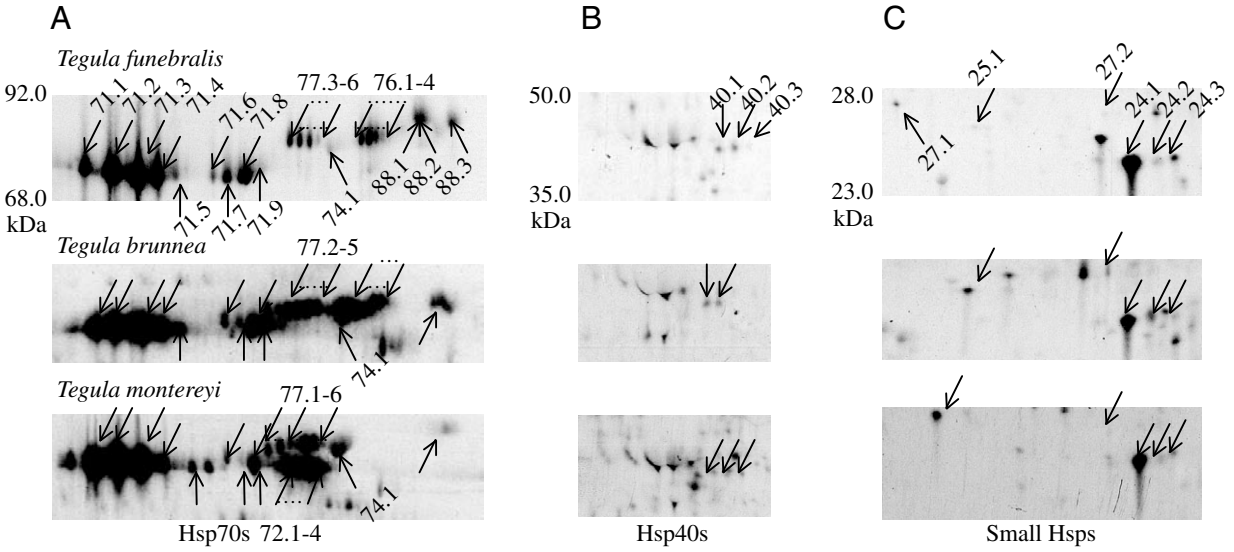

Fig. 2. Autoradiographs of newly synthesized ^{35}S-labelled proteins of (A) 70–88 kDa, (B) 40 kDa and (C) 24–27 kDa in *T. funebralis* (top), *T. brunnea* (middle) and *T. montereyi* (bottom). Proteins within a cluster are assigned according to (i) their approximate molecular mass and (ii) isoelectric point, pI (the higher the number the more basic the pI of the protein). The pattern and orientation of arrows displays matches of proteins between species. Note that not all Hsps were detected in all species, and not all Hsps were detected in all gels from a common incubation temperature within a species (for example, Hsp77.1-2 were not detected in case of *T. funebralis* for the particular gel shown here; additionally, not all Hsp88s were detected in *T. brunnea* and *T. montereyi*).

and 24 kDa (Fig. 1A,B). Although the exact identity of these proteins has not been determined (see below), almost all of these heat-induced proteins were found at or close to a molecular mass that typically represents a family of functionally related Hsps, e.g. Hsp70, Hsp77 and Hsp90. Additionally, under thermal stress, cells preferentially synthesize Hsps while inhibiting the synthesis of the majority of other proteins (Lindquist, 1993; Storti et al., 1980). The downregulation of non-Hsp synthesis is illustrated in Fig. 1A. I am therefore assuming that the majority of heat-induced proteins, and especially the highly expressed ones, are Hsps or variants thereof.

The three *Tegula* species showed several clusters of Hsps between 68 and 90 kDa (Fig. 2A): one cluster with nine proteins of 71 kDa contained the most highly expressed proteins. There was a single 74 kDa protein. Two clusters contained four proteins of 76 kDa (*T. funebralis* and *T. brunnea* only) and up to six of 77 kDa. There were two (*T. brunnea* and *T. montereyi*)

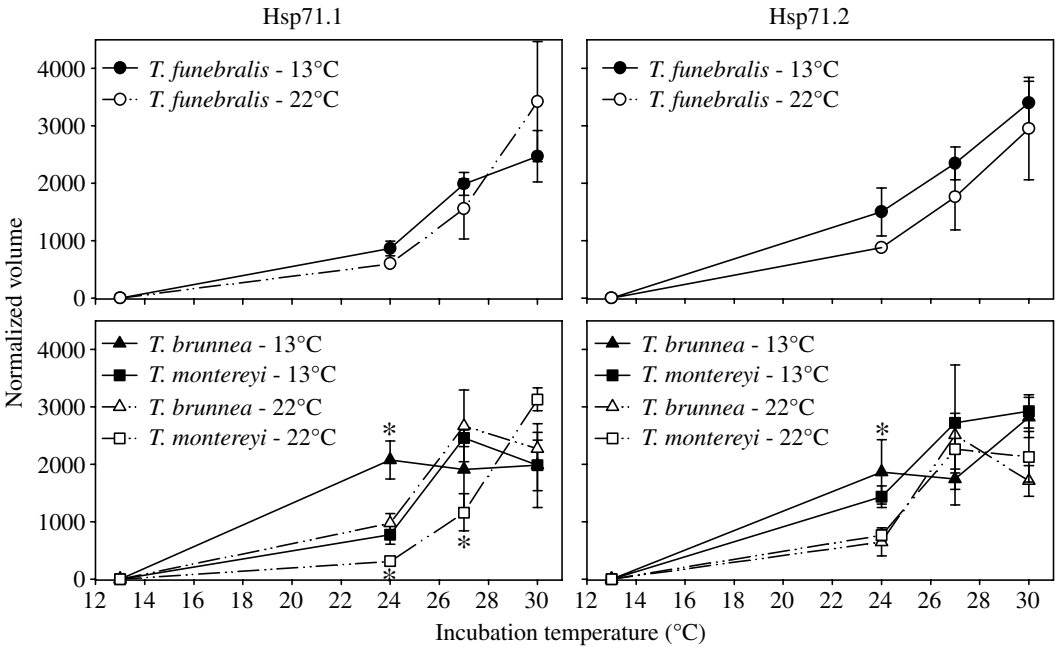

Fig. 3. Normalized volumes (relative to total spot volume within a gel) of acute synthesis of Hsp71.1 and Hsp71.2 in response to temperature in 13°C- and 22°C-acclimated specimens of *T. funebralis*, *T. brunnea* and *T. montereyi*. Values are means ± 1 S.E.M. **P*-value for the interaction effect of the specific acclimation and incubation temperature of either <0.05 or <0.10, located nearest the specific species symbol (see Table 1).

Table 1. *Probabilities for a main effect of acclimation (MEA) and an interaction effect of acclimation and incubation temperatures (24°C, 27°C and 30°C) on heat-shock protein synthesis*

	Tegula funebralis				*Tegula brunnea*				*Tegula montereyi*			
Hsp	MEA	24°C	27°C	30°C	MEA	24°C	27°C	30°C	MEA	24°C	27°C	30°C
24.1							0.1		0.05	0.05	0.05	0.05
24.2			0.05									
24.3									0.05	0.05	0.05	0.05
25.1	0.05							0.1		0.05	0.05	
27.1					N/A				N/A			
27.2										0.05		
40.1										0.1	0.05	
40.2							0.1					
40.3					N/A							
71.1						0.1				0.1	0.1	
71.2						0.1						
71.3										0.1		
71.4						0.05				0.05	0.05	
71.5						0.1						
71.6					0.05	0.1						
71.7												
71.8		0.05					0.05		0.05	0.05	0.05	0.05
71.9						0.05						0.1
72.1		N/A				N/A						
72.2		N/A				N/A						
72.3		N/A				N/A						
72.4		N/A				N/A					0.1	
74.1	0.05						0.05		0.05	0.05	0.05	0.05
76.1		0.05				0.05	0.05			N/A		
76.2						0.1	0.05			N/A		
76.3						0.05				N/A		
76.4						0.05				N/A		
77.1						N/A				0.05		
77.2						0.05	0.05					
77.3												
77.4				0.1		0.05						
77.5						0.1						
77.6						N/A						0.1
88.1					0.05		0.1	0.05			0.05	0.1
88.2						N/A					0.05	
88.3						0.05						

N/A, no detectable synthesis.

or three (*T. funebralis*) 88 kDa proteins. *T. montereyi* synthesized a cluster of four 72 kDa proteins, but lacked the 76 kDa cluster (Fig. 2A). *T. brunnea* did not express one of three heat-induced 40 kDa proteins (Fig. 2B). All species shared five proteins of about 24–25 kDa (Fig. 2C).

The effect of acclimation on acute Hsp synthesis

To examine the phenotypic plasticity of acute Hsp synthesis I acclimated snails of all three species to 13°C and 22°C, conditions within the seawater temperature range of *T. funebralis*, but at the high extreme of what *T. brunnea* and *T. montereyi* can tolerate. Of the >30 heat-induced proteins that I found in *Tegula*, Hsps71.1–71.4 were the most highly expressed (Figs 2A, 3). Acclimation decreased acute synthesis in three out of the four Hsp71s in the subtidal species, *T. brunnea* and *T. montereyi*, but not in the intertidal species *T. funebralis* (Table 1). The remaining four Hsp71s (Fig. 2A) were induced to one third or less of the level of Hsp71.1-4 (data not shown). Acclimation attenuated synthesis levels of these Hsps more in *T. brunnea* (Hsp71.5, 71.6 and 71.9) and *T. montereyi* (Hsp71.9) than in *T. funebralis* (Table 1). Four additional 71 kDa isoforms that were not shared by all species had more acidic isoelectric points (pI) than Hsp71.1 and were infrequently expressed (Figs 1B, 2A).

Hsp71.8 and 74.1 (Fig. 4) were heat-induced as well as constitutively expressed at 13°C (preliminarily confirmed with western analysis, data not shown). Acclimation to 22°C led to an increase in acute synthesis under heat-shock in both *T.*

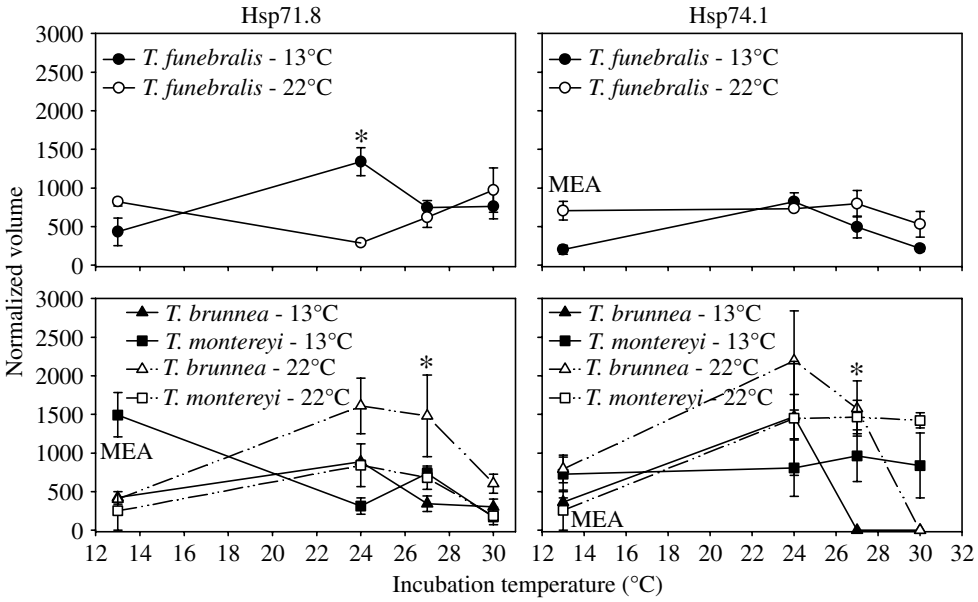

Fig. 4. Normalized volumes (relative to total spot volume within a gel) of Hsp71.8 and Hsp74.1 in response to temperature in 13°C- and 22°C-acclimated specimens of *T. funebralis*, *T. brunnea* and *T. montereyi*. Values are means ± 1 S.E.M. **P*-value for the interaction effect of the specific acclimation and incubation temperature of either ≤0.05 or ≤0.10, located nearest the specific species symbol (see Table 1). MEA indicates a main effect of acclimation (see Table 1 for additional *P*-values for the interaction effects for this species).

brunnea and *T. montereyi* (Hsp74.1 only; Table 1). The synthesis of both proteins changed with acclimation in *T. funebralis* (Table 1). Synthesis levels of Hsp71.8 decreased at an incubation of 24°C; Hsp74.1 levels increased overall (significant main effect for acclimation).

Acclimation had the greatest effect on synthesis of Hsp77s in *T. brunnea* (Hsp77.2; 77.4 and 77.5; Fig. 5), and a lesser effect in *T. montereyi* (Hsp77.1) and *T. funebralis* (Hsp77.4; Table 1). *T. montereyi* and *T. funebralis* expressed two additional proteins (Hsp77.1 and 77.6) that I did not detect in *T. brunnea* (Fig. 2A).

Hsp76.1–76.4 were only detected in *T. funebralis* and *T. brunnea* (Fig. 2A). Synthesis levels decreased after acclimation to 22°C in four proteins detected in *T. brunnea* but only in one (Hsp76.1) in *T. funebralis* (see Hsp76.3 in Fig. 5 and Table 1).

Gill tissue of *T. montereyi* expressed a unique cluster of four 72 kDa proteins. Acclimation led to the attenuation of Hsp72.4 synthesis only (Fig. 5 and Table 1).

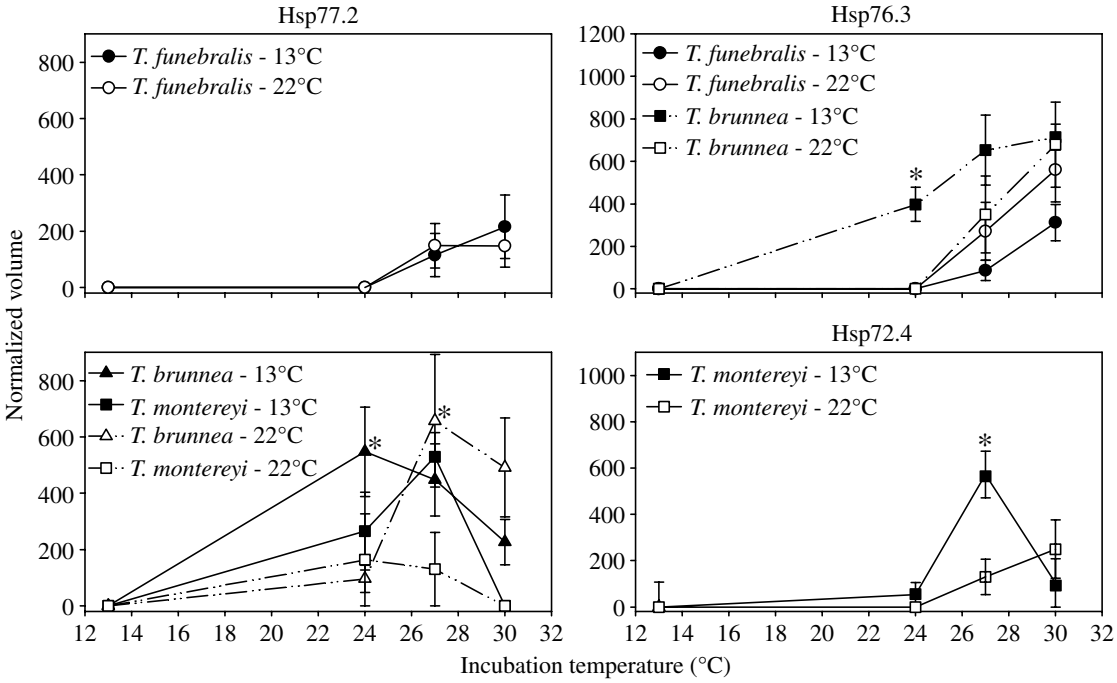

Fig. 5. Normalized volumes (relative to total spot volume within a gel) of Hsp77.2, Hsp76.3 (*T. funebralis* and *T. brunnea* only) and Hsp72.4 (*T. montereyi*) in response to temperature in 13°C- and 22°C-acclimated specimens of *T. funebralis*, *T. brunnea* and *T. montereyi*. Values are means ± 1 S.E.M. **P*-value for the interaction effect of the specific acclimation and incubation temperature of either ≤0.05 or ≤0.10, located nearest the specific species symbol (see Table 1).

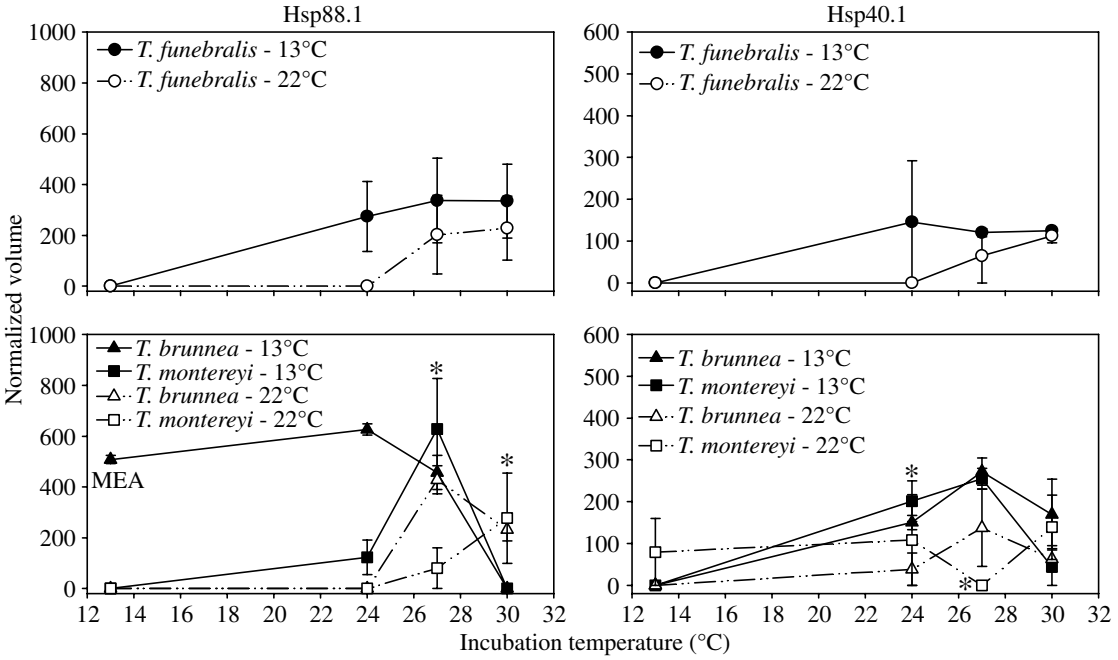

Fig. 6. Normalized volumes (relative to total spot volume within a gel) of Hsp88.1 and Hsp40.2 in response to temperature in 13°C- and 22°C-acclimated specimens of *T. funebralis*, *T. brunnea* and *T. montereyi*. **P*-value for the interaction effect of the specific acclimation and incubation temperature of either ≤0.05 or ≤0.10, located nearest the specific species symbol (see Table 1).

In response to heat, all species synthesized at least two proteins of about 88 kDa (Fig. 2A, not seen in all gels). Acclimation attenuated the synthesis of Hsp88.1 in *T. brunnea* and *T. montereyi*, but not in *T. funebralis* (Fig. 6 and Table 1). Synthesis of the two additional proteins, Hsp88.2 and Hsp88.3, was downregulated in *T. montereyi* and *T. brunnea*, respectively (Table 1).

Three proteins of about 40 kDa were synthesized in response to heat in *T. funebralis* and *T. montereyi* (Fig. 2B). *T. brunnea* did not synthesize one of the proteins (Hsp40.3). Acclimation attenuated the acute synthesis of Hsp40.2 in *T. brunnea* and of Hsp40.1 in *T. montereyi* (Fig. 6 and Table 1).

Hsp24.1 was the most highly expressed small Hsp and reached levels similar to the major Hsp71s (Figs 2C and 7). Acclimation affected its synthesis in all species except *T. funebralis*. However, whereas synthesis decreased overall after acclimation to 22°C in *T. montereyi*, in *T. brunnea* synthesis levels increased at an incubation temperature of 27°C following acclimation to 22°C. Acclimation changed the synthesis of Hsp24.2 and Hsp24.3 in *T. funebralis* and *T. montereyi*, respectively (Table 1). Acute synthesis of Hsp25.1 was significantly attenuated after acclimation to 22°C in *T. brunnea* (30°C incubation) and in *T. montereyi* (24°C and 27°C incubation); but overall upregulated in *T. funebralis* (Table 1). Hsp27.1 was only expressed in *T. funebralis* (Fig. 2C). Hsp27.2 was expressed in all three species and changed with increasing acclimation temperature in *T. montereyi* (Table 1).

Species differed not only in how acclimation affected acute

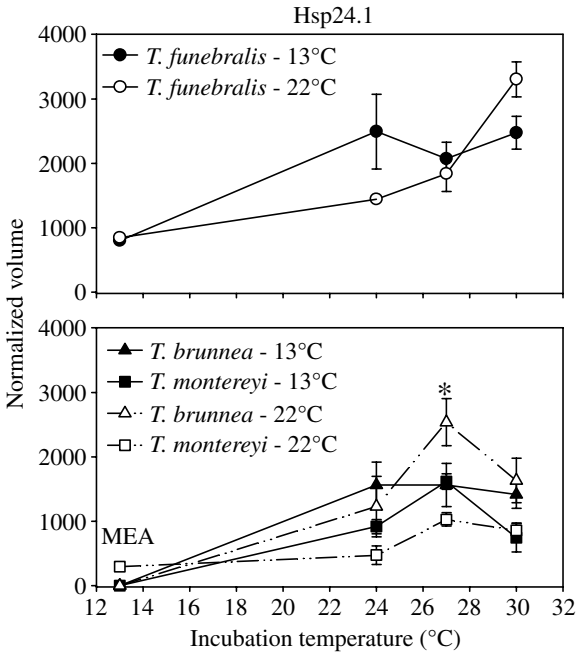

Fig. 7. Normalized volumes (relative to total spot volume within a gel) of Hsp24.1 in response to temperature in 13°C- and 22°C-acclimated specimens of *T. funebralis*, *T. brunnea* and *T. montereyi*. Values are means ± 1 S.E.M. **P*-value for the interaction effect of the specific acclimation and incubation temperature of either ≤0.05 or ≤0.10, located nearest the specific species symbol (see Table 1). MEA indicates a main effect of acclimation (see Table 1 for additional *P*-values for the interaction effects for this species).

Hsp synthesis but also in the T_{on} of Hsp synthesis within the fairly narrow range of incubation temperatures used in the present study, although the study was not designed to detect possible differences in T_{on} at lower temperatures (<24°C; Tomanek and Somero, 1999). For three out of the four shared Hsp77s *T. brunnea* and *T. montereyi* induced synthesis at 24°C, whereas *T. funebralis* did not initiate synthesis until 27°C after acclimation to 13°C and 22°C (Fig. 5). Following acclimation to 13°C, the synthesis of all four Hsp76s was induced at 24°C in *T. brunnea*, but at 27°C in *T. funebralis* (Fig. 5). Hsp40.1 showed a significant effect with an increase in incubation temperature to 24°C in *T. brunnea* and *T. montereyi*, and with an increase to 27°C in *T. funebralis* (Fig. 6).

Discussion

In *Tegula* congeners, phenotypic plasticity of the HSR is protein-specific and varies among species that differ in thermotolerance. Synthesis of almost all the major Hsps was attenuated after acclimation to 22°C (*vs* 13°C) in the heat-sensitive subtidal species, *T. brunnea* and *T. montereyi* (Figs 3–7 and Table 1). There were noticeable differences between the two species, however, specifically in the number of Hsp77s whose synthesis changed with acclimation. In contrast, in *T. funebralis* acclimation changed synthesis levels only for a small subset of Hsps (Hsp24.2, Hsp25.1, Hsp71.9, Hsp76.1 and Hsp77.4), including two constitutively expressed Hsps (Hsp71.8 and Hsp74.1), and not for the major Hsps (Hsp24.1, Hsp71.1-4, Hsp76.2-4 and Hsp77.1-6) (Figs 3–7 and Table 1).

The choice of acclimation temperatures (13°C and 22°C) was based on a previous study in which we used one-dimensional gel electrophoresis (Tomanek and Somero, 1999). In this study we found that an increase in acclimation temperature by 5°C can change T_{on} and the temperature of maximal Hsp synthesis (T_{peak}) for some Hsps. The upper acclimation temperature (22°C) was chosen because it is the highest temperature that the two subtidal species can tolerate over the 21-day acclimation period and is only a few degrees below temperatures at which *T. funebralis* shows the first signs of mortality when kept constantly submersed (personal observation). Although the results of the previous and the present study differ in detail, both show a greater shift in T_{on} (attenuation of acute Hsp synthesis) for the highly expressed Hsp70s in *T. brunnea* and *T. montereyi* than in *T. funebralis*. In the present study, however, I was able to resolve the bands of acutely expressed proteins into a complement of over 30 protein variants that are represented by spots that differ in molecular mass and isoelectric point (pI).

Variation within the complement

When compared to the Hsp complements of tropical fish of the genus *Poeciliopsis*, the eurythermal teleost *Gillichthys mirabilis*, *Drosophila* and intertidal limpets, *Tegula* snails show a surprisingly high number of Hsp variants (Garbuz et al., 2003, 2002; Kültz, 1996; Norris et al., 1997, 1995; Sanders et al., 1991; White et al., 1994). At this point I do not know if these variants are true isoforms (paralogous homologs) or represent post-translationally modified proteins. But a common molecular mass, proximate isoelectric points (pI), similar expression patterns and known post-translational modifications (PTM) of Hsps suggest that several of the proteins are more closely related to each other and are members of a common Hsp family. For example, Hsp71.1-4 are the most highly expressed Hsp70s, and the synthesis of three out of the four was significantly attenuated in *T. brunnea* and *T. montereyi* after acclimation to 22°C (Table 1). These proteins are likely paralogs of Hsp70.

Several highly induced Hsps are clustered around a similar molecular mass and pI (Hsp77s, 76s and 72s; Figs 1, 2). Since there is no report on the clustering of so many isoforms within an Hsp family, it is possible that some of these variants represent PTMs. For example, the mammalian BiP (or Grp78) binds to unfolded proteins in the endoplasmic reticulum and its function is in part regulated *via* phosphorylation (Gething, 1997); and a mammalian mitochondrial Hsp70 is characterized by its Ca^{2+}-dependent autophosphorylation activity (Leusteck et al., 1989). Phosphorylation is commonly observed to regulate the activity and cause the variation in synthesis patterns of small Hsps (Arrigo and Landry, 1994; Norris et al., 1997). The extent and role of PTMs must be addressed further to assess their importance for creating the patterns of Hsp variants in *Tegula* and their contribution to the differences in phenotypic plasticity within a cluster of Hsps.

Interspecific differences in phenotypic plasticity of Hsp synthesis were not only common between *T. funebralis* on the one hand and the two subtidal species on the other, but the plasticity of the Hsp77 cluster varied even between *T. brunnea* and *T. montereyi* (Fig. 2A and Table 1). After acclimation, the synthesis of three out of four variants changed in *T. brunnea*, but only one did in *T. montereyi.* Furthermore, these two species either expressed the Hsp76 (*T. brunnea*) or Hsp72 (*T. montereyi*) cluster (Fig. 2A), but not both.

Two constitutively expressed Hsps, 71.8 and 74.1, were also expressed during heat-shock (Fig. 4). I confirmed their identity with an anti-Hsp70 antibody that I had used previously (Tomanek and Somero, 2002). The synthesis of both proteins changed with acclimation in all three species; the direction of change differed among the species in case of Hsp71.8, but all species showed higher levels of Hsp74.1 synthesis after acclimation to 22°C (Fig. 4). Although acclimation affected the synthesis of several heat-induced proteins in *T. funebralis*, their expression levels were comparatively low. Thus, changes in the synthesis of Hsp71.8 and 74.1 suggest that the effect of acclimation on Hsps depends on differences in their expression pattern (constitutive and inducible *vs* inducible only).

Phenotypic plasticity and heat tolerance

Although *T. funebralis* tolerates higher temperatures (Tomanek and Somero, 1999), its ability to modify the stress response during thermal acclimation is limited in comparison to *T. brunnea* and *T. montereyi* (Figs 3–7 and Table 1). In

addition to the evidence presented here, previous work showed that *T. funebralis* does not adjust steady-state levels of two Hsp70 bands, Hsp90 and the heat-shock transcription factor 1 (HSF1) after acclimation (Tomanek and Somero, 2002). On the other hand, *T. funebralis* recovers from a heat-shock at a faster rate (Tomanek and Somero, 2000), has higher steady-state levels of HSF1 (Tomanek and Somero, 2002), survives thermally stressful low-tide periods better than *T. brunnea* (Tomanek and Sanford, 2003), and synthesizes Hsps over a much wider temperature range than the two subtidal species (Tomanek and Somero, 1999). All of these interspecific differences suggest that *T. funebralis* is adapted to cope with the greater thermal variation that it experiences in the mid-intertidal zone, but has a limited ability to modify the synthesis of the major stress proteins. It has been suggested that the function of acclimation is not '*to maximize performance at warm temperatures, but rather to protect against the possibility that the environment will become even hotter*' (Huey et al., 1999; Leroi et al., 1994). *T. funebralis* may have lost the capacity to modify the acute expression of many of its Hsps, in part because it is adapted to the frequent and extreme temperature changes that are typical for the mid-intertidal zone. By occupying a thermal zone that becomes warmer on a daily basis, for example during midday low-tide periods, *T. funebralis* may have opted for maximal possible biochemical protection from thermal insults, and thereby almost eliminated the need for any further modifications with increasing acclimation temperature. The interpretation of acclimation as a means by which to prepare for a warmer environment thus provides a conceptual framework to explain the limited acclimatory capacity in more eurythermal animals.

The limited plasticity of acute Hsp synthesis in *T. funebralis* also suggests a possible explanation for the observation that more heat-tolerant organisms are less capable of increasing tolerance to even greater heat stress. Clones and siblings of five organismal groups, including *Coelenterata*, *Arthropoda*, *Echinodermata* and *Amphibia*, which showed greater heat tolerance initially, acquired less of an increase in tolerance after acclimation to higher temperatures (Ushakov et al., 1977); strains and populations of *Drosophila* that were found or raised in warmer thermal habitats or temperatures during laboratory selection showed not only higher heat tolerance but also a limited short-term capacity to increase tolerance to greater heat stress, e.g. heat-hardening (Cavicchi et al., 1995; Hoffmann et al., 2003; Zatsepina et al., 2001); and more heat-tolerant porcelain crabs of the genus *Petrolisthes* show a lower capacity to acclimate the upper thermal limits of cardiac function (Stillman, 2003). To date, no other cellular mechanism has been proposed to explain this relationship.

Interspecific variation in Hsp expression and implications for vertical zonation

An important objective of this study and our previous studies has been to elucidate how the interspecific variation in Hsp expression patterns contributes to setting the vertical distribution limits of *Tegula* congeners. Our previous comparisons of the T_{on} of Hsp synthesis and the higher body temperatures that snails experience in the mid-intertidal zone suggested that *T. funebralis* activates the HSR frequently under natural conditions (Tomanek and Somero, 1999). One of the hallmarks of the HSR is the strong and preferential synthesis of Hsps at elevated temperatures, while the synthesis of non-stress proteins is suppressed (Lindquist, 1993; Storti et al., 1980). Fig. 1A illustrates the attenuation of synthesis of most non-stress proteins at a temperature that *T. funebralis* frequently experiences in the mid-intertidal zone (30°C). Thus, *T. funebralis* not only has to bear the costs of increased chaperoning activity but also of the disruption of protein homeostasis during midday low-tide periods that are most likely to activate Hsp synthesis. We know from studies on *Drosophila* and yeast that there are substantial metabolic costs associated with the HSR (Krebs and Feder, 1997; Sanchez et al., 1992). This may explain the slower growth rates shown by *T. funebralis* in comparison to *T. brunnea* and *T. montereyi* (Frank, 1965; Paine, 1969; Watanabe, 1982). Two-dimensional gel analysis improves the identification and detection of Hsp synthesis and will allow us to clarify when and to what extent the *de novo* synthesis of Hsps occurs under natural conditions, and thus to assess the costs of being able to cope with a thermal niche as extreme as the mid-intertidal zone.

The present study raises several new questions that have to be answered to better understand the complex role of the interspecific variation in Hsp synthesis and acclimation ability in setting limits to the thermal environment an organism can occupy. What do the many heat-induced protein variants represent: Hsp-families, Hsp-isoforms or their PTMs? What limits phenotypic plasticity on the cellular level: higher levels of HSF1, the interaction of a multi-chaperone complex with HSF1, or regulatory steps downstream of HSF1 binding to the Hsp promoter (Buckley et al., 2001; Morimoto, 1998; Tomanek and Somero, 2002)? Why does acclimation affect Hsps differently (e.g. Hsp77s), even among species that are similar in heat tolerance: are the likely explanations transcriptional effects or organelle-specific functions? What are the relevant PTMs of heat-induced proteins in *Tegula* and how do they contribute to the interspecific differences in heat tolerance? More specifically, are the Hsp76 and Hsp72 clusters homologous, and if not, how do these proteins differ and what is their function (thus explaining the interspecific differences in T_{on} of Hsp76 shown in Fig. 5)?

Another urgent issue is the question of how global climate change will affect organisms that differ in heat tolerance and phenotypic plasticity. Although more heat-tolerant organisms seem poised to cope better with an increase in average temperature and the occurrence of thermal extremes, their physiological limits are close to their maximal body temperatures (Somero, 2002) and they show a limited capacity to further modify these limits, so they may turn out to be particularly vulnerable to even a small increase in temperature.

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