AN ABSTRACT OF THE DISSERTATION OF

Lars Tomanek for the degree of Doctor of Philosophy in Zoology presented on September 2, 1999. Title: The Heat Shock Response and its Regulation in Congeneric Marine Snails (Genus *Tegula*) from Different Thermal Habitats: Implications for the Limits of Thermotolerance and Biogeographic Patterning.

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George N. Somero

Thermal stress sufficient to cause cellular damage activates the heat shock response, the enhanced expression of a group of molecular chaperones called heat shock proteins (hsp's). I compared phenotypic variation and its adaptive importance in the heat shock responses of several congeneric gastropod species (genus *Tegula*) that occupy thermal habitats differing in absolute temperature and change of temperature. Temperatures of onset (T_{on}), maximal level (T_{peak}) and inactivation (T_{off}) of new synthesis of hsp70 (and protein synthesis *per se*) were lowest during short-term heat stress in the temperate low-intertidal to subtidal *T. brunnea* and *T. montereyi*, intermediate in the temperate mid- to low-intertidal *T. funebralis* and highest in the subtropical intertidal *T. rugosa*. T_{off}'s correlated closely with thermotolerance limits. T_{off}'s of *T. brunnea* and *T. montereyi* were within the range of temperatures that are commonly experienced in the mid-intertidal by *T. funebralis*. T_{off} of the temperate *T. funebralis* was lower than the

highest temperatures experienced by the subtropical *T. rugosa*. For *T. funebralis* and *T. rugosa*, T_{off}'s were only slightly above their highest body temperatures. Thus, T_{off} is likely to play an important role in setting limits to thermotolerance and distribution. Acclimation to various temperatures changed T_{on} and T_{peak}, but not T_{off}. Field-acclimatized snails showed an attenuated heat shock response in comparison to laboratory-acclimated specimens, suggesting that factors other than temperature also regulate hsp synthesis. Onset, duration and intensity of the time course of the heat shock response following exposure to 30°C, a frequently experienced temperature for *T. funebralis* but not for *T. brunnea*, indicated that the mid-intertidal species, but not the subtidal species, can repair thermal damage and prepare for a subsequent exposure within a high-tide period. Genetically fixed interspecific and acclimation-induced intraspecific differences in T_{on} are likely to be in part regulated by an hsp70 isoform, hsp72. These results indicate that parts of the model of the cellular thermometer apply to laboratory acclimation. In summary, interspecific variation of features of the heat shock response appear to be of great importance in setting limits to thermotolerance and vertical distribution ranges along the subtidal to intertidal transition.

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The Heat Shock Response and its Regulation in Congeneric Marine Snails (Genus *Tegula*) from Different Thermal Habitats: Implications for the Limits of Thermotolerance and Biogeographic Patterning

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Lars Tomanek, Author

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The five years of my graduate studies have challenged me intellectually, helped me grow personally, rewarded me for my work and let me discover new questions in biology that I look forward to studying in the future. I completed my studies only because of the great support I received from numerous people in an atmosphere that allowed me to explore uninhibitedly my intellectual ideas in biology. I would like to thank Dr. Chuck Baxter and Dr. James Watanabe, who first told me about the *Tegula* snails and helped me to find and identify them. Jim deserves further thanks for his help with the statistical analyses. Dr. Michael Hellberg informed me about his phylogeny of the genus *Tegula* and thereby helped me to choose the right species for comparison. Several people were of great help during experimentation: Rachael Ream and George Somero helped with the metabolic labeling experiments, Hilary Waites did several of the enzyme thermal stability measurements and Shanshan Bao and Susan Han helped with the immunoblots. Special thanks go to Dr. Andrew Gracey for collecting *Tegula rugosa*.

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I
Dedicate
this Dissertation

to

Kalle, Johannes and Gregor

The Friends that Helped me
to Discover Nature

and to

Ruth

Whose Love Helped me to Accomplish my Studies

And to

My Mother Edith

Who Believed in me Living to Study Life

The Heat Shock Response and its Regulation in Congeneric Marine Snails (Genus *Tegula*) from Different Thermal Habitats: Implications for the Limits of Thermotolerance and Biogeographic Patterning

Chapter 1

Introduction

Temperature is one of the most ubiquitous of all physical challenges that the environment imposes on biological systems. Temperature affects all levels of biological organization – from biogeographic distributions to the stabilities of chemical bonds in biological macromolecules (Brown and Lomolino, 1998; Cossins and Bowler, 1987; Hochachka and Somero, 1984; Hoffmann and Parsons, 1991; Johnston and Bennett, 1996; Somero, 1997). The two main objectives of my thesis are, first, to demonstrate if and to what extent thermal properties of an important biochemical trait, the heat shock response, correlate with and limit the thermal niche that a species occupies and, second, to show that this correlation is likely to be maintained by natural selection. Natural selection targets the phenotype and thus acts when heritable phenotypic variation causes consistent differences in fitness among differing biological entities (Futuyma, 1998). It is important that the phenotypic variation that causes fitness differences can be

inherited, because otherwise natural selection would not have any effect on the evolution of this variation.

The Study of Adaptation

Phenotypic variation that is found to be associated with fitness differences would be called adaptive. The term adaptation can be applied to a trait that confers high fitness because of its current function regardless of its evolutionary origin (Reeve and Sherman, 1993) or, in a more restricted sense, only if the trait has been shown to have evolved specifically in response to a selective agent and that it is beneficial to the organism in regard to this agent (Harvey and Pagel, 1991). When this distinction was first proposed by Gould and Lewontin (1979), it was an important reminder to distinguish between variation that is solely based on phylogenetic distance, and therefore likely to be caused by chance, and variation that is based on selective pressures. Mayr (1983) responded by pointing out the "epistemological dilemma" that arises when we attempt to distinguish between a trait caused by chance or natural selection. Change (or phenotypic variation) by chance can not be disproved for any evolutionary process, but selection can be shown to be the probable cause of variation if the particular trait can be shown to be favored by selection, i. e. results in fitness differences under current conditions. Thus, in contrast to Mayr (1983), for Gould and Lewontin (1979) chance rather than selection constitutes the null hypothesis. Neither position can be taken in its entirety without encountering methodological problems. Often it is impossible to

delineate completely the evolutionary history of a particular trait. But Mayr's position (1983) encompasses the danger of inferring adaptive variation where traits differ because of chance alone.

I adopted a methodological approach that, first, reduces the probability of chance by comparing closely related biological entities, congeneric species, that share a similar biology but occupy differing thermal niches, and that, second, evaluates the evidence for selection by the trait's current contribution to fitness in context of each congener's ecology. Consistent variation in physiological and biochemical traits in regard to temperature among congeneric species occupying widely differing thermal niches is therefore likely to be favored by selection, and thus can be considered adaptive. Similar comparative studies among closely related biological entities, i. e. populations and congeneric species, have been an effective means to identify morphological, physiological, biochemical and molecular traits that are adaptive, because they are likely to have arisen due to the selective pressures imposed by differing thermal environments (Mitton, 1997; Powers and Schulte, 1998; Somero, 1997; Stillman, 1998). Although the analysis of adaptive differences among species within radiations that occurred over an evolutionary time period restricts our ability to control for many potentially confounding factors, the results ensure an evolutionarily realistic scenario that is controlled for "the totality of traits and qualities" which a genotype produces in a given environment (Dobzhansky, 1956). Therefore, by combining information on the heat shock or stress response and the thermal properties of proteins, I

hypothesized that a group of congeneric marine snails of the genus *Tegula*, which occupy widely differing thermal niches along the subtidal to intertidal transition along the Pacific coast of North America, would differ phenotypically in an adaptive fashion.

The Study System: The Genus *Tegula*

The genus *Tegula* (family Trochidae, order Archaeogastropoda, subclass Prosobranchia, class Gastropoda) arose approximately 15 million years ago and presently includes over 40 species. Species of this genus are found from the northern to the southeastern Pacific and in the Caribbean (Hellberg, 1998). Among the various clades of the genus are a Californian subtidal radiation (*T. regina*, *T. montereyi* and *T. brunnea*) and a Californian-Baja Californian intertidal radiation (*T. funebralis*, *T. gallina* and *T. rugosa*; Hellberg, 1998). Species of these two *Tegula* radiations thus occupy widely differing thermal microhabitats along the transition from the subtidal to the intertidal zone, and they differ in their latitudinal geographical distribution ranges. Three temperate *Tegula* congeners that differ in their geographical range occupy distinct vertical distribution ranges at Hopkins Marine Station (HMS) in Pacific Grove, California: *Tegula funebralis* can be found from the low- to the mid-intertidal at HMS and has the widest latitudinal range, from Vancouver Island, British Columbia, Canada, to central Baja California, Mexico (Abbott and Haderlie, 1980; Hellberg, 1998; Riedman *et al.*, 1981). *Tegula brunnea* and *T. montereyi* occupy the subtidal to low-intertidal zone at HMS, and

are distributed from Cape Arago, Oregon, USA, to the Channel Islands, California, USA, and from Sonoma County, California, to the Channel Islands, respectively (Abbott and Haderlie, 1980; Hellberg, 1998; Riedman *et al.*, 1981). A fourth species that I included in my comparison, the subtropical mid-intertidal *T. rugosa*, is endemic to the northern part of the Gulf of California (Hellberg, 1998).

Species of the genus *Tegula* have been the focus of ecological and developmental studies. Ecological studies either focused on the distribution limits of the intertidal *T. funebralis* (Byers, 1983; Byers and Mitton, 1981; Doehring and Phillips, 1983; Fawcett, 1984; Frank, 1975; Markowitz, 1980; Paine, 1969; Paine, 1971) or of subtidal *Tegula* congeners (Lowry *et al.*, 1974; Riedman *et al.*, 1981; Schmitt, 1985; Watanabe, 1983; 1984a; 1984b). These studies focused mainly on biotic factors such as predation, competition, food preference and recruitment as the potential mechanisms that limit vertical distribution ranges. It is known that *T. funebralis* undergoes a pelagic larval development and starts to metamorphose after approximately six days (Moran, 1997). Paine (1969) described a size-gradient for *T. funebralis* along its vertical distribution range. Larvae settled and smaller snails were found higher in the intertidal than their larger and therefore older conspecifics. Paine (1969) showed that this gradient was maintained in part by lower growth and reproduction rates that were observed for larger snails in higher sites of occurrence. Other studies, in contrast, showed that larvae first settle low in the intertidal and move up in response to the threat of predation by the asteroid *Pisaster* (Markowitz, 1980). The size-gradient was further shown to be inherent to

T. funebralis when tested under laboratory conditions (Doehring and Phillips, 1983). Such habitat preference is associated with polymorphism of specific enzyme genotypes (Byers, 1983; Byers and Mitton, 1981). Although these studies yielded insight into the role of biotic factors in structuring intraspecific patterns within the vertical distribution range, the underlying mechanisms that either set the upper or lower limits of vertical distribution in *T. funebralis* are still unknown. Preferences in vertical distribution ranges among subtidal *Tegula* congeners are mostly influenced by patterns of larval settlement (Watanabe, 1984b). Potential factors that influence the differences in vertical distribution ranges between subtidal and intertidal *Tegula* congeners have not been the focus of any study to date. Only one other biochemical study that compared protein thermal stabilities has recently focused on closely related species, porcelain crabs of the genus *Petrolisthes*, with such widely differing thermal niches along the subtidal to intertidal transition (Stillman, 1998).

Causes of Vertical Zonation on Rocky Shores

The distinct patterns of vertical distribution ranges in the rocky intertidal have been a focus of study at least since the beginning of this century. Much of the early work on the factors that limit vertical distribution ranges suggested that physical factors, e. g., temperature and desiccation (Baker, 1909; Broekhuysen, 1940; Newell, 1979; Southward, 1959), might set the upper distribution limits of many intertidal organisms. Although this is probably the case for most high-

intertidal animal species, upper limits of lower-occurring species are set by a more complex set of biological as well as physical factors, e. g. upper limits of higher- *versus* lower-occurring intertidal limpets (Wolcott, 1973). However, many intertidal algae can withstand physical conditions exceeding those characteristic of the extremes of their upper vertical limit, and grazing rather than physical stress has been demonstrated to set their upper distribution limits (Underwood, 1979; 1980).

In contrast to upper distribution limits, experimental studies by Connell (1961a; 1961b) and Paine (1966; 1969) demonstrated that the lower limits of barnacles in Scotland and mussel beds in Washington State, respectively, are likely to be determined by biological rather than physical factors, e. g. predation.

Although certain physical conditions in the environment were shown to potentially limit the vertical distribution range, many intertidal animals actively evade extreme conditions under natural conditions. In most animals, a thick shell that can either be closed (bivalves) or in which the main body can be withdrawn (some polychaete families, most gastropods) in combination with the secretion of a protective mucus layer and (or) evaporative cooling may prevent body temperatures from rising to lethal levels. However, because emersion exposes marine organisms to multiple stress factors, intertidal organisms reveal modifications of several physiological, biochemical and molecular processes. For example, aerial and aquatic respiration rates were correlated with temperature and emersion profiles in limpets according to intertidal sites, but were also dependent on feeding rates, which in turn correlated with height in the intertidal (for review see Branch, 1981;

Newell and Branch, 1980). Mitochondrial respiration rates and their acclimatory plasticity in regard to temperature have been analyzed in abalone species, genus *Haliotis* (Dahlhoff and Somero, 1993a). Several alternative anaerobic metabolic pathways are unique to intertidal organisms (for review see Zwaan and Mathieu, 1992). The homeostasis of membrane dynamics (Williams and Somero, 1996) as well as mechanisms of freeze-tolerance and -avoidance (for review see Storey and Storey, 1996) are important adaptations under the widely fluctuating thermal conditions that intertidal organisms experience. Nutritional status can provide a collective assessment of how various biotic and physical interactions can influence an organism's physiological performance and growth potential in the intertidal (Dahlhoff *et al.*, in preparation; Dahlhoff and Menge, 1996; Menge *et al.*, 1997). Temperature dependence of functional and structural properties of homologous enzymes varied between species occupying differing thermal niches in the intertidal (Dahlhoff and Somero, 1993b; Stillman, 1998). Studies of genetic polymorphism of intertidal organisms revealed the importance of metabolic pathways that are under strong selection, e. g. nitrogen metabolism *via* aminopeptidase-1 (Byers, 1983; Hilbish and Koehn, 1985) and aspartate aminotransferase (Johannesson *et al.*, 1995) or the metabolism of mannose *via* mannose-6-phosphate isomerase (Schmitt and Rand, 1999). The thermal stress that marine organisms experience during emersion has made intertidal animals a focus of studies of the importance of the heat shock response, a cellular stress response, under natural conditions

(Chapple *et al.*, 1998; Chapple *et al.*, 1997; Hofmann and Somero, 1995; 1996a; 1996b; Roberts *et al.*, 1997; Sanders *et al.*, 1991; Sanders *et al.*, 1992).

Surprisingly, most comparative physiological and biochemical studies have been limited to studying distribution patterns within the relatively homogenous thermal gradient of the intertidal zone, and have not investigated the influence of the very steep gradient of physical factors that characterizes the transition from sites of either frequent or no emersion, i. e. intertidal *versus* subtidal sites. This can partly be attributed to the lack of organismal groups that occupy these widely differing thermal niches, and that are closely related and thereby suited for comparison. A recent exception has been a comprehensive study of *Petrolisthes* congeners, a group of intertidal and subtidal porcelain crabs (Stillman, 1998; Stillman and Somero, 1996). These studies showed that temperate intertidal animals might be living close to their natural thermal maximum in regard to their heart rate (Stillman and Somero, 1996). It is unknown, however, if other physiological and biochemical processes also operate close to the upper thermal maximum and thereby limit the upper end of the vertical distribution range. By comparing several species of the genus *Tegula* that occupy the extremes along the steep thermal gradient from the subtidal to intertidal zone, I took advantage of a near-ideal study system to examine how phenotypic variation in the heat shock response underlies and (or) limits the vertical distribution range of intertidal animals. Thereby I followed the August Krogh principle (Krogh, 1929) that states

that nature provides an "ideal experimental system" to address a certain biological problem.

The Heat Shock Response

Discovery and function of the heat shock response

In 1962, Ritossa observed that increased temperatures changed the pattern of chromosomal puffs in the salivary glands of *Drosophila* (Ritossa, 1962). As temperature increased, puffs that were previously apparent disappeared and new ones appeared. The occurrence of "heat shock puffs" is rapid, and today we know that their occurrence represents an open chromosomal configuration that indicates the activation of gene transcription. Furthermore, the heat shock response is now also referred to as the stress response because it is activated by many diverse environmental, pathophysiological and developmental conditions (Ashburner, 1970; Morimoto, 1998). What was not known for a long time was what these diverse conditions had in common that caused cells to respond repeatedly in a predictable way. The nature of the heat-activated genes and their products was unknown until 1974, when Tissières and Mitchell first demonstrated that the occurrence of puffs coincided with the synthesis of specific proteins (Tissières *et al.*, 1974). What followed was the development of one of the most important research themes in biochemistry and molecular biology in recent decades. The response was soon seized upon as an opportune study system to elucidate the mechanisms of gene regulation. Thus, the heat induced *Drosophila* genes were

among the first eukaryotic genes to be cloned (Artavanis Tsakonas *et al.*, 1979; Craig *et al.*, 1979; Livak *et al.*, 1978; Moran *et al.*, 1979; Schedl *et al.*, 1978). The stretches of DNA upstream of the *hsp* genes, in the promoter region, which regulate the genes' transcription were characterized (Artavanis Tsakonas *et al.*, 1979; Craig *et al.*, 1979; Livak *et al.*, 1978; Moran *et al.*, 1979; Pelham, 1982; Pelham and Bienz, 1982; Schedl *et al.*, 1978) and the transcription factors that interact with these regulatory sequences were identified (Parker and Topol, 1984a; 1984b; Wu, 1980; 1984; 1985).

However, the functional properties of these heat shock proteins (hsp's) were unknown until the mid 1980s. Due to the fact that their synthesis could be induced by several stress factors, hsp's are also called stress proteins. This enigma was only solved by the development of what seemed at first an unrelated field, the folding and oligomerization of proteins. Largely due to the work of Anfinsen (Anfinsen, 1973), proteins were thought to fold spontaneously, with the primary amino acid sequence the only information required to complete the folding process successfully. Anfinsen himself cautioned that the *in vitro* experiments on which these conclusions were based might not represent the *in vivo* folding process in a crowded and highly complex cytoplasm. A study that showed that the protein nucleoplasmin is required for the assembly of nucleosomes provided the first evidence for a "molecular chaperone" (Laskey *et al.*, 1978), defined as a member of a group of unrelated protein families that catalyzes, by non-covalent interactions, the folding of another protein into its native conformation (Ellis, 1996b; Hendrick

and Hartl, 1993). Subsequent work confirmed the "chaperone concept" when it revealed that the chloroplast binding protein binds large subunits of the oligomeric chloroplast ribulose-bisphosphate carboxylase (Rubisco) that were subsequently transferred to the holoenzyme (Musgrove and Ellis, 1986). Pelham first speculated (Pelham, 1986) that hsp's could play a role in the folding and oligomerization of proteins. That hsp's function as molecular chaperones appeared likely only after two studies showed that, (i) the amino acid sequence of the chloroplast binding protein was 50% identical to GroEL (a protein that is necessary for the *gro*wth of the bacteriophage λ), the major prokaryotic hsp of about 60 kDa (Hemmingsen *et al.*, 1988), and, (ii) that GroEL was immunologically similar to heat shock proteins from plants and animal mitochondria (McMullin and Hallberg, 1988). When the type of experiments that Anfinsen conducted were repeated with Rubisco and GroEL added to the refolding buffer, the yield of folding was improved and aggregation after denaturation of Rubisco was reduced (Goloubinoff *et al.*, 1989). These findings led to the clarification of hsp function, and the role of hsp's as molecular chaperones has since been firmly established: Under non-stressful conditions they facilitate the folding, oligomerization, cross-membrane transport and activity of proteins (Becker and Craig, 1994; Bukau and Horwich, 1998; Ellis, 1996a; Ellis and van der Vies, 1991; Georgopoulos and Welch, 1993; Gething and Sambrook, 1992; Hartl, 1996; Hendrick and Hartl, 1993; Welch, 1992). Under stressful conditions hsp's prevent the denaturation of proteins, or facilitate the

renaturation or degradation of already denatured proteins (Parsell and Lindquist, 1993; Parsell and Lindquist, 1994; Sherman and Goldberg, 1996).

Homologies often, but do not always, cluster hsp's into families of a certain size class, e. g. the molecular mass of homologous isoforms of the hsp70 family varies from about 68 to 74 kDa. This pattern is consistent and allows one to infer functional properties of a hsp simply by its association with a certain size class. However, the mass range of homologous proteins can vary substantially, e. g. from 80 to 108 kDa in the case of the hsp90 family (Gething, 1997).

The most ubiquitous and important hsp isoforms that are induced by heat stress belong to the hsp70 family in most organisms (Parsell and Lindquist, 1994). Within the hsp70 family certain isoforms are synthesized only under normal conditions and, therefore, are constitutively expressed (heat shock cognates, hsc); others are only expressed under stress (inducible isoforms, hsp), and some are expressed equally under both conditions (Becker and Craig, 1994; Craig, 1992). However, because expression patterns of members of an hsp family that are encoded by distinct gene loci are only well defined for a few model systems, I will use "hsp" generically to refer to any member or groups of members of a family of hsp's.

Much of our understanding of hsp function has come from studies of the hsp70 family. In their role as molecular chaperones, members of the hsp70 family have been shown to possess a wide range of functions: Hsp70 (1) assists in the folding of newly synthesized proteins, (2) helps in assembling oligomeric proteins,

(3) translocates proteins across membranes, (4) facilitates the degradation of denatured proteins and (5) controls the biological activity of regulatory proteins (Bukau and Horwich, 1998). These processes are assisted by the cooperation of hsp70 with other hsp's, e. g. the J-domain containing hsp40 family that regulates the ATP-turnover rate and thereby the catalytic efficiency of hsp70 (Cyr *et al.*, 1994; Kelley, 1998). Isoforms of the hsp70 chaperone machinery are often found to be compartment- specific, e. g. in the cytoplasm, nucleus, mitochondria, chloroplasts, and endoplasmatic reticulum (Parsell and Lindquist, 1993).

Strong evidence for the role of hsp's in cellular thermotolerance was first provided for vertebrate cell lines, because cell lines of established laboratory model systems are more amenable to molecular analysis than whole organisms (Parsell and Lindquist, 1994). First, induction of thermotolerance correlated most closely with the synthesis of hsp70 (Li and Laszlo, 1985; Li and Werb, 1982). Second, selection of cell lines experiencing hyperthermic treatment yielded lines overexpressing hsp70 (Anderson *et al.*, 1989; Laszlo and Li, 1985). Third, microinjection of hsp70-antibodies into fibroblasts blocked the development of increased thermotolerance whereas microinjection of control antibodies did not (Riabowol *et al.*, 1988). Fourth, transgenic cell lines carrying extra *hsp70* gene copies were more thermotolerant than control cell lines (Angelidis *et al.*, 1991; Li *et al.*, 1991).

Although hsp90 is also synthesized during heat stress, it does not seem to prevent heat inactivation of proteins under *in vivo* conditions and, therefore, seems

to differ from hsp70 in its chaperoning function (Nathan *et al.*, 1997). *In vivo,* hsp90's contribution seems to keep various signal molecules, e. g. steroid receptors and tyrosine kinases, in a binding-competent conformation (Pratt, 1998). The complex regulatory role of hsp90, often in conjunction with other hsp's, is illustrated by recent studies that demonstrated the involvement of hsp90 in regulating several steps of the transcriptional activation of the heat shock response (Ali *et al.*, 1998; Zou *et al.*, 1998).

Of the several other hsp families, members of the yeast hsp100 family seem to be functionally closely related to hsp70, because downregulation of one of the two increases the importance of the other for thermotolerance (Sanchez *et al.*, 1993). The hsp60 chaperone machinery (the prokaryotic GroEL) that is found in the mitochondrial matrix and the chloroplast stroma (Bukau and Horwich, 1998) preferentially binds unfolded rather than native proteins and seems to facilitate their refolding. The hsp60 chaperone machinery also prevents the aggregation of already denatured proteins (Martin *et al.*, 1992). Small hsp's (15 – 30 kDa) also show chaperoning function and are synthesized during heat stress, however, their importance for thermotolerance is still unknown (Arrigo and Landry, 1994; Jakob and Buchner, 1994).

The protective function of heat shock proteins during thermal stress

The induction of the heat shock response is a protective measure to avoid cellular damage by heat or other stress conditions. An understanding of the

protective features of hsp's during stress provides insight into the cellular structures that are thermally perturbed and thereby trigger the signal that results in the induction of the stress response.

In an *in vitro* study it was shown that the prokaryotic hsp70 homolog DnaK can prevent the denaturation of RNA polymerase at high temperatures and renature the unfolded protein as well (Skowyra *et al.*, 1990). In an additional study, DnaK was shown to reactivate firefly luciferase *in vitro* as well as *in vivo*, with an enhancement of reactivation in the presence of the prokaryotic hsp40 DnaJ (Schroder *et al.*, 1993). Hsp's also play a role in the degradation of proteins. Some hsp's are actually proteases, e. g. the hsp104 of *E. coli*, ClpB, is a member of the protease Clp ATPase family (Parsell *et al.*, 1991). Hsp's also assist in proteolysis by keeping denatured proteins in an open conformation that facilitates recognition, ubiquitination and (or) proteolytic cleavage by the ubiquitin-dependent proteolytic pathway (Sherman and Goldberg, 1996). The ubiquitin-dependent degradation of proteins, a major proteolytic pathway in eukaryotes (Peters *et al.*, 1998), is strongly activated by heat shock (Parag *et al.*, 1987). Thus, the major functions of hsp's during thermal stress are to protect proteins from denaturation, refold already denatured proteins, and facilitate the degradation of aberrant proteins that can not be repaired.

Another feature of the heat shock response that protects cells from further thermal damage is the inhibition of synthesis of non-hsp's and the simultaneous preferential translation of hsp messages (Lindquist, 1980; 1981; 1993; Storti *et al.*,

1980). The inhibition of synthesis of non-hsp's redirects the translational activity entirely towards the synthesis of hsp's, and also may prevent further inappropriate interactions among newly synthesized proteins that are more prone to such interactions than hsp's are. The inhibition of synthesis of the normal suite of proteins is an interruption of protein homeostasis and thereby of energy production by various metabolic pathways, a process that in its overall consequences is likely to be energetically costly.

Several mutations in *hsp* genes provide insight into the *in vivo* role of hsp's for thermotolerance. Surprisingly, in view of the overall conservation of amino acid sequences in hsp's (Lindquist, 1986), mutational studies showed that the importance of hsp's for thermotolerance can vary among organisms. Although hsp70 is the major hsp in conferring tolerance to extreme thermal conditions in most organisms and specifically in *Drosophila*, hsp100 seems to play this role in the yeast *S. cerevisae* and *E. coli* (Parsell *et al.*, 1993). Such variation in the importance of hsp's for species' thermotolerance appears to be correlated with taxonomy, not the species' thermal niche.

Another important and recurring theme that arose from work on mutations was the role hsp70 seems to play in down-regulating the synthesis of other hsp's (Parsell and Lindquist, 1994). These observations led to a model of the "cellular thermometer" (Craig and Gross, 1991).

The regulatory function of heat shock proteins during transcriptional activation

To date, it is widely assumed that the heat shock response is activated by the denaturation of proteins during thermal stress. Following thermal denaturation of proteins hsp's that are already present in the cell are activated and thus, hsp's are redirected to a protective function away from their regulatory function as repressors of the induction of the heat shock response (Fig. 1.1). Hsp's repress the stress response under normal conditions by binding to the monomeric form of the heat shock factor (HSF; I will only refer to HSF1, one of the several known members of the HSF family, and the one best understood at present (Satyal and Morimoto, 1998; Wu, 1995)). Due to thermal stress hsp's are sequestered away from the HSF monomers, which can then form the HSF homotrimer that is competent to bind to the heat shock element (HSE), a regulatory promotor sequence that is typically found upstream of *hsp* genes (Pelham, 1982). This cascade of events results in the fast initiation of *hsp* gene transcription. Thus, the balance between hsp70, free HSF monomers, and denatured proteins was suggested to constitute the cellular thermometer (Craig and Gross, 1991). However, this interpretation of the cellular thermometer has now been extended to include several other hsp's, e. g., hsp90 and hsp40, that additionally act as repressors, and even some new members like the heat shock factor binding protein 1, HSPB1, which negatively modulates the response (Morimoto, 1998; Satyal *et al.*, 1998). All of these proteins are present in a multiprotein complex that binds to the HSF and thereby regulates the initiation of transcriptional activation in a yet unresolved way.

Figure 1.1. Regulatory steps in the activation of the heat shock response. Endogenous levels of hsp's of various size classes (e.g., hsp90, hsp70 and hsp40) bind to the heat shock transcription factor 1 (HSF1) and thereby repress the formation of the active (trimeric) form of HSF1. Heat shock or other environmental stress factors cause proteins to unfold and cause hsp's and HSF1 to dissociate. Consequently, hsp's are free to prevent further thermal denaturation and help refold already damaged proteins. HSF1 monomers can trimerize and subsequently bind to the heat shock element (HSE) to activate the transcription of *hsp* genes. Other regulatory elements within the promotor are the CCAT sequence and the TATA box. HSF1 has to be hyperphosphorylated (PPP) to become transcriptionally active ("+" indicates a positive regulatory role). During the transcriptional response several hsp's and a HSF1 binding protein (HSPB1) negatively regulate transcriptional activity ("-" indicates a negative or inhibitory regulatory role). The model of the cellular thermometer predicts that endogenous levels of hsp's, by binding to HSF1, can regulate the onset of the response. It is unknown, whether variation in the temperature of induction can also be based on differences in protein stability, either due to differential expression of proteins in various tissues or protein thermal stabilities that vary among orthologous homologs in different species. For further detail see text.

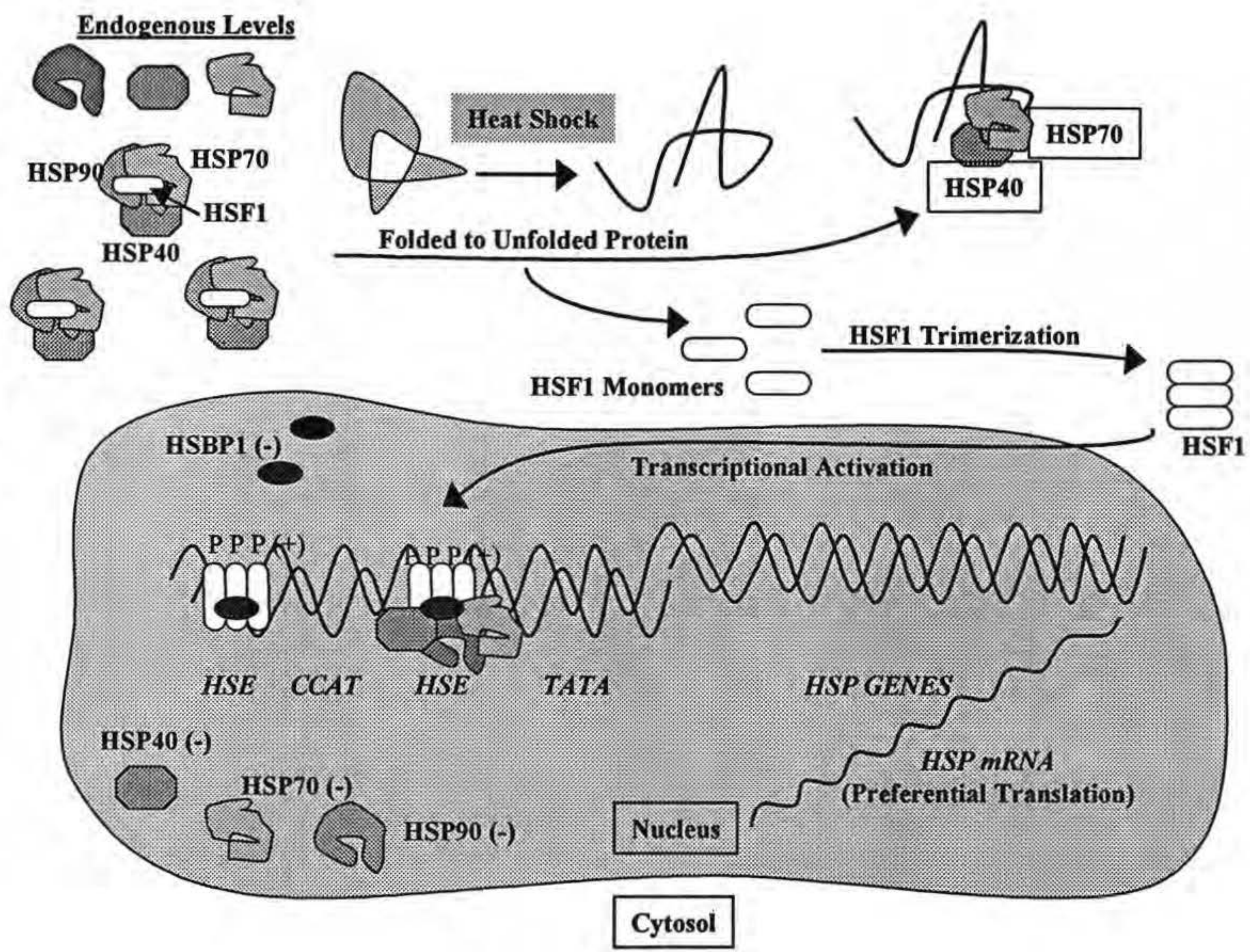

Figure 1.1

In addition to the recent observations regarding the regulatory role several hsp's seem to play during the induction of the heat shock response, Zhong *et al.* (1998) demonstrated that the HSF has an intrinsic regulatory activity that could represent an important component of a cascade of events that constitute the cellular thermometer. Triggered by heat or oxidants, purified HSF monomers can trimerize and subsequently bind to DNA without the presence of other hsp's (Rabindran *et al.*, 1994; Zhong *et al.*, 1998). The potential intrinsic regulatory capabilities of HSF were further highlighted by recent work on the prokaryotic HSF σ^{32}. Temperature-dependent changes in *hsf* mRNA secondary structure seem to control the activity of this transcription factor (Morito *et al.*, 1999). Despite this evidence for the HSF's role in self-regulating its trimerization, strong regulatory roles (as repressors) that have been attributed to hsp's are not excluded by these studies. A regulatory influence on the DNA binding activity of HSF by extrinsic components has been demonstrated on the cellular level by expressing the human HSF in *Drosophila* cells, which resulted in a shift of induction temperature from 42°, typical for human cell lines, to 37°C, the typical induction temperature in *Drosophila* cells (Clos *et al.*, 1993). Furthermore, induction temperature can vary within an organism. Mammalian male germ cells that grow at a lower temperature in the testis show a lower induction temperature in comparison to somatic cells (Morito *et al.*, 1999; Sarge, 1995; Sarge *et al.*, 1995).

Strong evidence indicates that hsp's are the most important repressors of the heat shock response: Hsp70 has been found to be bound to HSF trimers during attenuation of the transcriptional response (Abravaya *et al.*, 1992; Baler *et al.*, 1992; Mosser *et al.*, 1993; Shi *et al.*, 1998) and overexpression of hsp70 or hdj-1/hsp40 under non-stressful conditions prevents the expression of hsp's, probably by repressing the trimerization of HSF (Mosser *et al.*, 1993; Rabindran *et al.*, 1994; Shi *et al.*, 1998)). In addition, hsp90 has been shown to play a regulatory role in the interconversion of the HSF monomers to trimers and, therefore, in the transcriptional response (Ali *et al.*, 1998; Zou *et al.*, 1998).

Together, these observations suggest that variation in induction temperature may depend on HSF itself, the cell type (and therefore the specific cellular environment in regard to the proteins that are being expressed) and on levels of factors extrinsic to the HSF, e. g. hsp's, that seem to self-regulate the translation and transcription of their own genes. Different cell types are likely to differ in the proteins that they express. These proteins may in turn differ in their thermal stability and thereby reset the induction temperature within an organism (Sarge, 1995; Sarge *et al.*, 1995). These findings can be extended to hypothesize that interspecific differences in induction temperature are due to differentially set endogenous levels of hsp's or differing protein thermal stabilities. The term 'endogenous' describes the fact that the level of a particular hsp is likely to be caused by cellular processes that are affected by internal rather than external factors, e. g. the cell cycle instead of ambient temperature. The term is also used

when external factors affect hsp levels over a longer time period, e. g. weeks and month, thus also during acclimation or acclimatization to differing thermal conditions. Acclimatory changes in induction temperature observed within a species may also be based on changing endogenous levels of hsp's (Dietz and Somero, 1992; Roberts *et al.*, 1997). Although unlikely, changes in temperature-dependent protein expression patterns, with more thermally stable isoforms of the most temperature-sensitive proteins expressed at higher acclimation temperatures, may also shift induction temperature. Such a scenario would require that an organism evolve several separate genes for a homologous protein (so-called paralogous homologs) for at least a number of proteins. This has been demonstrated for the myosin light chain protein in carp (Crockford and Johnston, 1990) and the cytosolic form of malate dehydrogenase (Lin and Somero, 1995; Schwantes and Schwantes, 1982a; Schwantes and Schwantes, 1982b) and several others (Somero, 1995). However, for the majority of proteins that have been studied, no multiple paralogous isoforms were found, and if they were observed, an isoform served a tissue- rather than a temperature-dependent function (Somero, 1995).

The role of protein thermal stabilities in the induction of heat shock protein synthesis

The signal for the induction of the heat shock response is commonly held to be the accumulation of misfolded proteins. Experimental evidence supports this hypothesis: Amino acid analogs that resulted in non-native proteins induced the

synthesis of hsp's (Hightower, 1980; Kelley and Schlesinger, 1978), injection of aberrant proteins into *Xenopus* oocytes activated a *hsc70* reporter gene (Ananthan *et al.*, 1986), and a mutant λ repressor induced hsp expression (Parsell and Sauer, 1989). These findings correspond with observations following heat shock which indicate that cytoskeletal proteins are among the first cellular structures that are damaged by heat (Welch and Suhan, 1985). However, the structural integrity and function of several other cellular structures are also severely compromised by heat (Parsell and Lindquist, 1994; Welch and Suhan, 1985). Among the more sensitive cellular structures are membranes, which show changes in surface morphology, an increased bilayer fluidity, and the aggregation of membrane proteins (Parsell and Lindquist, 1994). Therefore, membrane lipoproteins have also been proposed to transduce the signal that initiates the stress response (Carratu *et al.*, 1996; Horvath *et al.*, 1998).

Proteins are only marginally stable at physiological temperatures, because rather than being optimized for the most thermally stable conformation, proteins seem to evolve a balance between conformational flexibility and stability (Jaenicke, 1991; Somero, 1995; 1997). A flexible conformation is required for high catalytic efficiency, but a protein that is too flexible would be too unstable to maintain its proper binding conformation. Because of the balance between stability and flexibility that is necessary for optimal catalytic function, proteins function best under normal physiological temperatures. Thus, proteins can become unstable and unfold at temperatures within a species' thermal range. During unfolding

predominantly hydrophobic side chains that are normally buried inside the protein are exposed ("thermal breathing") and tend to interact inappropriately with hydrophobic side chains of other proteins, causing widespread thermal damage in the heat stressed cell.

Protein thermal stabilities among some orthologous homologs have been shown to correlate with a species' commonly encountered temperature range, or adaptation temperature, e. g. in pyruvate kinase (Low and Somero, 1976), actin (Swezey and Somero, 1982), lens crystallins (McFall Ngai and Horwitz, 1990) and cytosolic malate dehydrogenase (Dahlhoff and Somero, 1993c). However, a broader comparison of homologous proteins among closely related species that differ in thermotolerance is still lacking. In addition, such data are unavailable in any species for which the heat shock response has been characterized under field or laboratory conditions. Consistent interspecific differences among protein thermal stabilities may not only restrict the range of acclimatory plasticity that can be observed for a species, but also may underlie interspecific variations in induction temperature that are maintained even after acclimation to a wide range of temperatures.

The Ecological and Evolutionary Importance of Heat Shock Proteins

Heat shock proteins, thermotolerance and biogeography

Several major questions remain that concern the ecological and evolutionary importance of hsp's. How does the heat shock response explain

differences in thermotolerance at the organismal level? Furthermore, how does the heat shock response vary under differing ecological conditions on an evolutionary time-scale, i. e. a time scale long enough for selection to act on variation; over several weeks, for acclimatory effects to occur; and over minutes to hours, to assess the immediate protective capacity of hsp synthesis? And how is the phenotypic variation that occurs on these time-scales regulated?

Before I review the evidence for the importance of hsp's for thermotolerance on the organismal level, I would like to stress that other biochemical processes and structures are of equal or even greater importance for thermotolerance in various cases under widely differing ecological conditions. Examples include protein conformation and function, the dynamics of membranes and their lipid composition, compatible osmolytes and their protein stabilizing effects, the proteolytic machinery and its importance in eliminating denatured proteins, and at the cold end of the temperature scale, antifreeze and ice-nucleating molecules and their role in freeze-tolerance or freeze-avoidance (Cossins and Bowler, 1987; Gross, 1996; Hochachka and Somero, 1984; Hoffmann and Parsons, 1991; Johnston and Bennett, 1996; Parsell and Lindquist, 1993; 1994; Somero, 1997; Somero and Yancey, 1997).

Although evidence of the important role that hsp expression plays in thermotolerance comes from many studies on unicellular organisms or cell lines of multicellular organisms, the evidence on the organismal level is more difficult to demonstrate, partly due to the difficulties in manipulating organisms genetically.

Most organisms are not amenable for genetic studies, and it is therefore necessary that comparisons of differently thermally adapted organisms and studies of the few "model" systems that can be genetically manipulated complement each other.

The importance of hsp's for thermotolerance on the organismal level was illustrated by experiments in which *Drosophila* embryos that contained 12 extra copies of an hsp70 isoform encoding gene displayed an elevated expression of hsp70 and showed enhanced thermotolerance in comparison to a control strain that included the excision without the additional *hsp70* genes (Welte *et al.*, 1993). However, even the control (excision) strain carried 10 almost identical *hsp70* wild-type genes. Differences in thermotolerance were most pronounced in 6 hour-old embryos. 12 hour-old embryos of both strains did not differ in thermotolerance. Although limited to an early developmental stage, these experiments provided the first direct evidence that the expression of hsp70 influences organismal thermotolerance. Further studies illustrated that larval- and pupae-stages with extra copies overexpress hsp70 and, compared to the excision strain, better survive heat stress that both life-stages may experience under natural conditions (Feder *et al.*, 1996). Although such genetic studies represent a novel and important approach to the study of organismal thermotolerance, they may be limited to intraspecific comparisons alone. Comparisons among different genomes that carry a gene insertion, an experiment in which it would be difficult to control for position effects among species due to technical constraints, could not infer that changes in phenotypic variation are due to the effect of the inserted *hsp* gene(s) alone, because

the genomes potentially differ in other ways that can not be controlled for. This problem could partly be remedied by comparing closely related species that differ in a controlled fashion in their *hsp* genes by insertion, but share a substantial homology among their genomes. However, such experiments can be found by comparing congeneric species that show phenotypic variation in the heat shock response. Thus, comparisons of transgenic species can not substitute for species comparisons in elucidating the importance of phenotypic variation for thermotolerance during a species' evolutionary history.

Although the *Drosophila* studies illustrate the beneficial effects of overexpressing hsp's, other studies indicate that overexpression of hsp's can lower survival and slow development in *Drosophila* larvae under normal conditions that do not induce heat shock (Feder *et al.*, 1992). Larvae that vary naturally in their expression of hsp's show the same trends (Krebs and Feder, 1997). In another example, yeast strains that do not express hsp100 grow faster than the wild-type strain under some nutritional conditions (Sanchez *et al.*, 1992). This may be explained by the high ATP costs that the expression and activity of hsp's entail. Denatured proteins that are targeted by hsp's have to be refolded or proteolytically degraded, and both processes are energetically costly. The preferential translation of hsp's, even if transient, disrupts protein homeostasis and may interrupt the production of ATP.

To date, the most important source of information on how variation in the expression of hsp's results in thermotolerance on the organismal level comes from

comparisons among species that are distributed along environmental gradients (for review see (Feder and Hofmann, 1999). Importantly, an organism encounters a multitude of biotic and abiotic challenges in its environment and it is difficult to discern for which the expression of hsp's is mostly employed. However, due to the large amount of information that is available on hsp's, temperature can be deduced to be the major, although not the sole, environmental factor inducing the heat shock response under natural conditions. The relative importance of temperature as an environmental factor inducing the response under natural conditions is likely to depend on a combination of several factors, for example, the thermal features of the environment, the developmental stage and, of course, the evolutionary history of a species. To date, only one study on freshwater cnidarians (polyps) of the genus *Hydra* has comprehensively investigated the importance of interspecific phenotypic variation in the induction of the stress response and the thermal features of the niches that species occupy (Bosch *et al.*, 1991; Bosch *et al.*, 1988; Gellner *et al.*, 1992).

Several features of the heat shock response have been shown to correlate positively with stress levels discerned from environmental gradients (Feder and Hofmann, 1999). Species differ in the onset, peak and cessation temperature of the response and the level of constitutively expressed hsp's (Feder and Hofmann, 1999). They also differ in the number of hsp isoforms that are expressed (Norris *et al.*, 1995; White *et al.*, 1994). Geographically disjunct species of taxonomic groups that occupy widely differing environments, e.g. deserts and ocean coast, vary in

their response (Dietz and Somero, 1993; Gehring and Wehner, 1995; Hofmann and Somero, 1996a; Ulmasov *et al.*, 1992) as do species that inhabit differing thermal niches on a microscale, e. g. within the intertidal zone (Sanders *et al.*, 1991). Intraspecifically, the heat shock response varies seasonally in fish (Dietz and Somero, 1992) and mussels (Chapple *et al.*, 1998; Roberts *et al.*, 1997). Despite many studies that attempt to relate natural temperature regimes to the expression of hsp's, it is not known if hsp's are expressed "routinely, occasionally, or seldom" under natural conditions (Feder and Hofmann, 1999). Interspecific differences in the frequency with which the response is induced are going to influence the energetic costs that are devoted towards coping with thermal stress in a given thermal niche, and these costs are likely to affect the thermal limits in which a species can occur. Furthermore, information on interspecific differences in acclimatory plasticity of the heat shock response is still sparse. Such differences may be crucial in influencing a species thermal niche width and may determine if a species is relatively steno- or eurythermal.

Regulation of the phenotypic variation of the heat shock response

Several studies conducted under field conditions have found that relatively higher endogenous levels of hsp's (associated with warmer acclimatization temperature) shift the induction of the stress response towards higher temperatures (Dietz and Somero, 1992; Roberts *et al.*, 1997). Thus, these studies supported the general concept of the cellular thermometer. Differences in acclimatory plasticity

of induction temperature and endogenous levels of hsp's among species varying widely in thermotolerance are basically unknown. To date, the genetically fixed differences in thermal stabilities of proteins, a factor that may set the limits of acclimatory plasticity in induction temperature, have only been surveyed for one enzyme (Fields and Somero, 1997) in one species for which we also know the effect of acclimation on hsp expression (Dietz and Somero, 1992). Although stabilities of orthologous homologues tend to correlate positively with adaptation temperature (Somero, 1995), it is not known if protein thermal stabilities invariably are higher in more warm-adapted species and thereby could partly explain higher induction temperatures.

Phenotypic variation among species in the induction temperature of the heat shock response may therefore depend on two factors: Variation that is not amenable to acclimatory changes may be set by genetically fixed interspecific differences in protein thermal stabilities, or in genetically fixed differences in endogenous levels of hsp's that regulate the induction of the stress response. In contrast, variation that is amenable to acclimatory change may mainly be based on changing endogenous levels of hsp's only. Genetically fixed and acclimation-induced variation may be regulated by different hsp's or by different isoforms, e.g., in case of hsp70. To date these questions have not been addressed comprehensively for any species group.

Phenotypic Variation of the Heat Shock Response in *Tegula* Congeners

Species of the genus *Tegula* that differ in distribution ranges along a latitudinal gradient, as well as along a steep temperature gradient along the subtidal to intertidal transition, provided a powerful study system to address the importance of phenotypic variation in the heat shock response. Phenotypic variation was controlled for acclimatory plasticity among differently adapted, acclimated and acclimatized specimens of several closely related congeners. A comparison of these congeneric species and the characterization of their thermal niche allowed me to examine the role of the heat shock response under natural temperature conditions. Furthermore, differently acclimated specimens provided insights into how acclimatory plasticity of the induction temperature of the stress response is regulated among *Tegula* species that occupy widely varying thermal habitats.

Chapter 2

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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Abstract

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's). Variation in the heat shock response among differently thermally adapted species and differently acclimated conspecifics may contribute to adaptive variation in heat tolerance. Here we compare the heat shock responses of 4 species of marine snails of the genus *Tegula* that occupy thermal niches that differ in absolute temperature and range of temperature. We examined effects of short-term heat stress and thermal acclimation on synthesis of hsp's of size classes 90, 77, 70 and 38 kDa by measuring incorporation of ^{35}S-labeled methionine and cysteine into newly synthesized proteins in gill tissue. We compared three important characteristics of the heat shock responses of these congeners: (i) Temperatures at which enhanced synthesis of hsp's first occurred (T_{on}), (ii) temperatures of maximal induction of hsp synthesis (T_{peak}), and (iii) temperatures at which hsp synthesis was heat-inactivated (T_{off}). Each of these temperatures was 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*. The upper thermal limits of hsp synthesis (and protein synthesis *per se*) correlated closely with thermotolerance limits. In *T. brunnea* and *T. montereyi*, synthesis of hsp's ceased at temperatures below body temperatures that *T. funebralis* commonly experiences in the mid-intertidal zone. Similarly,

synthesis of hsp's by *T. funebralis* ceased at temperatures below those encountered by *T. rugosa*. Thermal limits to synthesis of hsp's may play important roles in determining organismal thermal tolerance and, thereby, biogeographic patterning. Acclimation of snails to 13°, 18°, and 23°C shifted T_{on} and T_{peak}, but did not affect T_{off}. The heat shock responses of field-acclimatized snails were generally reduced in comparison to laboratory-acclimated snails. Overall, despite the occurrence of acclimatory plasticity in the heat shock responses of these snails, genetically fixed differences exist which reflect the species' separate evolutionary histories and which may play important roles in setting their thermal tolerance limits and, thereby, their distribution patterns.

Introduction

The heat shock response has received an enormous amount of study, especially since the discovery that heat shock proteins (hsp's) (also termed stress proteins) are a subset of the large family of molecular chaperones that function in all types of cells to ensure the proper folding and compartmentation of proteins (Bukau and Horwich, 1998; Feige *et al.*, 1996; Gething, 1997). Following a heat shock, hsp's act to prevent aggregation of heat-damaged proteins and to facilitate their renaturation. The more than fourteen thousand publications currently existing on molecular chaperones and the heat shock response document the effort directed at understanding the heat shock response and its role in thermotolerance (Bukau and Horwich, 1998; Feder *et al.*, 1996; Feder and Krebs, 1998; Parsell and

Lindquist, 1994; Parsell *et al.*, 1993). As the recent review of Feder and Hofmann (1999) emphasizes, however, surprisingly few of these studies have addressed questions about the expression of hsp's under natural (field) temperature conditions, or about variation in the heat shock response among species that may contribute to establishing differences in their thermal tolerance limits.

Among the important and largely unanswered questions that need to be addressed are the following: What characteristics of the heat shock response differ among species adapted to different thermal niches? How do such important characteristics as the onset temperature of enhanced synthesis of hsp's (T_{on}), the temperature of maximal hsp expression (T_{peak}), and the upper thermal limit for synthesis of hsp's (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 much 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's differ within a species? Does expression of different classes of hsp's 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. Establishing a correlation between biogeographic patterning and variation in physiological traits that play a critical role in establishing thermal tolerance ranges may help to provide

a firm basis for interpreting and predicting the effects of climate changes due to global warming.

To address the above questions, we have initiated studies of the heat shock response in marine snails belonging to the genus *Tegula*. Snails of this genus represent a potentially powerful study system for examining issues related to the role of temperature adaptation in biogeographic patterning. 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 (Hellberg, 1998; Riedman *et al.*, 1981; Watanabe, 1984b). In this study, we examined four congeners from temperate and subtropical habitats. *Tegula brunnea* and *T. montereyi* are low-intertidal to subtidal cool-temperate species which seldom face exposure to air (emersion) and are thus 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 near 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 reflect

phylogenetic distance (Hellberg, 1998). Comparisons of congeneric species often provide an effective means of identifying potentially adaptively important physiological and biochemical changes that are instrumental in governing their biogeographic patterning (Dahlhoff and Somero, 1993a; 1993c; Fields and Somero, 1997; Graves and Somero, 1982; Hofmann and Somero, 1996a; Holland *et al.*, 1997; Stillman and Somero, 1996).

Using field-acclimatized and laboratory-acclimated specimens of *Tegula*, we determined the effects of acute thermal exposure on rates of incorporation of ^{35}S- methionine/cysteine into proteins in gill tissue. Because of the high capacity of gill tissue of soft-bodied marine invertebrates to accumulate dissolved free amino acids (Wright, 1988), this tissue is an excellent study system for examining temperature effects on protein synthesis. We quantified the amount of new synthesis of hsp's 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 acclimatory plasticity in their heat shock responses, the four congeners have genetically fixed differences in their heat shock responses and in their upper thermal limits of protein synthesis that may play important roles in establishing and maintaining 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. 2.1A. *Tegula funebralis* has the widest latitudinal range (Fig. 2.1B), 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* is found from Cape Arago, Oregon, USA (43°21'N), to the Channel Islands, California, USA (34°00'N). *Tegula montereyi* occurs from Sonoma County, California (38°17'N), to the Channel Islands (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*, 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 sizes of specimens were similar among 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, Ohio, model 44006; accuracy of ±0.2°C) into the interior of the shell and subsequently covered the

opening with silicone sealant. The thermistor's extension was connected to a Stow Away XTI (Onset, Massachusetts) temperature data logger (temperature accuracy greater than ±0.7°C), which was placed in a submersible case (Ikelite). The shells were then glued to rocks with an epoxy resin (A-788 Splash Zone Compound, Z-SPAR, California) near sites where *Tegula* is abundant during low tide, and temperatures were recorded continuously from 22 March to 16 April 1996. Temperature data were recorded for *T. brunnea* from a site 0.08 m below Mean Low Low Water (MLLW), and for *T. funebralis* 0.51 m above MLLW.

To determine how closely the temperatures of the gelatin-filled shells simulated those of live snails, we measured the body temperatures of live snails by inserting a thermistor as far as possible into the mantle cavity. Temperatures in the continuously monitored gelatin-filled shells differed by less than 1°C from temperatures determined during shorter-term measurements of live snails (unpublished data). Henceforth we will refer to temperatures of gelatin-filled shells as 'body temperatures'.

Tidal data for Monterey Bay were obtained from the HMS website (www-marine.stanford.edu/HMSweb/Tides.txt), and sunrise and sunset times from a US Navy website (www.usno.navy.mil/). Average sea water temperature at 16 m depth approximately 300 m offshore of the Hopkins Marine Life Refuge was measured in spring 1996 with a Stow Away XTI data logger (unpublished data of Dr. J. J. Leichter, Woods Hole Oceanographic Institution).

Thermal tolerance measurements

Two thermal tolerance studies were conducted. In the first, which was designed to measure differences among field-acclimatized specimens of the 3 temperate-zone congeners, specimens of *T. funebralis*, *T. brunnea*, and *T. montereyi* were collected in mid-August 1996 and placed in a recirculating aquarium containing 14°C seawater (SW). Within 48 h of collection, we assessed thermal tolerance by raising the incubation temperature by 1°C every 12 min, up to a maximal temperature of 44°C. When each target temperature was reached, 20 snails of each species were removed from the aquarium and immediately checked for survival by prodding the underside of the foot to see if a withdrawal reaction occurred.

The second thermal tolerance study was designed to allow comparison between whole snail thermal tolerance and thermal resistance of protein synthesis in gill tissue. 13°C-acclimated (32 – 44 d) specimens of *T. funebralis* and *T. brunnea* were exposed for 2.5 h to 30°, 33°, 36° and 39°C or 30°C and 33°C, respectively (n = 20 for each temperature), to mimic conditions used for the incubation of isolated gill tissues. Survival of snails was determined as described above by testing for the foot withdrawal response.

Thermal acclimation

Specimens of the 3 temperate-zone species were collected for the acclimation experiment in mid-July 1997 and either immediately used for

radiolabeling experiments (field-acclimatized control group) or kept in temperature-controlled (13°, 18° and 23°C) circulating SW aquaria for 30 to 34 d. *Tegula rugosa* was collected in mid-July 1998, and acclimated at 23°C for 30 d. Specimens were kept constantly immersed and fed regularly with freshly collected giant kelp (*Macrocystis pyrifera*).

Heat shock protocol and tissue preparation

We dissected gill tissue under non-heat shock inducing conditions (13°C and 23°C SW for temperate and subtropical species, respectively) and immediately placed the tissue into plastic microcentrifuge tubes containing 0.2 µm filtered SW 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 that were pre-heated to the desired incubation temperature.

Samples of gill tissues for all species were incubated at 13°, 21°, 24°, 27°, 30° and 33°C (n = 5 for each temperature). Additionally, gills from acclimated *T. funebralis* were incubated at 36°, 38° and 39°C (13° and 23°C-acclimated specimens only) and *T. rugosa* samples were incubated at 36°, 38°, 40° and 42°C. Field-acclimatized *T. funebralis* were incubated at 13°, 24°, 27°, 30°, 33°, 36° and 38°C. After incubation for 2.5 h, tissues were placed at 13°C (23°C for *T. rugosa*) for 15 min before ^{35}S-labeled methionine/cysteine was added (NEN) to the tube. The duration of the thermal exposure we applied represents a minimum exposure

period during which snails experience high temperatures when emersed in the mid-intertidal. Thus the duration is conservative in regard to detecting possible interspecific differences. Higher concentrations of labeled amino acids were used for *T. brunnea* and *T. montereyi* (0.33 mCi ml^{-1}; 30.0 to 45.0 mg wet weight) than for *T. funebralis* and *T. rugosa* (0.22 mCi ml^{-1}; 15.0 to 25.0 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 we washed the gill tissue in ice-cold SW, 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 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. No proteolytic activity was detected for this homogenization procedure. To determine the amount of incorporated ^{35}S-amino acids into newly synthesized proteins, we pipetted aliquots (5 or 10 µl) of gill supernatant onto a GF-C glass-fiber filter (Whatman) and allowed them to air dry. To remove unincorporated ^{35}S-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 (RT), and finally

rinsed the filters in 95% ethanol. The filters were dried at RT, and counts per min (cpm) of incorporated ^{35}S-amino acids were quantified in a scintillation counter.

Gel-electrophoresis and fluorography

For most tissue samples we loaded ~500 000 cpm onto each lane of 10% SDS (sodium dodecyl sulfate)-polyacrylamide gels. The gels were run first at 25 mA for 1 h 20 min (stacking gel) and then at 30 mA for 2 h 30 min (resolving gel). Because we obtained significantly lower incorporation rates for gill tissue exposed to higher temperatures (33°C for *T. brunnea* and *T. montereyi*, 36°, 38° and 39° for *T. funebralis* and 40° and 42°C for *T. rugosa*) we loaded only 100 000 cpm 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 EN^{3}HANCE (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 (500 000 cpm) or 18 h (100 000 cpm). We corrected for the fact that 500 000 cpm samples emitted 2.23 times more counts over 8 h than did samples with 100 000 cpm over 18 h. All levels of hsp expression are given after this correction, that is, after normalization to 500 000 cpm at 8 h exposure time.

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

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 the temperatures at which significant new synthesis of hsp's first occurred (T_{on}), the temperatures of their maximal expression (T_{peak}), and the upper thermal limits for their synthesis (T_{off}), we developed a quantification protocol that used the intensity of the relevant hsp's 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. Intensities of this band at other temperatures were expressed relative to the intensity at 13°C for all classes of hsp's. We emphasize that all ^{35}S-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 hsp's are named according to molecular mass, but have not been further characterized with respect to the number of isoforms present or homology with hsp's of other species.

Comparisons of hsp band intensities were performed using a 1-sided Dunnett test after a one-way analysis of variance (ANOVA; SYSTAT software,

Systat, Inc.). For the ANOVA, data were log-transformed and incubation temperature was used as the independent variable, while expression level of hsp's was used as the dependent variable. We describe the first temperature at which band intensity was significantly higher ($p \leq 0.05$) than band intensity of the 13°C-control group (23°C in case of *T. rugosa*) as the T_{on} of the synthesis of a particular hsp. To reduce clutter in Figs. 2.5 –2.8, 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 zone species of *Tegula* examined in this study have distinct vertical distributions along the intertidal-to-subtidal transition at HMS (Fig. 2.1A). *Tegula funebralis* occurs highest and is the dominant snail in the mid- to low-intertidal zones. *Tegula brunnea* occurs in the low-intertidal to subtidal zones near the shoreward side of kelp beds. *Tegula montereyi* mainly occupies intermediate depths within the kelp bed, but is also found near the canopy (Watanabe, 1984b); personal observation). The transition from the zone that *T. funebralis* dominates to one that *T. brunnea* dominates is quite abrupt (personal observation). The subtropical *T. rugosa* occupies the mid-intertidal zone. Figure 2.1B displays the geographical distribution ranges.

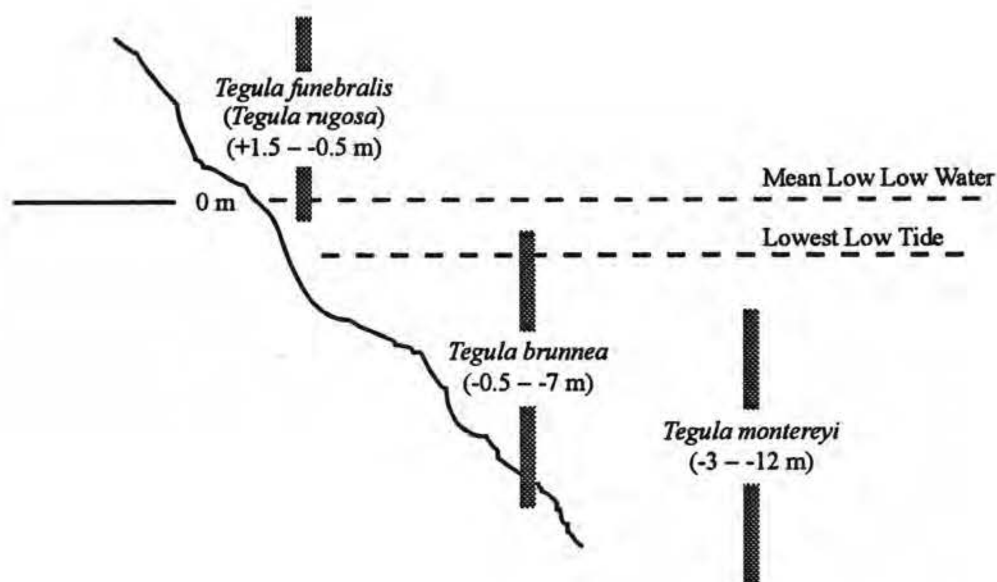


Figure 2.1A. Vertical distribution ranges of three temperate snail species of the genus *Tegula* (*T. funebralis*, *T. brunnea* and *T. montereyi*) along the intertidal-subtidal transition at Hopkins Marine Life Refuge, Pacific Grove, California (after Riedman *et al.*, 1981; Watanabe, 1984). The subtropical *Tegula rugosa* has a vertical distribution range similar to *T. funebralis*.

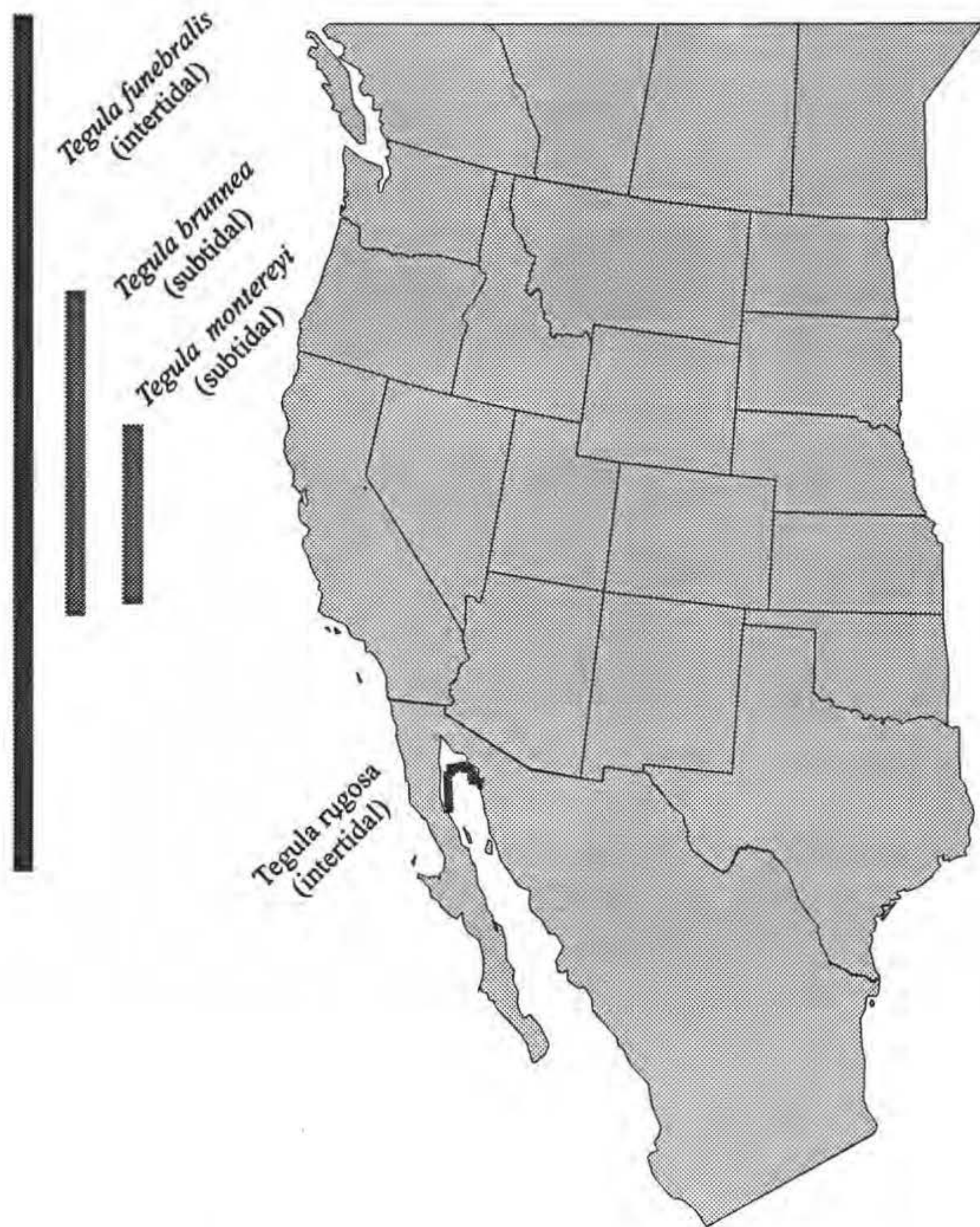


Figure 2.1B. Biogeographical distribution ranges of four snail species of the genus *Tegula* (*T. funebralis*, *T. brunnea*, *T. montereyi* and *T. rugosa*) along the Pacifc coast of North America (Morris *et al.*, 1980; Ricketts *et al.*, 1985; Watanabe, 1984; Hellberg, 1998).

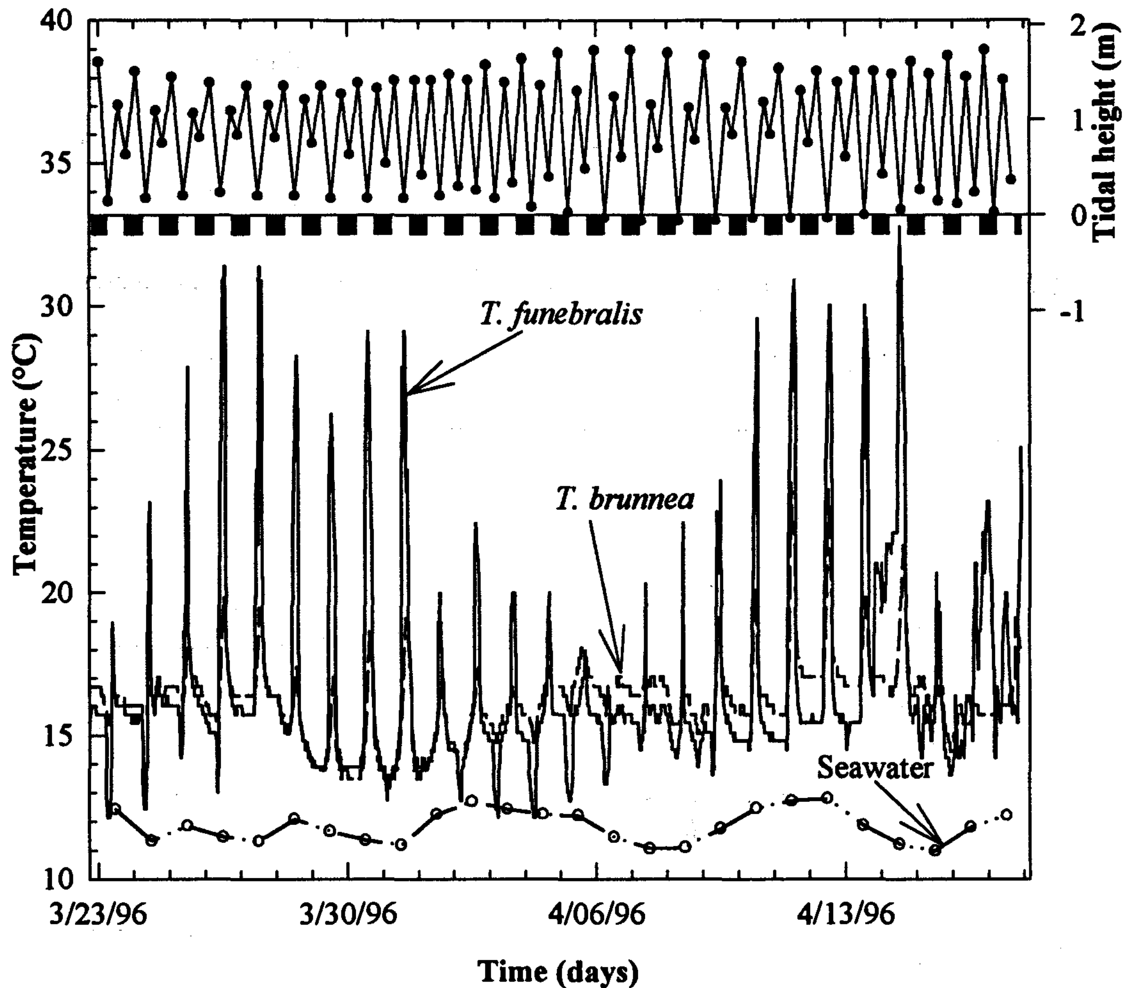


Figure 2.2. Field body temperature measurements of gelatin-filled shells of *T. funebralis* (black solid line) and *T. brunnea* (gray dashed line) from different intertidal heights (0.51m above Mean Low Low Water (MLLW) and 0.08m below MLLW, respectively) at Hopkins Marine Life Refuge, Pacific Grove, 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 gray dashed line with open circles.

To evaluate the role of the heat shock response under field conditions, it is important to determine the ranges of body temperature and the rates of heating that occur under natural habitat conditions. *In situ* body temperatures of *Tegula* congeners reflect the species' vertical distribution ranges and the effects of the tidal cycle. Figure 2.2 displays a continuous series of temperature measurements of gelatin-filled shells of *T. funebralis* and *T. brunnea*, obtained at intertidal sites within each species' vertical distribution range, during a spring tide series in 1996. Also shown are tidal and day-night cycles, as well as the average seawater temperature at 16 m depth off HMS. Temperatures in the continuously monitored gelatin-filled shells differed by less than 1°C from temperatures determined during shorter-term measurements of live snails (unpublished data; see Materials and Methods). The vertical heights that were chosen (0.51 m above Mean Low Low Water (MLLW) for *T. funebralis* and 0.08 m below MLLW for *T. brunnea*) represent upper thermal extremes for these two species at HMS. For *T. brunnea* this height represents the upper limits 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 (personal observations).

Body temperatures for both species varied with the daily tidal rhythm (Fig. 2.2), but maximal changes in body temperature during a tidal cycle were more than twice as great for *T. funebralis* (~19°C) as for *T. brunnea* (~7°C). The maximal

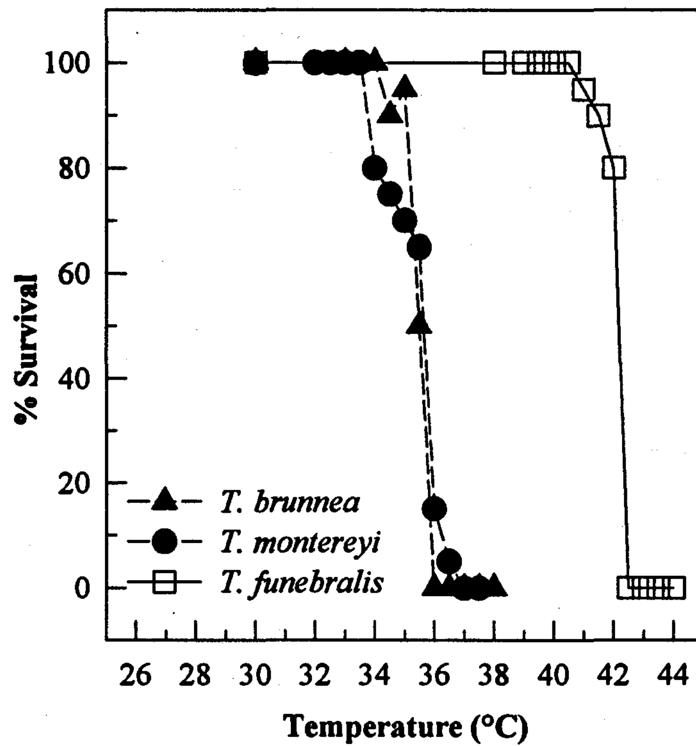


Figure 2.3. Percent survival of field-collected specimens (mid-August 1996) of three species of the genus *Tegula* (*T. funebralis*, *T. brunnea* and *T. montereyi*) after temperature exposure. The increase in temperature was 1°C every 12 min (n = 20 for each data point).

body temperatures measured for *T. funebralis* (~33°C) are about 10°C higher than those for *T. brunnea* (~24°C). *Tegula funebralis* frequently experienced temperatures in the high 20's°C and low 30's°C (Fig. 2.2), but *T. brunnea* experienced temperatures as high as the low 20's°C only during three extreme midday low tides (11, 12, and 14 April; Fig. 2.2). Because *T. funebralis* is distributed as far south as mid-Baja California, Mexico, whereas *T. brunnea* occurs only as far south as the Channel Islands, California, USA, we predict that the *in situ* body temperatures of these two species at their environmental extremes may actually differ by more than 10°C.

We also observed longer-term periodicity linked to the timing of low tides. For example, the series of high body temperatures that was observed during daytime low tides for *T. funebralis* during the last week of March (Fig. 2.2) was followed by an 8 – 10 d 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 solar heating of the animals' dark shells at low tide would be likely to raise the snail's body temperatures even higher.

To determine if the differences in vertical distribution and field body temperature of the three temperate zone congeners were correlated with differences in heat tolerance, we assessed 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. 2.3). Under this heating regimen, the temperature of 50% mortality (LT_{50}) of *T. funebralis* was 42.5°C, markedly higher than the LT_{50} of 36.0°C for *T. brunnea* and *T. montereyi*.

Effect of acclimation I: Interspecific differences

The autoradiographs in Fig. 2.4 show the patterns of protein synthesis, at a common incubation temperature of 13°C, following exposure of isolated gills from 13°- and 23°C-acclimated *T. funebralis* and *T. brunnea* to several temperatures between 13°C and 36°C. We used densitometric analysis of such autoradiographs to generate Figs. 2.5 – 2.8, 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 are seen for all four size classes of hsp's. First, the temperature at which increased synthesis of a particular hsp first occurs (T_{on}) tends to be positively correlated with normal habitat (adaptation) temperature. For example, comparisons of 13°C-acclimated *T. funebralis* and *T. brunnea* (Figs. 2.4 and 2.5) show that T_{on} of hsp70 occurred at 27° and 24°C, respectively. For hsp 90, comparisons of the four species (23°C acclimation

Figure 2.4. Autoradiographs showing effects of incubation temperature on incorporation of ^{35}S-methionine/cysteine into proteins of gill tissue of *T. funebralis* (A) and *T. brunnea* (B) acclimated to either 13° or 23°C. For each incubation temperature, duplicates of a single snail's gill sample were loaded on a 10% SDS-polyacrylamide gel. ^{14}C molecular weight markers are shown in the far left lane of each autoradiograph. All lanes were loaded with 500 000 cpm and exposed for 8 h to pre-flashed x-ray film.

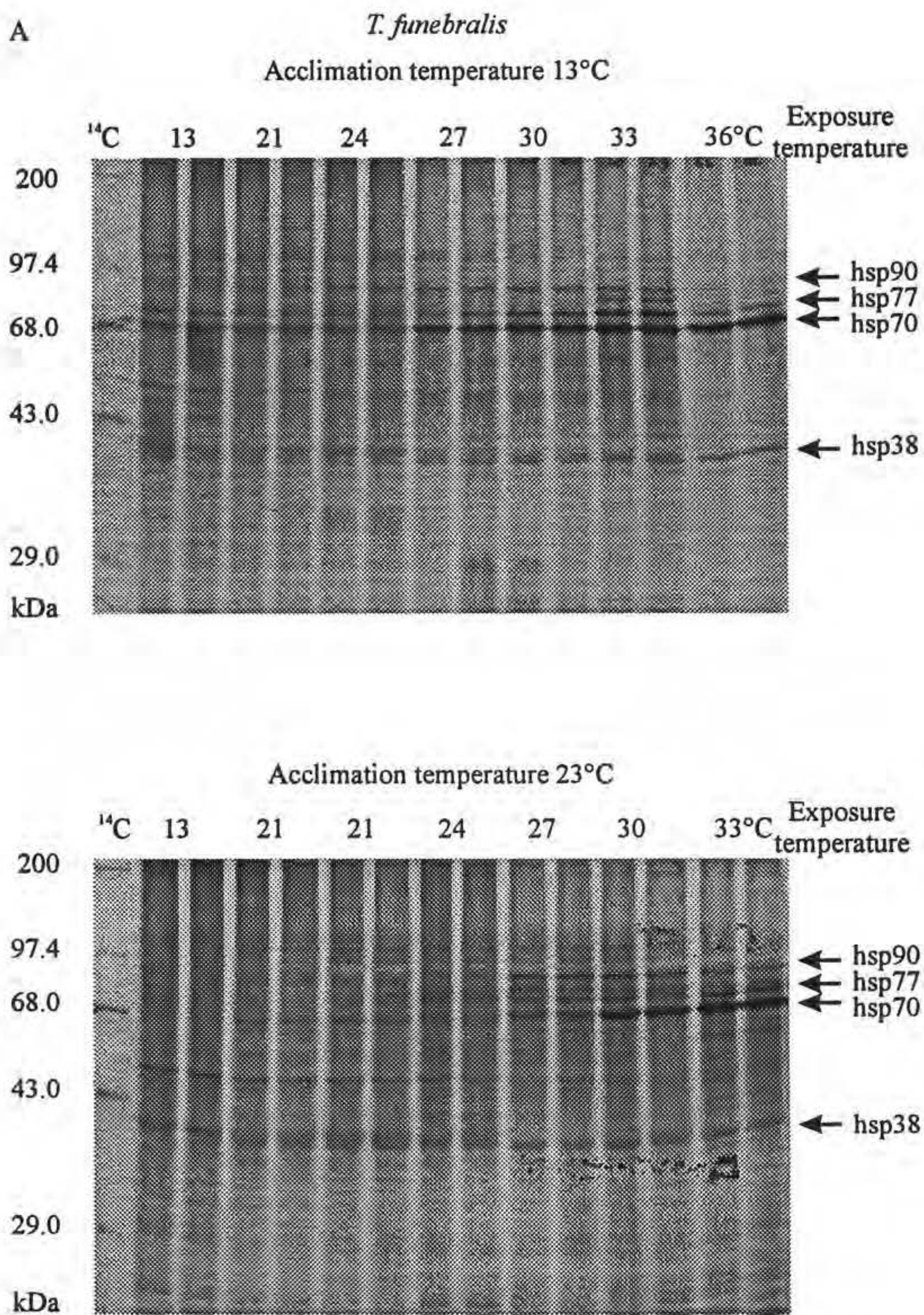
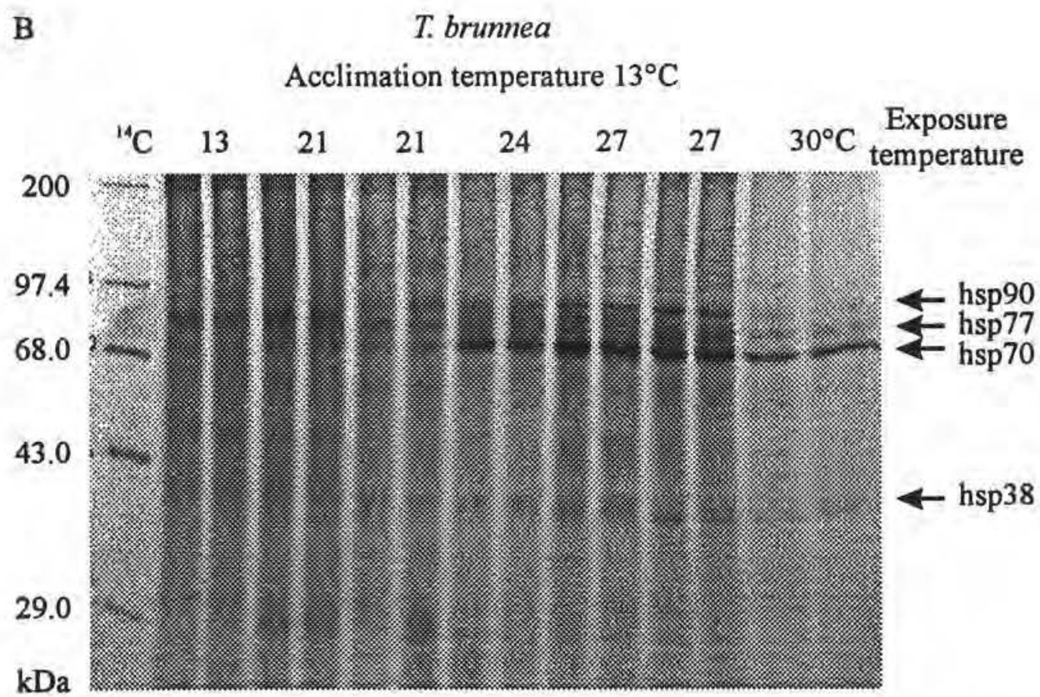


Figure 2.4



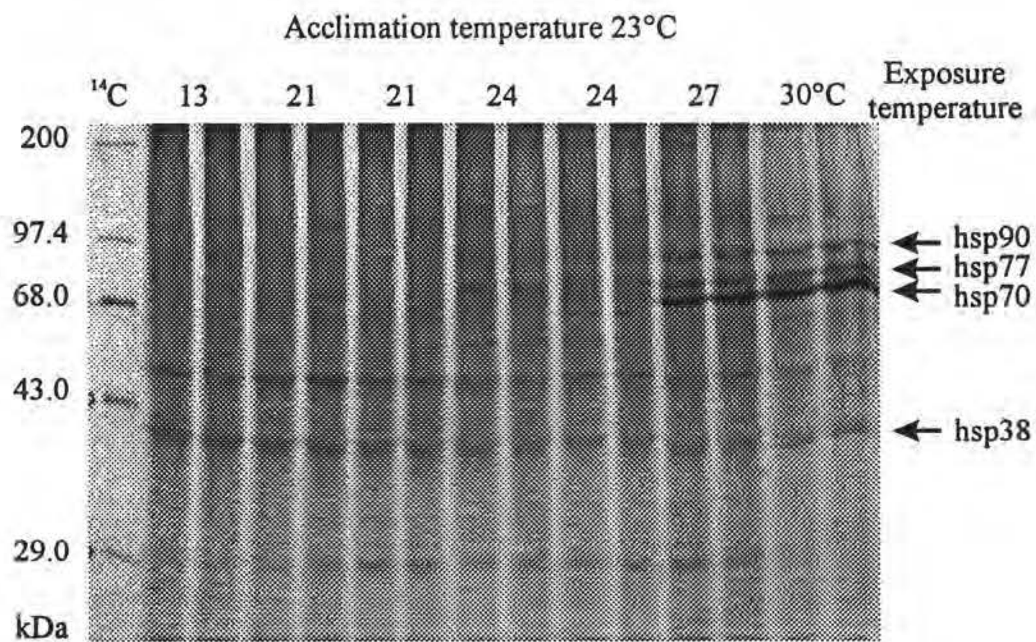


Figure 2.4 (continued)

Figure 2.5. Relative induction (to 13°C control group) of hsp70 in three temperate *Tegula* congeners from the mid- to low-intertidal (*T. funebralis*) and low-intertidal to subtidal (*T. brunnea* and *T. montereyi*) after laboratory-acclimation at 13°, 18° and 23°C for 30-34 d and field-acclimatization (July 1997). Data are also given for 23°C-acclimated *T. rugosa* (23° control group). T_{on} indicates the onset temperature, T_{peak} the temperature of maximal induction and T_{off} the cessation temperature of hsp synthesis. Data are means ± 1 S.E.M. (n = 5 for all data points except n = 4 for 13°C-panel: 36° *T. f.*; 23°C-panel: 33° *T. f.*, 13°C *T. b.*; field: 27°C *T. f.*, 13° and 30°C *T. m*).

Figure 2.6. Relative induction (to 13°C control group) of hsp38. Data are means ± 1 S.E.M. (n = 5 for all data points except for 13°C-panel: 36° (n = 4) *T. f.*; 18°C-panel: 13° (n = 4), 21° (4), 24° (3), 27° (4) and 30° (3) *T. m.*; 23°C-panel: 33° (4) *T. f.*; field: 27° (4) *T. f.*, 13°(4) and 30° (4) *T. m.*).

Figure 2.7. Relative induction (to 13°C control group) of hsp90. Data are means ± 1 S.E.M. (n = 5 for all data points except for 13°C-panel: 36° *T. f.*; 23°C-panel: 33° *T. f.*; field: 27° and 33° *T. f.*, 13° *T. m.* for which n = 4).

Figure 2.8. Relative induction (to 13°C control group) of hsp77. Data are means ± 1 S.E.M. (n = 5 for all data points except 13°-panel: 36° *T. f.*; 23°C-panel: 33° *T. f.*; field: 27° *T. f.*, 13° and 30° *T. m.* for which n = 4).

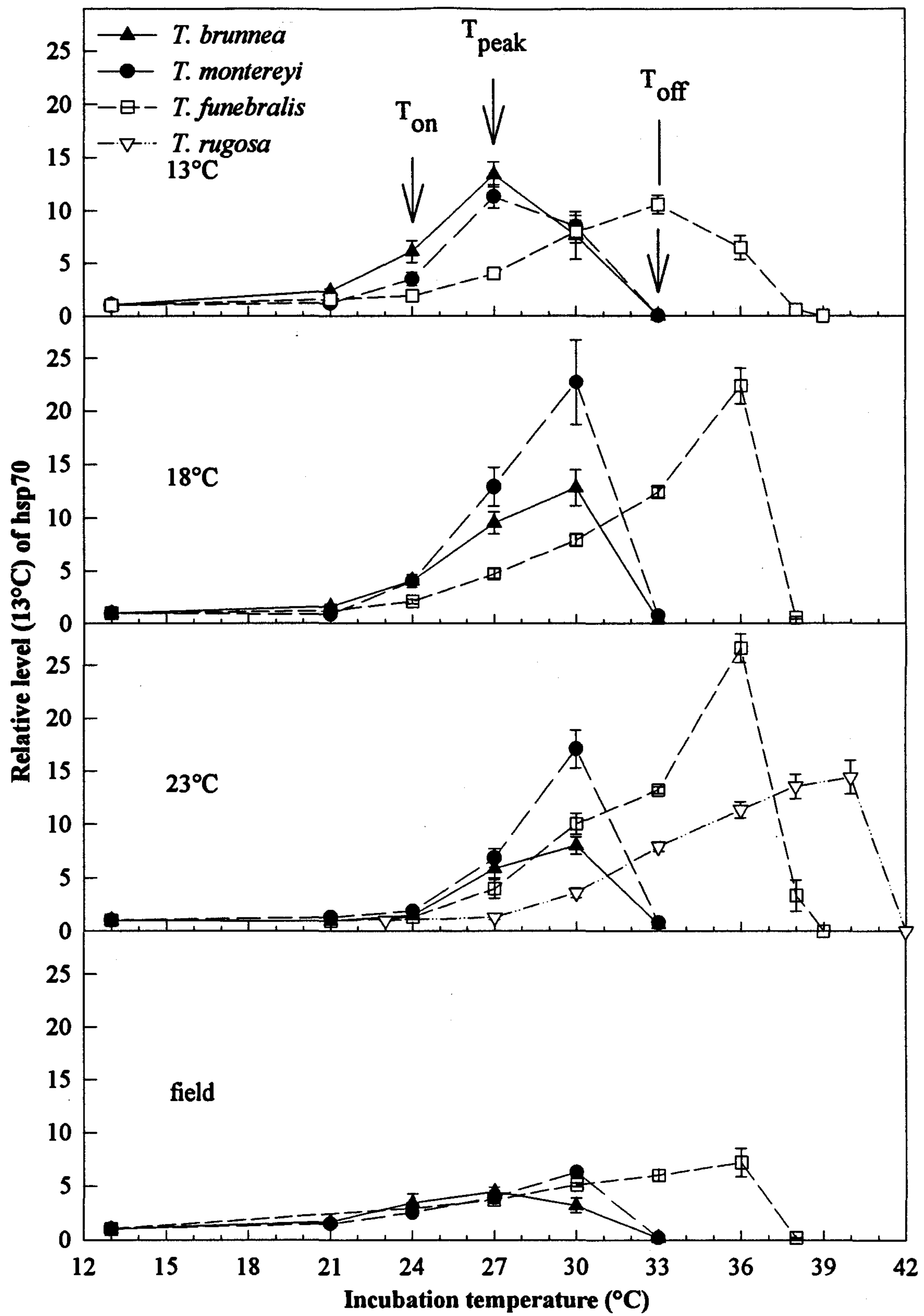


Figure 2.5

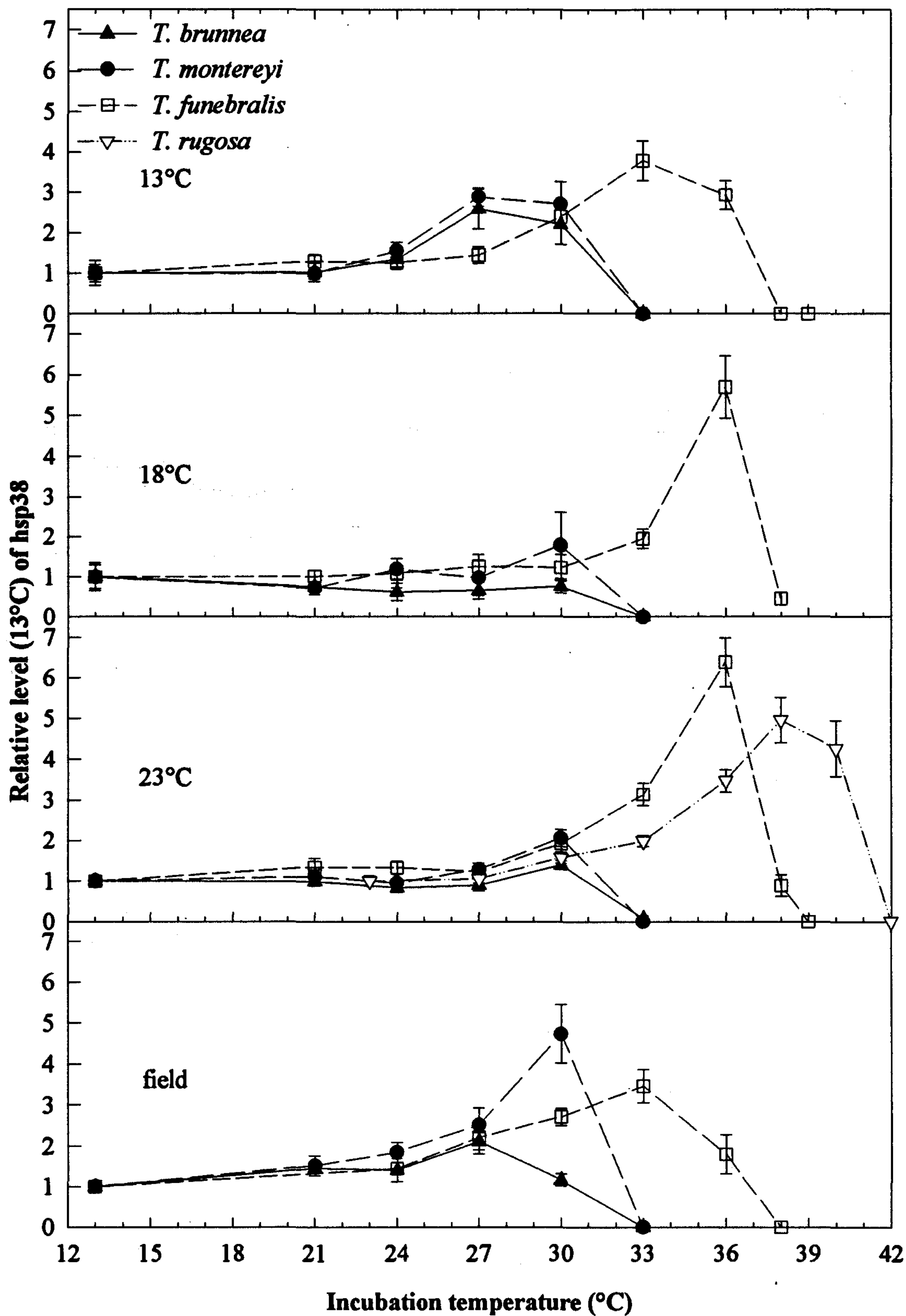


Figure 2.6

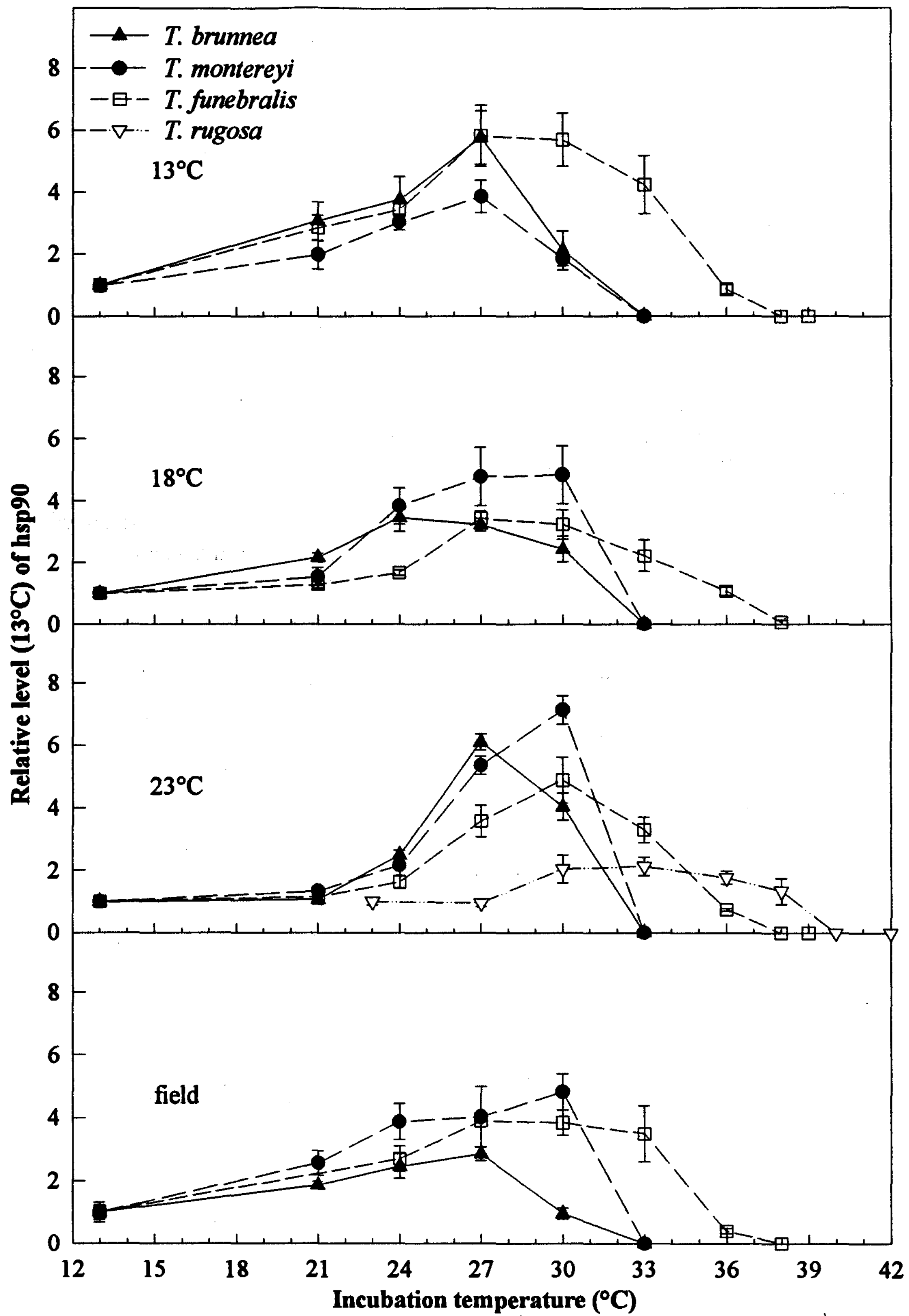


Figure 2.7

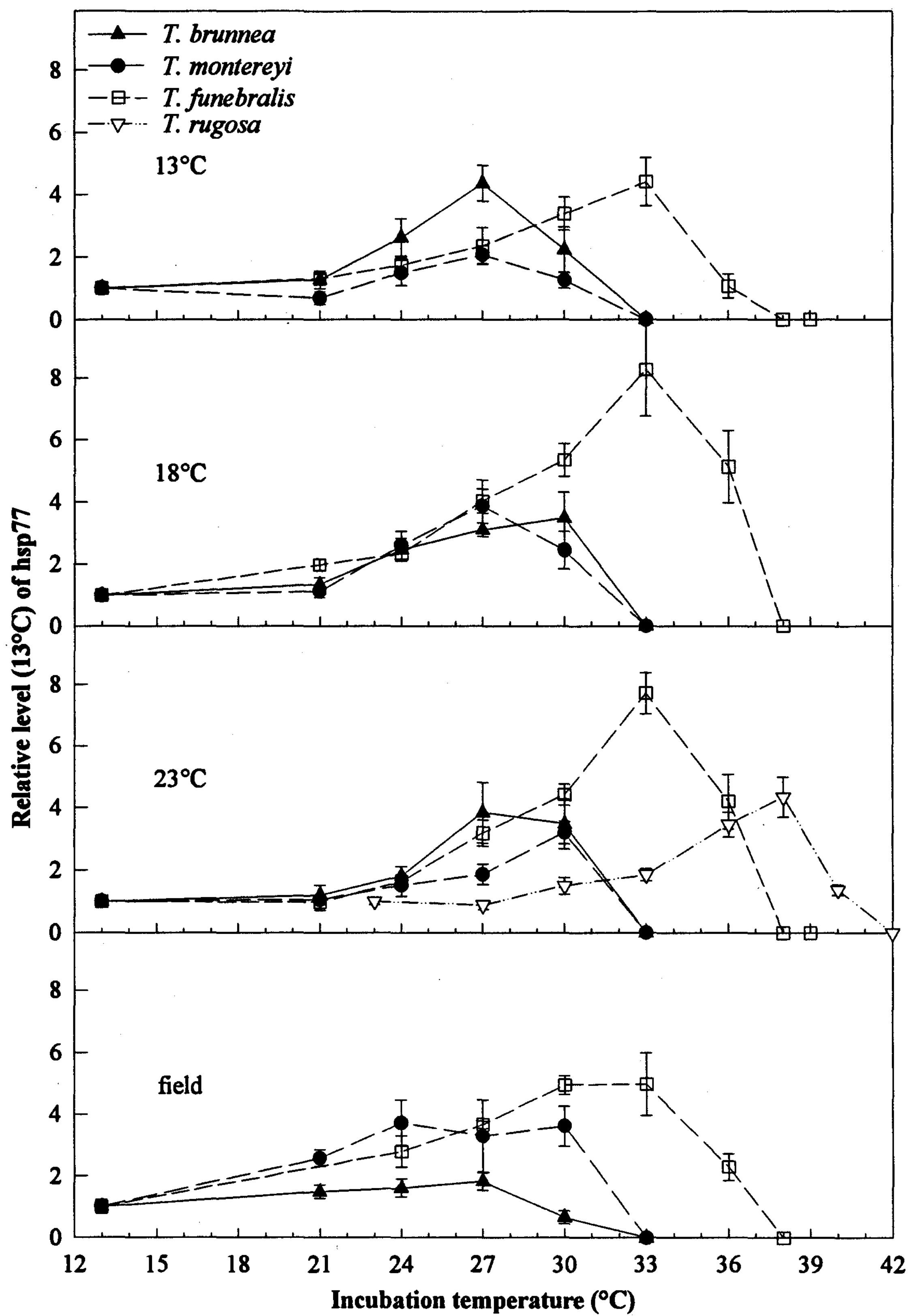


Figure 2.8

groups) show a T_{on} of 24°C in *T. brunnea* and *T. montereyi*, 27°C in *T. funebralis*, and 30°C in *T. rugosa* (Fig. 2.7).temperature. For example, comparisons of 13°C-acclimated *T. funebralis* and *T. brunnea* (Figs. 2.4 and 2.5) show that T_{on} of hsp70 occurred at 27° and 24°C, respectively. For hsp 90, comparisons of the four species (23°C acclimation groups) show a T_{on} of 24°C in *T. brunnea* and *T. montereyi*, 27°C in *T. funebralis*, and 30°C in *T. rugosa* (Fig. 2.7).

Second, the temperature at which maximal synthesis of hsp's occurred (T_{peak}) often correlated positively with adaptation temperature. This was seen for hsp70 for all species over all acclimation temperatures (Fig. 2.5). It was additionally found for hsp38 (Fig. 2.6) and hsp77 (Fig. 2.8), but not consistently for the expression of hsp90 (Fig. 2.7).

Third, among all classes of hsp's, a positive correlation was found between adaptation temperature and the maximal temperature at which hsp synthesis, as well as protein synthesis *per se* (T_{off}), occurred (Figs. 2.5 – 2.9). The T_{off}'s were near the upper limits of the thermal tolerance ranges for *T. funebralis* and *T. brunnea* (Figs. 2.9 and 2.10). This is shown by the organismal heat tolerance data in Fig. 2.10, which were obtained using an incubation temperature regimen similar to that employed in the protein synthesis experiments. Additionally, the difference between temperate intertidal and subtidal species in T_{off} (39° *versus* 33°C) was similar to the interspecific differences in survival temperature, 6.5°C, obtained using the protocol shown in Fig. 2.3. Furthermore, in *T. brunnea* and *T. montereyi*, protein synthesis was blocked at temperatures (30° to 33°C) that *T. funebralis*

Figure 2.9. Expression of newly synthesized proteins at highest thermal exposures in specimens of *T. brunnea* and *T. montereyi* (A), *T. funebralis* (B) and *T. rugosa* (C) that were acclimated to either 13° or 23°C. Arrows indicate the major hsp's of size classes 90, 77, 70 and 38kDa. 100 000 cpm per lane were loaded on a 10% SDS-polyacrylamide gel and exposure of the gel to pre-flashed X-ray film was 18 h for all temperature incubations except for the 30°C lanes in *T. brunnea* and *T. montereyi*, which were loaded with 500 000 cpm and exposed for 8 h to pre-flashed film. ^{14}C molecular weight markers are shown in the far left lane of the autoradiographs for *T. brunnea*, *T. montereyi* and *T. funebralis*.

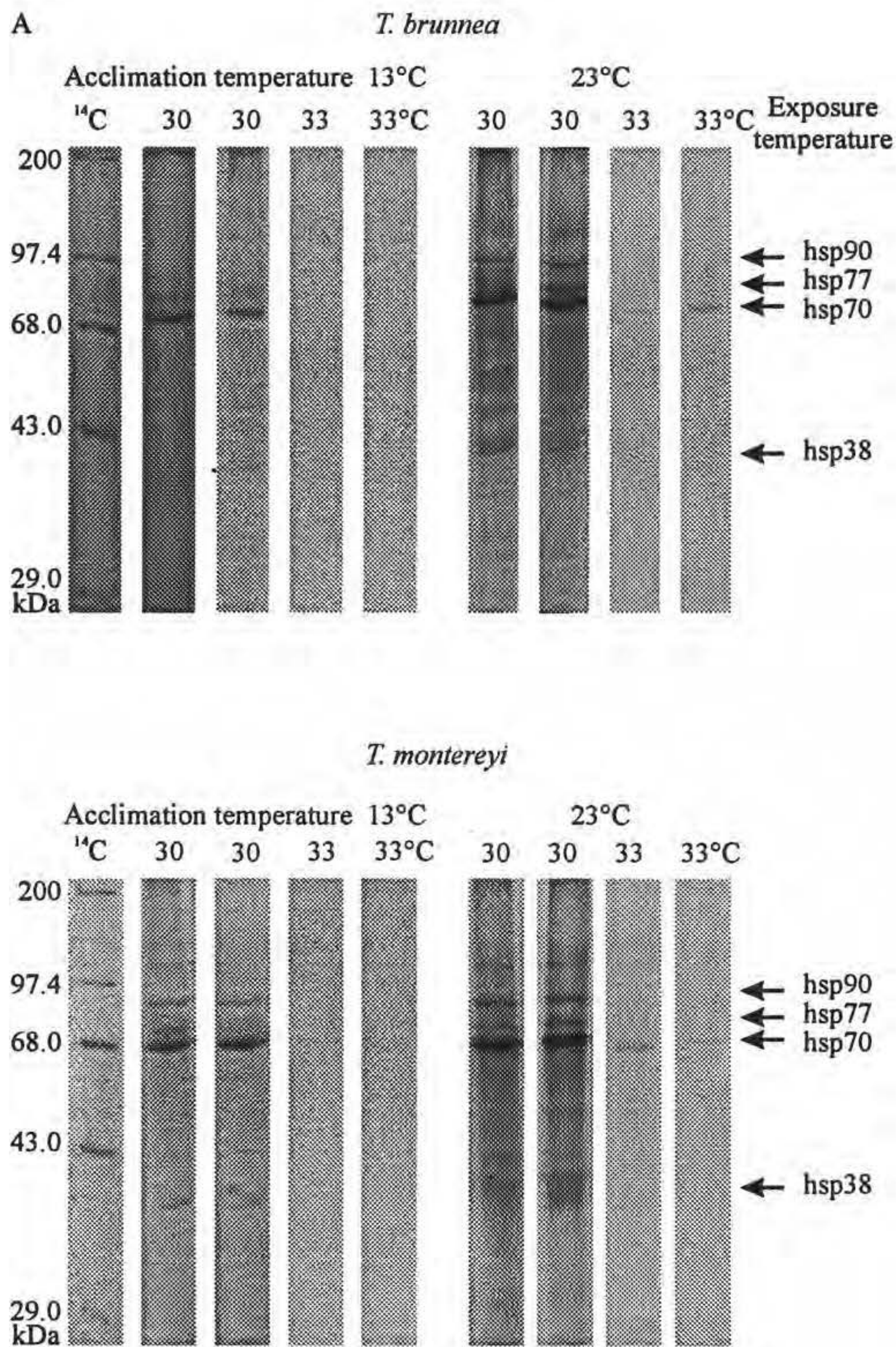
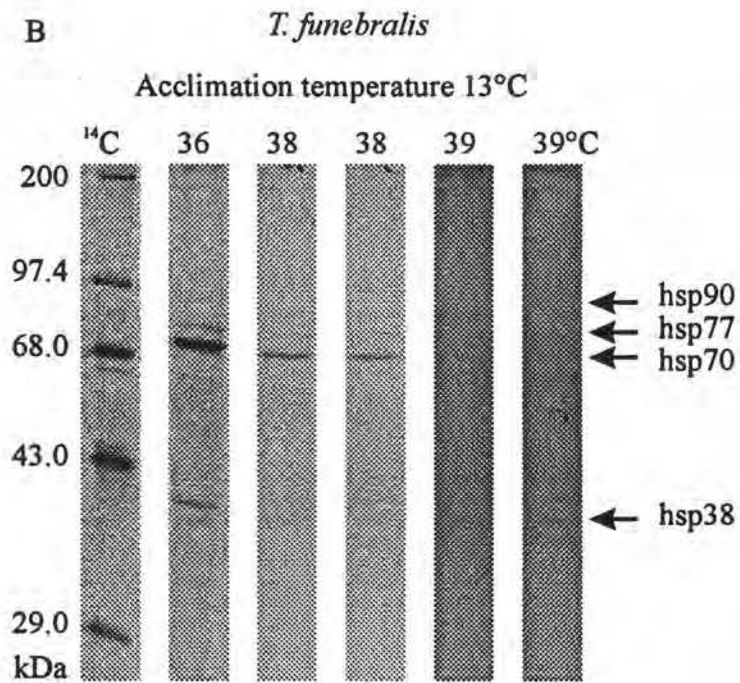


Figure 2.9



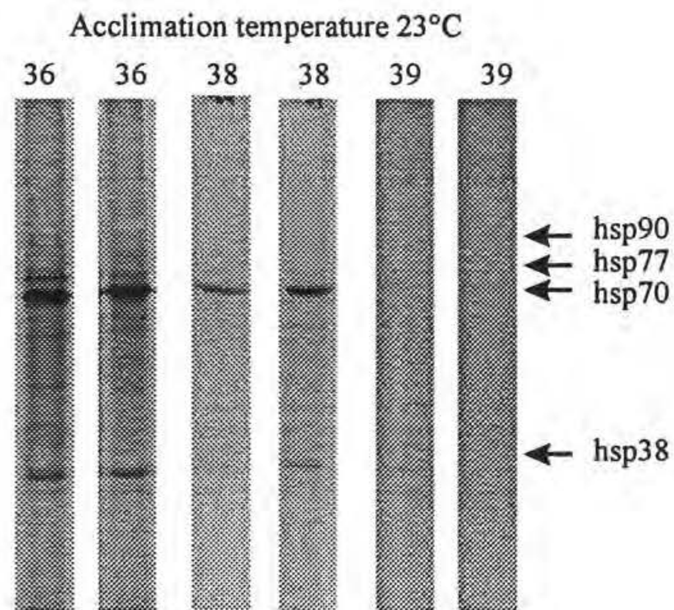


Figure 2.9 (continued)

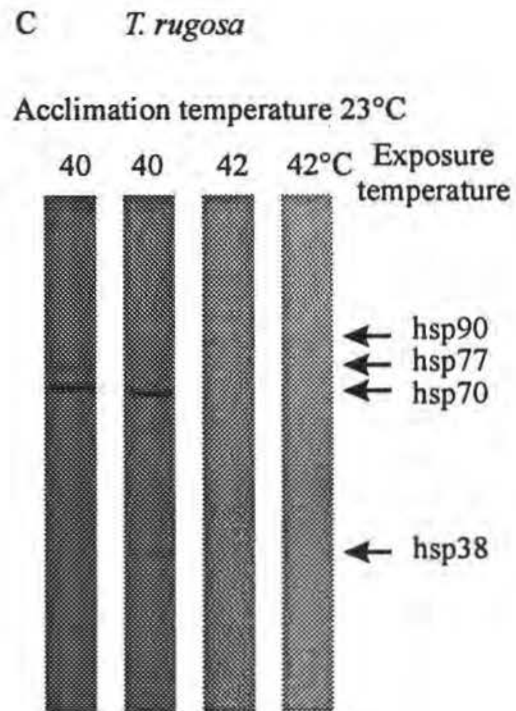


Figure 2.9 (continued)

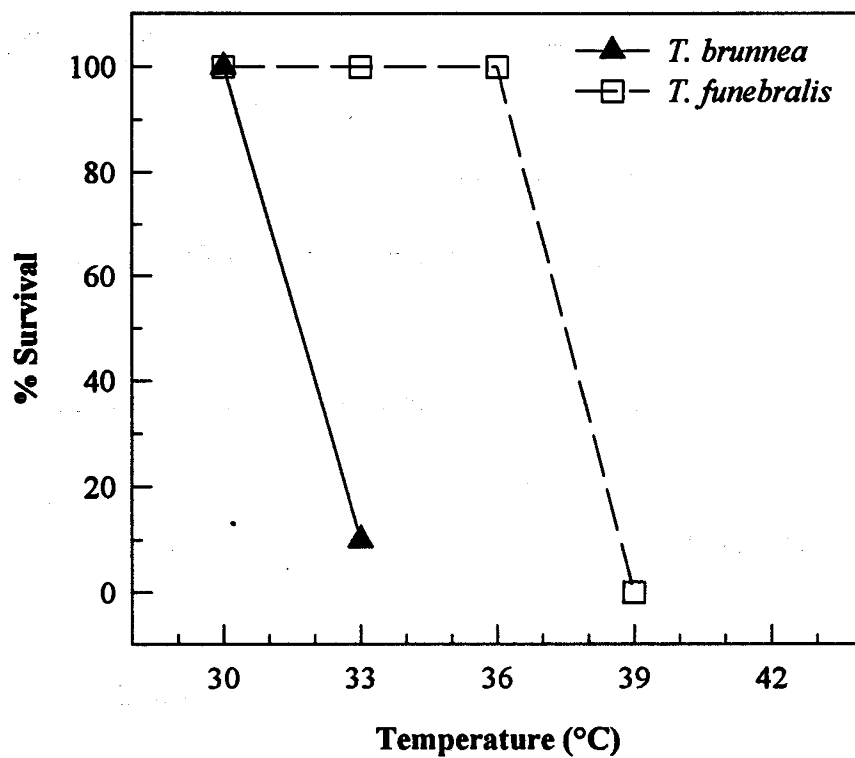


Figure 2.10. Survival of 13°C-acclimated (5-7 weeks) *T. funebralis* and *T. brunnea* after exposure for 2.5 h at different temperatures (n = 20 for each time point). Survival was assessed within 30 min after exposure by examining the withdrawal response of the foot.

experienced frequently during the spring (Fig. 2.2). 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*.

Interestingly, T_{off} showed no response to acclimation (Figs. 2.5 – 2.9). This finding suggests that the process(es) that set the thermal tolerance limits for protein synthesis is (are) not amenable to temperature-acclimatory modification. This apparently genetically fixed thermal limit may be an important mechanism in establishing biogeographic patterning, as discussed below.

Differences among the four species were also noted in the relative intensities of induction of the different size classes of hsp's. For example, for hsp90, comparisons among the 23°C-acclimated snails showed that *T. brunnea* and *T. montereyi* increased synthesis most, whereas *T. rugosa* increased synthesis least (Fig. 2.7). In contrast, the increase in synthesis of hsp38 was highest in *T. rugosa* and *T. funebralis* and lowest in *T. brunnea* and *T. montereyi* (Fig. 2.6). For hsp77, relative synthesis was highest in *T. funebralis* (Fig. 2.8). These variable patterns of hsp expression among species suggest that heat stress does not activate a common 'heat shock program' in the four congeners, but rather leads to unique patterns of expression in each species.

Effects of acclimation II: Hsp-specific expression patterns

To examine the phenotypic plasticity of the heat shock response, we acclimated specimens of the 3 temperate zone species to 13°, 18°, 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*.

For *T. brunnea* and *T. montereyi*, an increase in acclimation temperature from 13° to 23°C led to an increase in T_{on} of hsp70 from 24° to 27°C (Figs. 2.4 and 2.5). T_{peak} of hsp70 in these two species shifted from 27° to 30°C as acclimation temperature rose from 13° to 18°C (Fig. 2.5). No additional increase in T_{peak} was seen following acclimation to 23°C. *Tegula funebralis* showed no acclimation-induced changes in T_{on} or T_{off} for hsp70 (Figs. 2.4 and 2.5). However, T_{peak} of hsp70 increased from 33° to 36°C as acclimation temperature increased from 13° to 18°C. As in the case of *T. brunnea* and *T. montereyi*, no additional changes were observed when the acclimation temperature was increased from 18°C to 23°C (Fig. 2.5).

For hsp38, acclimation had a marked effect on the responses of *T. brunnea* and *T. montereyi*. Although 13°C-acclimated specimens of both species exhibited two-to three-fold increases in synthesis of hsp38 at 27°C, induction of hsp38 synthesis in the 18°C- and 23°C-acclimated specimens was much less intense (Fig. 2.6). *Tegula funebralis* showed enhanced synthesis of hsp38 at temperatures above 30°C in all acclimation groups, and the intensity of synthesis varied less than two-fold among the acclimation groups.

For hsp90, *T. funebralis* exhibited a shift in T_{on} and an 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 (Fig. 2.7). An increase in acclimation temperature from 18°C to 23°C did not change this response 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. 2.8).

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.

Variation was also observed among size classes of hsp's within a species at a common acclimation temperature. For example, 13°C-acclimated *T. funebralis* induced hsp90 at 24°C, hsp70 at 27°C and hsp38 and hsp77 at 30°C (Fig. 2.5 – 2.8). 13°C-acclimated *T. brunnea* and *T. montereyi* induced hsp70 and hsp90 at 24°C, but hsp38 and hsp77 at 27°C. In general, hsp's were induced in the following order (lowest to highest induction temperatures): hsp90 < hsp70 < hsp77 < hsp38.

At higher heat shock-inducing temperatures, synthesis of proteins other than hsp's was typically much reduced, especially in 13°C-acclimated snails (Figs. 2.4 and 2.9). However, in all 3 temperate species, increasing acclimation temperatures restored to some degree the synthesis of non-hsp's at higher temperatures, e.g. at 30°C in *T. brunnea* and *T. montereyi* (Fig. 2.9). Thus, acclimation to higher temperatures allowed synthesis of non-hsp's to occur at higher levels at the

maximal temperatures at which synthesis was possible, even though the T_{off}'s were not altered by acclimation. Synthesis of hsp's was also increased at the highest temperatures at which synthesis was possible. In some cases, the maximal levels of hsp synthesis shifted towards higher temperatures (Figs. 2.4 – 2.9).

Field-acclimatized specimens versus laboratory-acclimated specimens

Because the vast majority of studies of the heat shock response have been conducted with laboratory-acclimated specimens or cell lines (Feder and Hofmann, 1999), it is important to determine whether data obtained under these conditions reflect the heat shock responses of field-acclimatized organisms. To this end, we conducted a parallel series of labeling experiments using freshly collected (July) specimens of *T. brunnea*, *T. montereyi*, and *T. funebralis*. Because 13°C and 18°C were within the environmental ranges of all 3 species (Fig. 2.2), we restrict comparisons of the field-acclimatized snails to snails subjected to these two acclimation temperatures.

As shown in Figs. 2.5 – 2.8, the heat shock responses of field-acclimatized snails both resembled and differed from those of the laboratory-acclimated conspecifics. T_{off}'s 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 between acclimatized and acclimated snails was found for hsp70. For all 3 species, field-acclimatized specimens appeared to

induce hsp70 only about one-fourth to one-third as strongly as snails from the 13° and 18°C acclimation treatments. However, this apparently lower intensity of induction in field-acclimatized snails is in large measure a consequence of the normalization procedure used to compare intensities of induction following treatment at different temperatures. Thus, whereas the intensity of induction of hsp70 in the field-acclimatized snails did not vary with treatment temperature as much as in the case of the laboratory-acclimated snails, the absolute levels of hsp70 synthesis in fact were higher in the 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 experiments) provides a means for evaluating how the heat shock response varies among treatment temperatures, but 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 the heat shock responses of these four congeners of *Tegula* suggest that, despite some acclimatory plasticity in this response, genetically fixed differences exist among the species in (i) temperatures at which hsp expression is first induced (T_{on}), (ii) temperatures at which hsp expression attains its maximal intensity (T_{peak}), and (iii) the upper thermal limits of hsp synthesis (T_{off}), as well as

protein synthesis in general. Each of these interspecific differences has implications for biogeographic patterning and 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 among the four species. In their natural habitats, the two temperate zone species with subtidal to low-intertidal distributions, *T. brunnea* and *T. montereyi*, are much less likely to experience temperatures that elicit enhanced expression of hsp's than are the two congeners that occur in the low- to mid-intertidal region, *T. funebralis* and *T. rugosa*. For example, during the 26 d period of midday low tides between late March and mid-April, 1996 (Fig. 2.2), on at least 11 days body temperatures of *T. funebralis* may have exceeded 27°C, the T_{on} for certain hsp's, e.g., hsp70 and hsp90 (in 13°C-acclimated specimens, Figs. 2.5 and 2.7). During this same 26 d period, on only a single day did the body temperature of *T. brunnea* reach a value as high as 24°C, the T_{on} for hsp70 and hsp90 in 13°C-acclimated specimens. Although we collected no field temperature data for *T. rugosa*, the facts that (i) water temperatures in its shallow intertidal habitat rise to at least 36°C in summer (Dietz and Somero, 1992) and (ii) absorption of solar radiation by the dark shell of the snails is apt to increase body temperatures above air temperature, which may exceed 40°C, suggest that this intertidal snail must commonly activate the heat shock response *in situ*.

Interspecific differences in the frequency with which the heat shock response is induced could be associated with significant differences among these

species in how temperature affects their energy budgets. Although it is not possible to quantify precisely the energy consumed in activating the heat shock response, it is clear that this process is costly (Heckathorn *et al.*, 1996; Sanchez *et al.*, 1992). Energy is required for synthesis of hsp's, and the chaperoning activity of most hsp's requires hydrolysis of ATP. Over-expression of hsp's can significantly decrease fitness (Feder *et al.*, 1992; Krebs and Loeschke, 1994), possibly as a consequence of the energy costs associated with the heat shock response and the preferential synthesis of hsp's, at the expense of synthesis of other types of proteins, at elevated temperatures (Figs. 2.4 and 2.9). Therefore, the species of *Tegula* that inhabit the low- to mid-intertidal zone may face substantially higher energy costs related to synthesis and use of hsp's and more frequent interruption of synthesis of non-hsp proteins than their lower-occurring congeners. The fraction of ingested energy that can be allocated to growth and reproduction thus might be lower in higher-occurring, more heat-stressed species. The costs entailed in activating the heat shock response may contribute to the upper limits of vertical distribution of intertidal invertebrates.

A correlation between induction of the heat shock response at natural (habitat) temperatures and variability of the thermal habitat has also been reported in a comparison of congeneric freshwater cnidarians (polyps) (Bosch *et al.*, 1991; Bosch *et al.*, 1988; Gellner *et al.*, 1992). Bosch and colleagues found a greatly attenuated level of hsp synthesis in the stenothermal *Hydra oligactis* relative to the eurythermal *H. vulgaris* and *H. magnipapillata*. The former species is common in

lakes and fast-flowing rivers where it undergoes conspicuous fluctuations in abundance when changes in water temperature occur (the upper temperature at which it disappears from its habitat is 22°C). In contrast, the two latter species are found in habitats that are characterized by a wider temperature range (7°-29°C), e.g. shallow freshwater ponds, and they do not undergo conspicuous seasonal changes in abundance. In *H. vulgaris* and *H. magnipapillata* synthesis of hsp's is detected by 22°C, a temperature apparently commonly seen by both species, whereas synthesis of hsp's is hardly detectable at any temperature in *H. oligactis*. Interspecific differences in levels of hsp synthesis were recently traced to a reduced stability of hsp70 mRNA in the stenothermal *H. oligactis* (Brennecke *et al.*, 1998).

A second major difference found among the four congeners of *Tegula* is the upper thermal limit for synthesis of hsp's and other proteins. Protein synthesis of the two temperate species occurring lowest in the intertidal zone, *T. brunnea* and *T. montereyi*, was heat-inactivated between 30° and 33°C. These temperatures are commonly experienced in the field by *T. funebralis* (Fig. 2.2), and are temperatures at which synthesis of some hsp's, for instance, hsp70 (Fig. 2.5), is maximal in this species. The thermal sensitivity of protein synthesis by *T. brunnea* and *T. montereyi* may play a role in preventing these two species from occurring in the mid-intertidal region inhabited by *T. funebralis*. Thermotolerance measurements (Fig. 2.10) 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 may be maximal in the case of 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. 2.5 – 2.9).

Further evidence that heat sensitivity of protein synthesis may play a role in establishing thermal tolerance limits and, thereby, contributes 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* found a 2°C difference in T_{off} between mid- and high-intertidal species (Sanders *et al.*, 1991). Comparisons of a heat-adapted ant (genus *Cataglyphis*) from the Sahara with a temperate ant (genus *Formica*) revealed a 6°C higher T_{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, 1996a). These observations, taken in conjunction with the differences observed among *Tegula* congeners, provide evidence that adaptive changes in T_{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: (i) Variations in the thermal responses of a given size class of hsp in differently acclimated or acclimatized individuals, and (ii) differences in response among size classes of hsp's within a particular treatment group. This variation indicates that no fixed 'heat shock program' is induced during thermal stress in either different congeneric species or differently acclimated or acclimatized individuals of a single species.

The first type of plasticity, in which T_{on} and T_{peak} of hsp synthesis, and the intensity of synthesis vary with acclimation, has been observed in other species, including eurythermal goby fishes (Dietz and Somero, 1992) and intertidal mussels (Roberts *et al.*, 1997). In the present study, these types of variation are best illustrated by the synthesis of hsp70 and hsp38 in gills of *T. brunnea* and *T. montereyi*. In both of these low-intertidal to subtidal species, initial T_{on} and T_{peak} of hsp70 increased as acclimation temperature rose. Intensity of expression of hsp38 was greatly attenuated with increasing acclimation temperature.

Acclimation-induced changes in the heat shock response generally did not 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 temperature was raised from 18° to 23°C may reflect the

infrequency with which these two species encounter temperatures as high as 23°C (Fig. 2.2). However, *T. funebralis*, which frequently encounters seawater temperatures above 23°C at its southern distribution limit, also did not show any 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 acclimation effects on congeners of abalone (genus *Haliotis*) also found that the range of temperatures over which acclimatory changes occurred were narrower than the ranges of habitat temperatures (Dahlhoff and Somero, 1993a).

Despite the variation in T_{on} of hsp synthesis due to acclimatory history, the fact that interspecific differences in T_{on} typically remained at any temperature of acclimation suggests fixed genetic differences are important in setting the temperatures of induction of hsp synthesis. These genetically based differences could be due to (i) the gene regulatory factors that establish the set points for induction of hsp synthesis (Craig and Gross, 1991; Morimoto, 1998; Morimoto and Santoro, 1998) or (ii) interspecific variations in thermal stability of cellular proteins (see Chapter 4 and 5, respectively).

The model of Craig and Gross (1991) for expression of the *hsp70* gene stresses the importance of binding equilibria among heat-unfolded proteins, hsp70, and heat shock factor (HSF), a gene regulatory protein that helps modulate expression of the *hsp70* gene. According to this model, when bound to hsp70, HSF

is unable to migrate to the nucleus and activate transcription of the *hsp70* gene. Under heat stress, when thermally induced unfolding of proteins occurs, hsp70 dissociates from HSF and binds to denatured proteins, thereby freeing HSF and leading to induction of synthesis of additional hsp70. Acclimation-induced changes in T_{on} for hsp70 synthesis thus could be a consequence of changes in the ratio of hsp70 to HSF. Although we are unaware of any data suggesting that HSF varies in concentration in differently acclimated ectotherms, several studies have shown that levels of hsp70 rise during warm acclimation (Hofmann and Somero, 1995; Roberts *et al.*, 1997). Higher levels of hsp70 would mean that more hsp70 can bind to denatured proteins and this would raise the temperature at which the HSF is released and the heat shock response induced. In the congeners of *Tegula*, we also found higher endogenous levels of hsp70 in warm-acclimated specimens (Chapter 4). Intriguingly, other hsp's that play a role in the induction of the heat shock response, e.g. hsp90 (Morimoto, 1998; Morimoto and Santoro, 1998), also show a positive correlation between T_{on} and higher endogenous levels (Dietz and Somero, 1992). Differences in endogenous levels of hsp's may be the main factors establishing acclimatory plasticity as well as evolutionary variation among the species.

Besides endogenous levels of hsp's, the thermal stabilities of proteins may establish interspecific differences in the T_{on} of the heat shock response. For most proteins examined, orthologs from warm-adapted species have higher thermal stabilities than orthologs from cold-adapted species (reviewed in Somero, 1995)).

On this basis alone, one would predict both a positive correlation between evolutionary adaptation temperature and T_{on}, and a limit in the extent to which interspecific differences in T_{on} could be eliminated by thermal acclimation. A survey of five metabolic enzymes among the three temperate *Tegula* congeners did not show a consistent correlation between thermal stabilities and adaptation temperature (Chapter 5). However, the proteins that are disrupted first by a heat shock are conjectured to be structural rather than metabolic proteins, e.g. intermediate filaments of the cytoskeleton (Parsell and Lindquist, 1994). In general, although hardly the sole factor, endogenous levels of hsp's may explain interspecifc and acclimation-induced intraspecific variation in T_{on} in *Tegula* congeners.

Variations in T_{on} and T_{peak} were also observed when different size classes of hsp's were compared within a species. For a given acclimation group, T_{on}'s for different size classes of hsp's varied by several degrees Celsius. For instance, in 23°C-acclimated *T. brunnea* and *T. montereyi*, induction of hsp90 was observed at 24°C, whereas induction of hsp70 and hsp38 was evident by 27° and 30°C, respectively (Figs. 2.5 – 2.7). The typical order in which hsp's were induced with increasing incubation temperature was hsp90, hsp70, hsp77 and, lastly, hsp38.

Acclimation affected T_{on}'s and T_{peak}'s differently among size classes of hsp's. Whereas hsp70 might show a large change in thermal response with acclimation, another hsp, for instance, hsp77, might exhibit a relatively constant synthesis profile across acclimation treatments, e.g. in *T. brunnea* and *T. montereyi*

(Figs. 2.5 and 2.8). Differences between field-acclimatized and laboratory-acclimated conspecifics also were noted among size classes of hsp's (Figs. 2.5 – 2.8). For some classes of hsp's, field-acclimatized specimens exhibited a greatly attenuated response, notably in the case of the most strongly expressed hsp, hsp70 (Fig. 2.5). However, this attenuation in response may have been largely a consequence of the normalization procedure used in these experiments. Because normalization was based on the amount of synthesis occurring in the 13°C-incubated preparations, higher levels of synthesis observed in field-acclimatized in comparison to laboratory-acclimated gills could mask the intensity of the heat shock response. The higher levels of protein synthesis observed in the field-acclimatized snails could be due to at least two factors. First, field acclimatization might support higher levels of protein synthesis due to better nutritional conditions. If this were the case, then higher amounts of synthesis of non-hsp proteins with masses similar to those of hsp's could mask the intensity of induction of hsp's. Second, field-acclimatized snails may have synthesized higher levels of hsp's due to exposure to conditions such as desiccation or hypoxia, that would not have affected the laboratory-acclimated specimens.

One additional effect of acclimation merits emphasis, the increased ability of gills from the most warm-acclimated specimens to synthesize proteins at the highest incubation temperatures (Fig. 2.9). The synthesis of hsp's during heat shock blocks the synthesis of non-hsp's in some organisms due to 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 indicate for the first time 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.

In summary, the diversity of synthetic patterns seen among species and among acclimation and acclimatization groups indicates that the heat shock responses of these snails are not a stereotypical, fixed 'program' that involves induction of a common battery of hsp's expressed with the same relative intensities. Instead, wide variation was found in the relative intensity, T_{on}, and T_{peak} of the different classes of hsp's, as functions of species and acclimation temperature. The bases of the differences in relative amounts of synthesis and T_{on}'s are undoubtedly complex, and likely are based on the different roles played by the various classes of hsp's (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 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's in the heat shock response before we can fully understand the importance of the inter- and intraspecific differences observed in this and other studies.

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. A study of field-acclimatized and laboratory-acclimated mussels (*Mytilus californianus*) also found marked differences between these two treatments (Roberts *et al.*, 1997). In common with the results reported for congeners of *Tegula*, summer-acclimatized mussels showed a greatly attenuated hsp70 response to temperature. These observed differences between field- and laboratory-based studies suggest that the complex set of abiotic factors other than temperature present in field settings, such as 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 hsp's can affect

conclusions. If constitutive synthesis of a particular class of hsp is high even in specimens not subjected to laboratory heat shock, as in the case of the gills from field-acclimatized snails incubated at 13°C, then the intensity of induction of hsp's at hsp-inducing temperatures may be under-estimated. In the present study, some of the apparent attenuation in the heat shock responses of field-acclimatized snails may have been due to a high constitutive level of synthesis at 13°C, especially in the case of hsp70.

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 have adaptive value and that play important roles in establishing 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 these species at a higher risk from global warming.

Chapter 3

The Time Course of Synthesis of Heat Shock Proteins in Congeneric Marine Snails (Genus *Tegula*) from Different Thermal Habitats: Relationships to Severity of Thermal Stress and Tidal Period

Abstract

Temporal patterning and magnitude of synthesis of heat shock proteins (hsp's) in relation to severity of thermal stress are important, yet poorly understood, aspects of the heat shock response in natural populations. I examined the synthesis of hsp's of size classes 90, 77, 70 and 38 kDa during recovery at 13°C after a 30°C exposure over a 50 h time period in marine snails (genus *Tegula*) that inhabit widely differing thermal habitats. Synthesis was measured by quantifying the incorporation of ^{35}S-labeled methionine and cysteine into newly synthesized proteins in gill tissue. In the mid- to low-intertidal *T. funebralis*, which frequently experiences 30°C, synthesis of hsp's was greatly enhanced immediately after heat stress, reached maximal levels 1-3 h into recovery, and returned to pre-stress levels by 6 h, except for hsp90 (14 h). In contrast, in the low-intertidal to subtidal *T. brunnea*, for which 30°C represents a near-lethal heat stress, enhanced synthesis of hsp's was observed 2-14 h after heat stress, reached maximal levels after 15-30 h, and returned to pre-stress levels in case of hsp90 (50 h) and hsp77 (30 h) but not in case of hsp70 and hsp38. Exposures to 30°C under aerial (emersion) in comparison

to aquatic (immersion) conditions resulted in much higher levels of hsp synthesis in *T. brunnea*, but not in *T. funebralis*. The delayed onset, higher magnitude, and prolonged period of hsp synthesis in *T. brunnea* suggest that the upper limit of its distribution range may be set by the thermal perturbation of proteins and the energetic costs of a severe heat shock response.

Introduction

The heat shock response, a cellular stress response in which heat shock proteins (hsp's) that are molecular chaperones are synthesized, occurs in almost all cell types (Feige *et al.*, 1996; Gething, 1997; Parsell and Lindquist, 1993; Parsell and Lindquist, 1994). Hsp's prevent protein damage caused by thermal or other stress, can refold already misfolded proteins or direct them to proteolytic degradation, and are viewed as critical for species' thermotolerance (Feder and Hofmann, 1999; Morimoto, 1998; Parsell and Lindquist, 1993; Parsell and Lindquist, 1994; Satyal and Morimoto, 1998). Several studies have shown that the stress response is frequently activated in some organisms near the upper temperatures that they experience (Feder and Hofmann, 1999; Hofmann and Somero, 1995; Roberts *et al.*, 1997; Tomanek and Somero, 1999) and that the magnitude of hsp synthesis is proportional to the severity of the stress (DiDomenico *et al.*, 1982b; Lindquist, 1993). Thus, the frequency and the amount of hsp synthesis should be proportional to the severity of the thermal stress. In

turn, more eurythermal species should show an overall less intense stress response to a given heat shock inducing exposure than stenothermal species.

Such differences in the intensity and the time course of the heat shock response, I hypothesized, may be of great importance for species in the intertidal zone, where they undergo exposures to large increases in temperatures (up to 20°C) that can reoccur within 24 h during periods of midday low tides (Tomanek and Somero, 1999). The time available to repair and prepare for heat damage is thus limited and the time course of hsp synthesis is therefore likely to be of great importance for species' thermotolerance. Temperature exposures that are experienced during low tide periods (emersion) have been shown to activate the heat shock response in bivalves of the genus *Mytilus* (Hofmann and Somero, 1995; Roberts *et al.*, 1997) and gastropod snails of the genus *Tegula* (Tomanek and Somero, 1999).

To investigate further the importance of the heat shock response for species' thermotolerance in the intertidal zone, I followed the time course of hsp synthesis after a 30°C exposure in the subtidal to low-intertidal *T. brunnea* and the low- to mid-intertidal *T. funebralis*, which differ in thermotolerance by 6.5°C (Tomanek and Somero, 1999). 30°C is frequently experienced by *T. funebralis*, but represents a near-lethal temperature for *T. brunnea*. Thus, by comparing these two congeners I addressed the following questions: What features distinguish a heat shock response (at 30°C) near the upper lethal temperature of hsp synthesis and therefore close to the limits of thermotolerance (as in the case of *T. brunnea*) from a normal

heat shock response at a temperature that is commonly experienced (as in the case of *T. funebralis*)? Does the intensity of the heat shock response and time required to recover from thermal damage correspond adaptively with the thermal features of the environment? Do different size classes of hsp's differ in their expression patterns within a species? To what extent does the time course (in comparison to a single time point measurement) of the heat shock response represent an experimentally important variable in elucidating the differences between the heat shock responses of *Tegula* congeners (Tomanek and Somero, 1999; Chapter 2)? In general, these questions address the importance of evolutionary or genetically fixed variation in the heat shock response that are instrumental in limiting thermotolerance and thereby distribution.

To elucidate genetically fixed phenotypic differences, I used laboratory-acclimated specimens of both species and followed the stress response over a 50 h time period after 2.5 h of 30°C heat shock by measuring incorporation of ^{35}S-methionine and -cysteine into newly synthesized proteins in gill tissue, which readily accumulates dissolved amino acids (Wright, 1988). I quantified the relative amount of new synthesis of putative hsp's belonging to four size classes, 90 kDa (hsp90), 77 kDa (hsp77), 70 kDa (hsp70), and 38 kDa (hsp38). The results presented here show that the time courses and magnitude of hsp synthesis during recovery from heat stress differ markedly between these species, and suggest that these differences play a role in determining the vertical distribution patterns of the two congeners.

Materials and Methods

Distribution, collection site, and maintenance of organisms

Tegula brunnea, which inhabits the subtidal to low-intertidal zone, occurs in the eastern Pacific Ocean from Cape Arago, Oregon, USA (43°25'N) to the Channel Islands, California, USA (34°00'N) (Abbott and Haderlie, 1980; Riedman *et al.*, 1981; Watanabe, 1984b). *Tegula funebralis*, which is restricted to the low- to mid-intertidal zone, occurs over a wider latitudinal distribution range, from Vancouver Island, British Columbia, Canada (48°25'N), to central Baja California, Mexico (28°00'N) (Abbott and Haderlie, 1980; Riedman *et al.*, 1981).

Specimens of both species were collected at the beginning of November 1997 at HMS of Stanford University in Pacific Grove, California, USA (36°36'N, 121°54'W) and kept at 13°C for a 29 d period. Specimens were kept constantly immersed and fed regularly with freshly collected giant kelp (*Macrocystis pyrifera*).

Survival of whole snails after heat shock

Laboratory-acclimated (13°C) specimens of both species were exposed to 30°C for 2.5 h and survival was assessed afterwards at 13°C over a 50 h time period by prodding the underside of the foot. If the withdrawal response did not occur, the snail was regarded as dead.

Heat shock protocol and tissue preparation: Time course of hsp expression

13°C-acclimated specimens of *T. funebralis* and *T. brunnea* were exposed to aerated seawater (SW) of 13°C (control group) and 30°C for 2.5 h. This period of exposure is much shorter than the full period of emersion frequently encountered at low tide in the mid-intertidal zone (see Chapter 2). Thus the thermal stress I applied was not extreme for *T. funebralis*, but was for the subtidal *T. brunnea*, which rarely gets emersed. After exposure to 30°C snails were returned to 13°C for 0 (control and treatment group), 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 14, 29 and 50 h. At each time point, gill tissue was dissected from 5 individuals of each species (dissections were completed within a 30 min period) under non-heat shock temperature conditions (13°C). Then, gill tissue was placed into 13°C-pre-equilibrated microcentrifuge tubes containing 0.2 μm filtered SW with 10 mmol l^{-1} glucose and ^{35}S-methionine and –cysteine (NEN), and incubated for 4 h under aeration. Higher concentrations of labeled amino acids were used for *T. brunnea* (0.33 mCi ml^{-1}; 30.0 to 45.0 mg wet weight) than for *T. funebralis* (0.22 mCi ml^{-1}; 15.0 to 25.0 mg) to compensate for higher tissue mass and lower uptake rates in the former species. Thus, levels of hsp's quantified in the autoradiographic analysis represent the sum of 4 h of synthesis after the indicated time of recovery. Homogenization of gill tissue, preparation of samples for autoradiography, SDS-polyacrylamide electrophoresis and fluorography all followed the protocol in Tomanek and Somero (1999) (Chapter 2).

Because temperatures that can induce heat shock are experienced in the intertidal zone under both aquatic (tidepools) and aerial (emersion) conditions, I tested how the heat shock responses of *T. funebralis* and *T. brunnea* differed under these two conditions of exposure. I compared levels of hsp synthesis between snails that I exposed to 30°C in SW (time point 0) with 13°C-acclimated snails that I exposed to 30°C air temperature for 2.5 h on moist paper substrate. Gill tissue of snails was dissected immediately after the exposure and radiolabeled for 4 h under aeration. All other steps followed the procedures described in Tomanek and Somero (1999) (Chapter 2).

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

Film images were digitized with a scanner (Sharp JX-330) and analyzed with image analysis software (ImageMaster 1D, Version 2.01, Pharmacia). I express all hsp band intensities relative to the respective band intensity of the 13°C control to account for potential differences in the overall level of protein synthesis between the two species. Levels of hsp synthesis therefore express the increase or decrease of synthesis relative to the control (13°C) group (see Chapter 2).

Note that hsp's are named according to molecular mass, but it has not been shown that the hsp's are homologous with other well characterized members of other hsp's of the same size class. Additionally, using one-dimensional electrophoresis, I could not exclude the possibility that hsp bands that were assigned to a specific size class contain more than one hsp homolog.

Hsp band intensities among different recovery times within a species were compared to the 13°C control group by using a 1-sided Dunnett test after a one-way analysis of variance (ANOVA; SYSTAT software, Systat, Inc.). For the ANOVA, data were log-transformed and time of recovery was used as the independent and hsp band intensity as the dependent variable. The first time at which levels of hsp were significantly different ($p \leq 0.05$) from the 13°C-control level represents the onset of synthesis of that hsp. The first time at which the level of hsp synthesis returned to, and remained at, a level of hsp expression not different from the 13°C-control is defined as the time of recovery (= completion of the heat shock response). For the comparison of hsp band intensities after exposure to 30°C under aquatic or aerial conditions I used a Student t-test to compare means.

Results

Time course of hsp expression during recovery from heat stress

I first established that a 30°C exposure has greatly different effects on the survival of *T. brunnea* and *T. funebralis* (Fig. 3.1). *Tegula brunnea* showed almost complete mortality over a 50 h period following exposure, whereas *T. funebralis* did not show any mortality. Therefore, comparisons of the heat shock responses of the two species might reveal how the temporal patterning and intensity of hsp synthesis is related to the severity of the heat stress. 30°C is a temperature that is frequently experienced by *T. funebralis* in its intertidal habitat (Tomanek and Somero, 1999). With incoming high tide, *T. funebralis* that just experienced heat

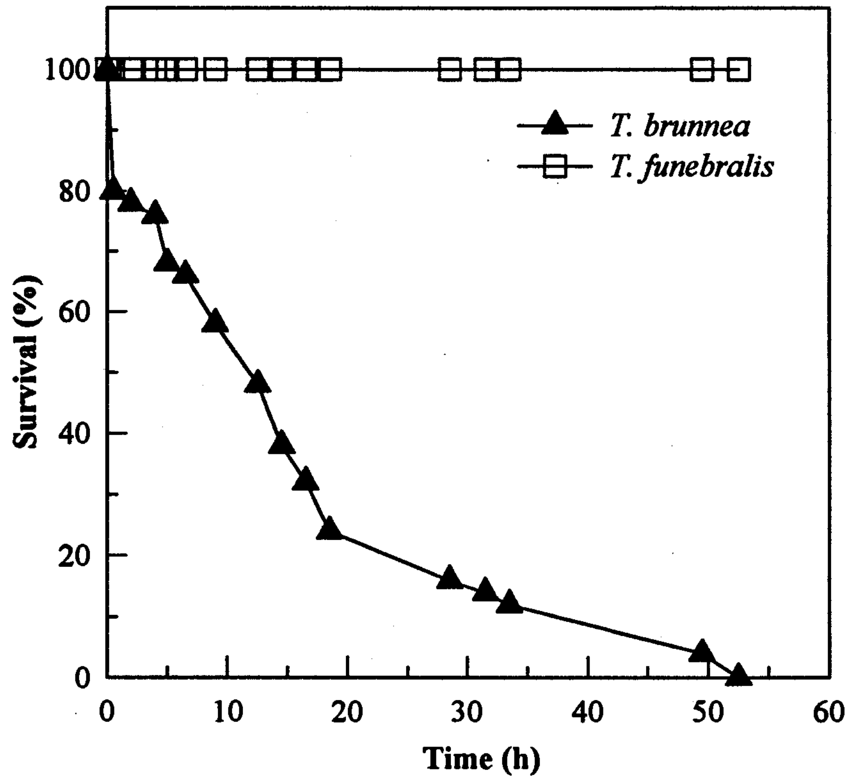


Figure 3.1. Time course of survival at 13°C of 13°C-acclimated *T. funebralis* and *T. brunnea*, after exposure to 30°C for 2.5 h (n = 50 for each species).

Figure 3.2. Autoradiographs showing the time course of incorporation of ^{35}S-methionine and –cysteine into proteins of gill tissue of *T. funebralis* (A) and *T. brunnea* (B) during 50 h of recovery at 13°C following a 2.5 h exposure at 30°C. Lanes represent *de novo* synthesis of proteins within 4 h after a certain time of recovery. For each time point, duplicates of a single snails' gill sample were loaded on a 10% SDS-polyacrylamide gel. ^{14}C molecular weight markers are shown in the far left lane of each autoradiograph. All lanes were loaded with 500 000 cpm and exposed for 8 h to pre-flashed X-ray film.

A

T. funebralis

Acclimation temperature 13°C

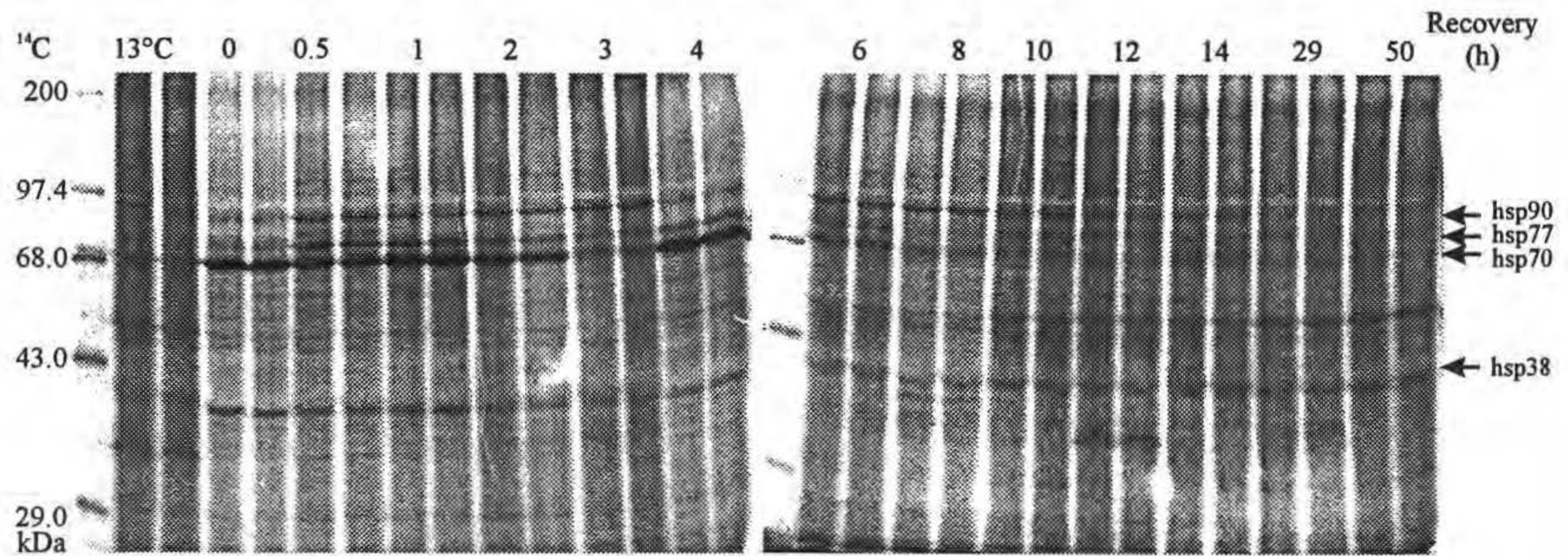


Figure 3.2

B

T. brunnea

Acclimation temperature 13°C

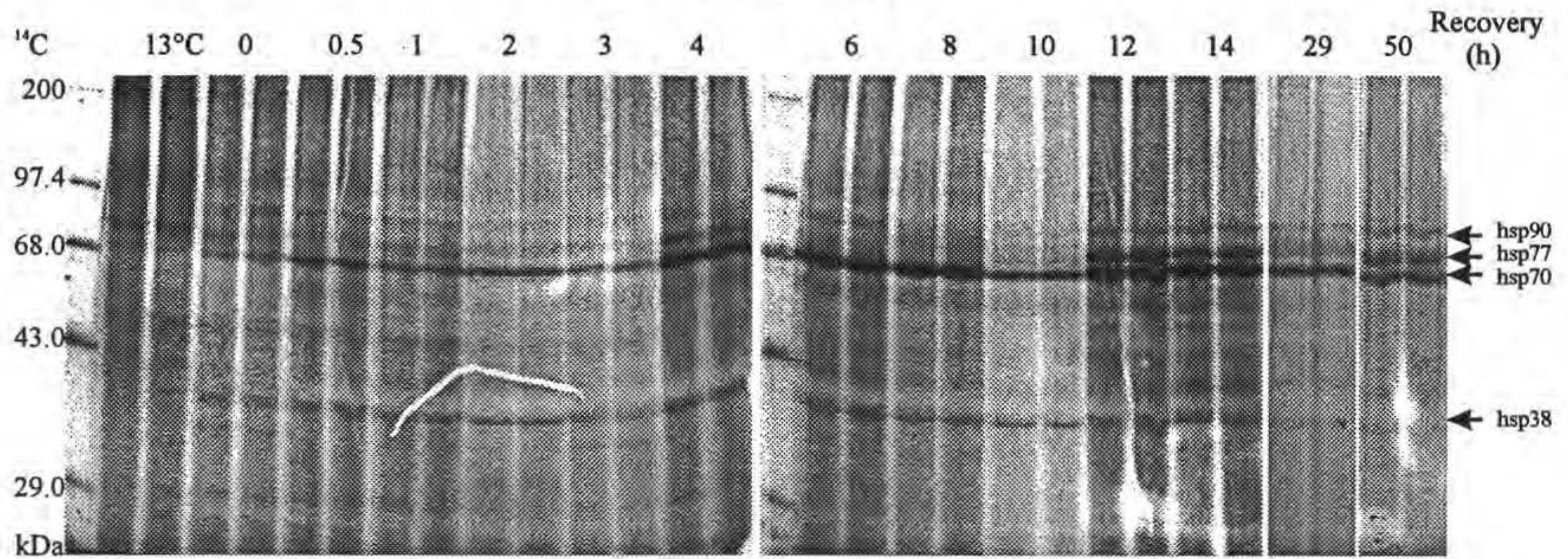


Figure 3.2 (continued)

Figure 3.3. Time course of synthesis of proteins of size classes 70kDa (hsp70), 38kDa (hsp38), 90kDa (hsp90) and 77kDa (hsp77) at 13°C in 13°C-acclimated *T. funebralis* and *T. brunnea* after a 2.5 h exposure to 30°C. Hsp levels are given relative to the 13°C control group and represent *de novo* synthesis of hsp's within 4 h of labeling after a certain time of recovery. Data are means ± 1 S.E.M. (n = 5 for all data points except for *T. brunnea*: 13°C control group (n = 4), 1 h (n = 4), 4 h (n = 4, n = 5 for hsp77), 10 h (n = 4 for hsp70 and hsp77, n = 3 for hsp90), 12 h (n = 4 only for hsp77) and 50 h (n = 4 for 38) time point).

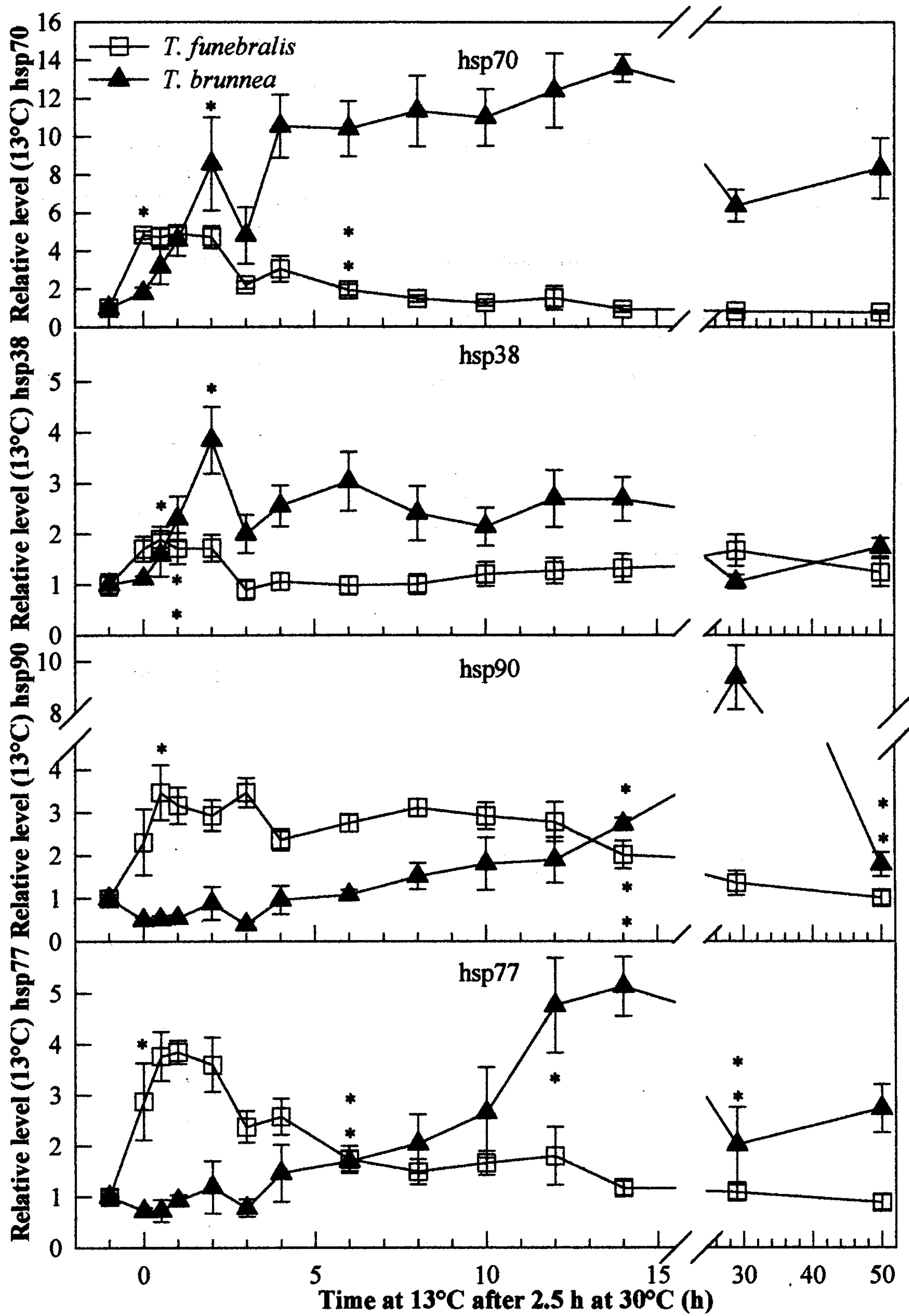


Figure 3.3

shock inducing temperatures get immersed and return to temperatures that allow recovery, a response that is likely to occur in nature (Hofmann and Somero, 1996b).

The autoradiographs given in Fig. 3.2 show the patterns of protein synthesis during the 50 h recovery period for *T. funebralis* and *T. brunnea*. As predicted, the two species differed significantly in the temporal patterning and intensity of the heat shock response. The onset of hsp synthesis was consistently more rapid for *T. funebralis*. For all size classes of hsp's, induction in *T. funebralis* was noted at the earliest time point and the heat shock response was maximal after 1-3 h of recovery (Figs. 3.2 and 3.3). The rate of decrease in the intensity of the response varied among hsp's (Fig. 3.3). For hsp70, synthesis decreased to control levels (=a 2.5 h exposure at 13°C followed by 4 h of labeling) after 6 h of recovery. Synthesis of hsp38 was only significantly different from control levels 30 min after exposure. For hsp77 and hsp90, synthesis fell to control levels after 6 and 14 h, respectively. The stress response in *T. funebralis* therefore can be initiated and largely completed within the time period of immersion following an extreme midday low tide.

In *T. brunnea* induction of hsp's occurred more slowly and seemed to be inhibited early during recovery, especially for hsp90 and hsp77 (Figs. 3.2 and 3.3). Hsp70 and hsp38 were significantly induced by 2 h of recovery. The maximal intensity of hsp synthesis was uniformly higher in *T. brunnea*. For instance, whereas synthesis of hsp70 was enhanced approximately five-fold in *T. funebralis*,

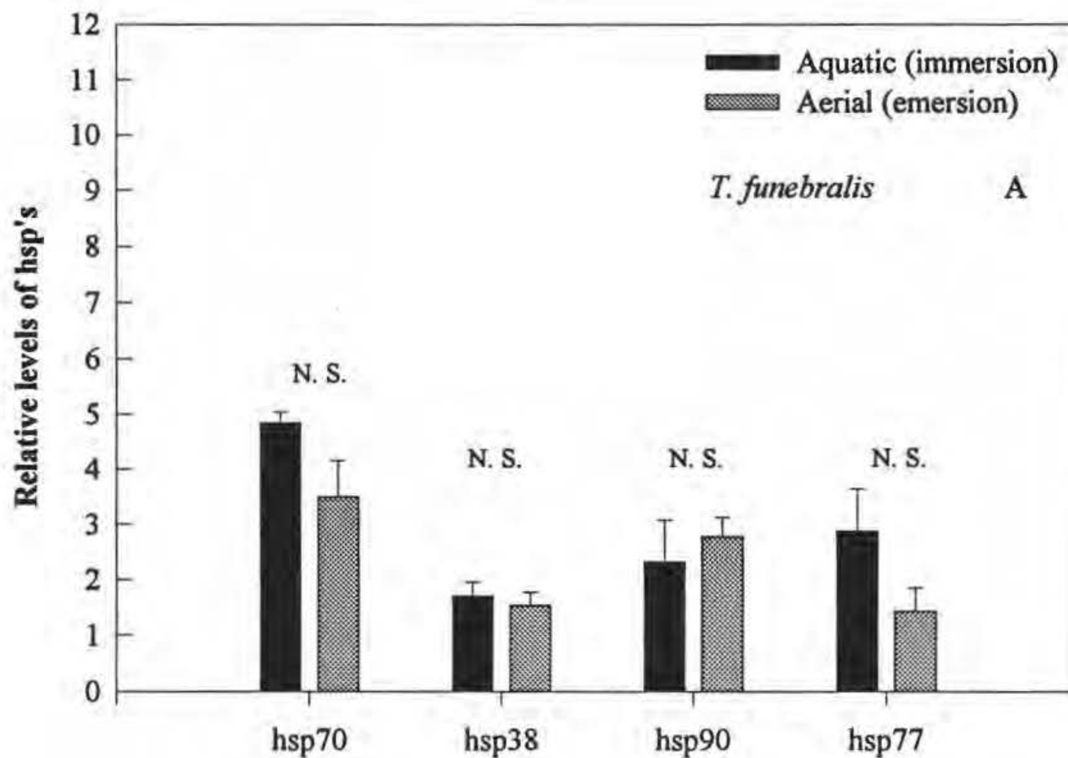


Figure 3.4. Levels of newly synthesized proteins of size classes 70 kDa (hsp70), 38 kDa (hsp38), 90 kDa (hsp90) and 77 kDa (hsp77) at 13°C in 13°C-acclimated *T. funebralis* (A) and *T. brunnea* (B) after a 2.5 h exposure to 30°C under aquatic and aerial conditions. Hsp levels are given relative to the 13°C control group and represent *de novo* synthesis of hsp's within 4 h of labeling with ^{35}S-methionine/ -cysteine. Data are means ± S.E.M. (n=5 for all data points). N.S. = non significant; * : $p \leq 0.05$.

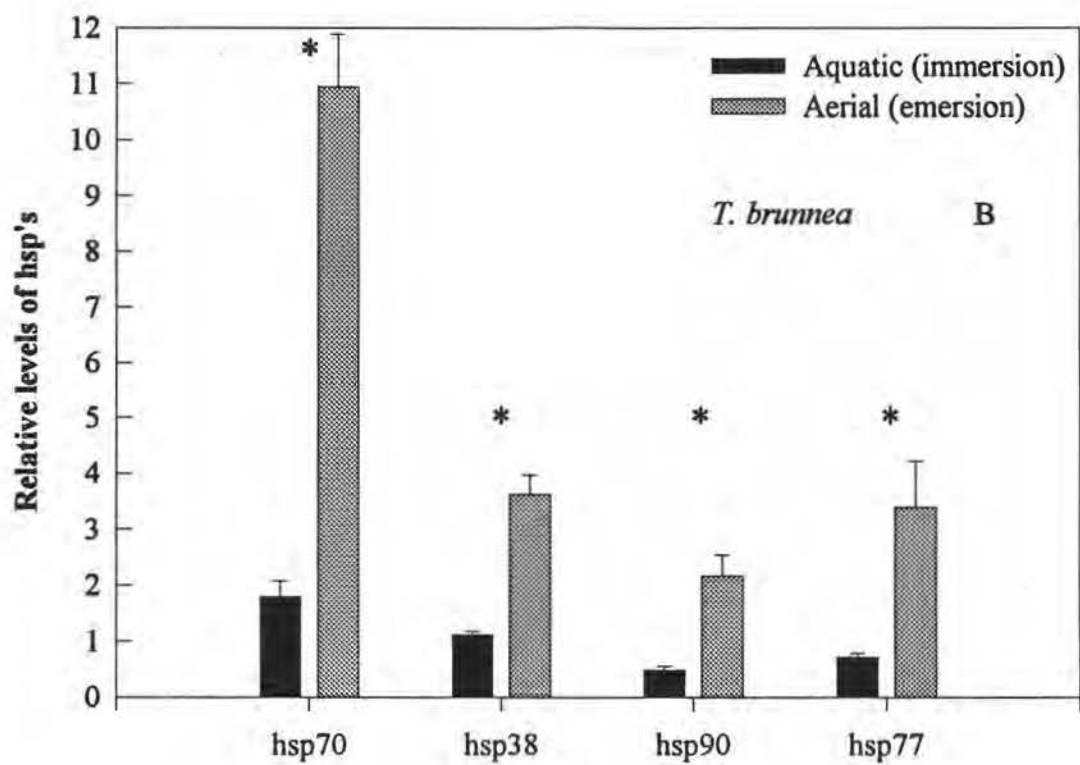


Figure 3.4 (continued)

an approximately 14-fold induction occurred in *T. brunnea* by 15 h of recovery. The delayed induction of synthesis, the prolonged period of synthesis, and the higher intensity of synthesis in *T. brunnea* are all consistent with a higher level of stress from exposure to 30°C compared to *T. funebralis*. In addition, note that gills of *T. brunnea* exhibited a stronger pattern of preferential synthesis of hsp's throughout recovery than *T. funebralis* (Fig. 3.2).

Heterogeneity in the temporal patterning of synthesis was not only observed between species but also among size classes of hsp's within a species. In *T. funebralis*, hsp70, hsp38 and hsp77 are synthesized for a shorter period than hsp90. In *T. brunnea*, hsp70 and hsp38 are induced much more rapidly (after 2 h) than hsp90 and hsp77 (14 and 12 h, respectively).

Heat shock responses under aquatic versus aerial conditions

Thermal conditions that can induce heat shock are experienced in tidepools and under aerial conditions during low-tide in the intertidal zone. To determine how the heat shock responses of *T. funebralis* and *T. brunnea* compare under both conditions, I conducted an experiment in which I exposed 13°C-acclimated specimens of both species to a 30°C heat shock under aquatic (see recovery experiment, 0 h time point) and aerial conditions (Fig. 3.4A and B). As would be predicted by the differing heat-conducting properties of water and air, the rate of heating differed between treatments: Body temperatures of snails transferred from 13°C SW to 30°C SW increased by about 4.50°C/min; temperatures of snails

transferred to humid air increased by about 0.42°C/min. Thus, body temperatures of snails increased about 10 times faster in water than in air.

In case of *T. funebralis*, I found no significant differences in levels of hsp synthesis between aerial (gradual) and aquatic (rapid heating) conditions for all hsp's ($p<0.05$). In *T. brunnea* levels of hsp expression were three- to four-times higher in case of hsp38, hsp77 and hsp90, and up to seven-times higher for the synthesis of hsp70 under aerial in comparison to aquatic conditions ($p<0.05$).

Discussion

Interspecific differences in the heat shock response and vertical zonation along the transition from the subtidal to the intertidal zone

The subtidal to low-intertidal *T. brunnea* and the mid- to low-intertidal *T. funebralis* differed in the time of initiation, the duration, and the intensity of the heat shock response following exposure to 30°C. Exposure to 30°C under aerial and aquatic conditions affected *T. brunnea*, but not *T. funebralis*, largely because of differences in the rate of heating that snails experienced under these conditions. Thus, features of the time course of hsp synthesis are greatly dependent on the severity of the stress, which in turn depends on the rate of heating. *T. funebralis*, which frequently experiences 30°C in the mid-intertidal zone (Tomanek and Somero, 1999), activated and completed the heat shock response faster and synthesized lower levels of hsp's than its subtidal congener. With the heat shock response largely completed after 6 h, specimens of this species are, thus, able to cope with protein damage that can be inflicted by a midday low tide before the re-

occurrence of the next low tide. The ability to repair the consequences of and to prepare for a subsequent heat shock within a high tide period is likely to greatly affect the ability of *T. funebralis* to inhabit the intertidal zone, especially in comparison to its subtidal congener. At least when the rate of heating is rapid, the infrequently emersed *T. brunnea* is unable to successfully complete hsp synthesis during a typical re-immersion period following heat stress, and may not be able to withstand a subsequent exposure.

These interspecific differences in the heat shock response are likely to play an important role in establishing the vertical distribution ranges of these two species. Within a very narrow zone (0.5 m or less) the low-intertidal to subtidal *T. brunnea* replaces *T. funebralis* as the dominant *Tegula* species. Snails within the vertical range of *T. brunnea* are infrequently emersed during low tide periods and therefore rarely obtain body temperatures that induce heat shock (Tomanek and Somero, 1999). The interspecific differences presented in this study and our previous investigations that considered only a single time point in the recovery period, suggest that *T. brunnea* could not cope with exposure to thermal conditions that are commonly experienced by its higher-occurring congener. Because experimental exposure conditions were chosen conservatively in regard to the degree of heat stress that is encountered in the intertidal zone (Tomanek and Somero, 1999), I conjectured that the differing features of the heat shock response were likely to be factors that limit vertical distribution.

Possible sites of thermal damage that are of particular importance in limiting the thermal range of the heat shock response are suggested by the differing levels of hsp synthesis following exposure to emersed (aerial) *versus* immersed (aquatic) i. e., gradual *versus* rapid, heating conditions. Previous studies on *Drosophila* showed that the rate of heating to the exposure temperatures plays an important role in the kinetics of the stress response, i.e., slower heating rates allow *Drosophila* cells to extend their temperature range of hsp synthesis (DiDomenico *et al.*, 1982b; Lindquist, 1980). In contrast, rapid heating rates block transcription and thereby limit the temperature range of hsp synthesis. These observations may in part explain why *T. brunnea* synthesized higher levels of hsp's under aerial conditions (=slower heating rates; Fig. 3.4) and why the induction of the heat shock response was delayed in *T. brunnea* following exposure to aquatic conditions (=rapid heating rates, Figs. 3.2 and 3.3). To allow enough transcription and translation of *hsp* message to occur before critical temperatures are reached may ultimately determine if a cell can cope with the thermal stress. Hsp70 is often found to be bound to RNA, which may indicate that hsp70 is necessary to stabilize its own message, the transcriptional and (or) the translational apparatus to secure its own transcription and translation at the highest temperatures (Beck and De Maio, 1994; DiDomenico *et al.*, 1982a).

The relatively greater severity of the 30°C exposure to *T. brunnea* is also indicated by the inhibition of synthesis of most non-hsp's over much of the recovery period, but especially at the 29 h and 50 h time point (Fig. 3.2).

Commonly it is observed that mRNA's of hsp's are preferentially translated following extreme heat stress (Lindquist, 1980; 1981; 1993; Storti *et al.*, 1980). The preferential translation of hsp's is likely to disrupt protein homeostasis and therefore the production of ATP. This and the observation that the repair and the degradation of damaged proteins is energetically costly (Feder *et al.*, 1992; Heckathorn *et al.*, 1996; Krebs and Loeschke, 1994) may together contribute significantly to defining the limits of a species' thermal niche.

Experimental design influences the observed properties of the heat shock response

The present as well as a previous study (Tomanek and Somero, 1999) illustrate that the heat shock response in an organism is dependent on several factors, most importantly (i) the previous thermal history (acclimation and acclimatization state) of an organism, (ii) the magnitude of the temperature exposure relative to the thermal history, (iii) the rate with which the thermal stress is applied, and (iv) the time(s) at which hsp synthesis is measured following thermal stress. All of these factors of the experimental design have an influence on the observed properties of the heat shock response.

In a previous study (Tomanek and Somero, 1999) I reported interspecific differences in the heat shock response of identically acclimated specimens of several *Tegula* species according to thermal niche. However, levels of hsp synthesis were only compared at a single time point during recovery. The time course of the heat shock response revealed additional patterns of interspecific

differences that could not be detected by comparing a single time point. Differences in the time of induction, and the duration and the magnitude of the response could be detected only after we followed the time course over an extended time period (50 h). These features of the heat shock response differed significantly between the lower- and higher-occurring *Tegula* congeners and are likely to be very important for limiting thermotolerance and thereby for establishing limits of vertical distribution.

Chapter 4

The Role of Hsp70 Isoforms in Regulating Interspecific and Acclimation-Induced Intraspecific Variation in the Induction Temperature of the Heat Shock Response in Congeneric Marine Snails (Genus *Tegula*) from Different Thermal Habitats

Abstract

Heat shock proteins (hsp's) have been implicated in the negative regulation of the transcription of their own genes by inhibiting the induction of the heat shock or stress response until increasing levels of stress require higher levels of hsp's. Although hsp70, a stress protein with a molecular mass of 70 kDa, is only one of the members of a multiprotein complex that regulates the induction of the stress response, there is strong evidence that endogenous levels of hsp70 directly repress induction by interacting with the heat shock transcription factor. The role of endogenous levels of hsp70 in regulating interspecific and acclimation-induced intraspecific variation in the induction of the stress response is still unresolved. Here I examined *via* immunodetection the regulatory role of low (hsp72) and high molecular mass (hsp74) hsp70 isoforms in gill tissue among several *Tegula* congeners that inhabit widely differing thermal habitats. Acclimatory changes indicate that endogenous levels of hsp72 may negatively regulate the induction of the stress response. Levels of hsp72, but not hsp74, were higher at lower acclimation temperatures in the mid- to low-intertidal *T. funebralis* than in the low-

intertidal to subtidal *T. brunnea* and *T. montereyi*, in accordance with higher temperatures of induction of the heat shock response in the former species. Hsp72, but probably not hsp74, also seems to regulate acclimation-induced intraspecific changes in induction temperature that I observed for *T. brunnea* and *T. montereyi*. Neither levels of hsp72 (and hsp74) nor induction of the stress response changed with acclimation in *T. funebralis*. Additionally, I examined acclimatory changes for both isoforms under varying thermal conditions that were either slightly lower than temperatures that induce the heat shock response (13° → 23°C) or temperatures at which the response was induced (18° → 28°C). Under both acclimation conditions, hsp72 displayed greater changes and seemed to reflect temperature variation more closely than hsp74. Thus, endogenous levels of hsp72 seem to play a greater regulatory role than levels of hsp74 in setting interspecific and acclimation-induced intraspecific variation in induction of the stress response. These results therefore support the hypothesis that the "cellular thermometer" regulatory process based on levels of hsp's operates during laboratory acclimation.

Introduction

Thermal and other environmental stress can activate the heat shock response, the enhanced synthesis of a group of proteins, the heat shock proteins (hsp's), that are members of several unrelated families of molecular chaperones (Bukau and Horwich, 1998; Feige *et al.*, 1996; Gething and Sambrook, 1992). Hsp's are induced mainly due to the denaturation of proteins, and act by preventing

further denaturation and by facilitating refolding of already damaged proteins (Parsell and Lindquist, 1993, 1994). The activation of the heat shock or stress response is tightly regulated and has been proposed to be one of the steps of the "cellular thermometer" (Craig and Gross, 1991; Morimoto, 1998). Generally, a multiprotein complex that is mainly composed of hsp's is thought to repress the activation of the heat shock response by binding to the monomeric form of heat shock transcription factor 1 (HSF1). When proteins are denatured due to stress, hsp's, e. g. hsp70, are sequestered away from the multiprotein complex. The inactive monomeric form of HSF1 can now convert to the active trimeric form, which binds to the heat shock element (HSE), a DNA regulatory sequence upstream of *hsp* genes, and can thus initiate the activation of the heat shock response.

The observation that the amount of *hsp* gene expression correlated positively with the severity of the stress and negatively with the quantity of hsp70 synthesized in *Drosophila* (DiDomenico *et al.*, 1982b) first implied an autoregulatory role of hsp's in the transcription of their own genes. Subsequent evidence from mutations in hsp70's of *Sacchromyces cerevisae* and *Escherichia coli* that led to overexpression of hsp's suggested a model for the cellular thermometer in which hsp70 acts as the main repressor of the transcription of its own genes *via* its interaction with HSF1 (Craig and Gross, 1991). Transiently altered levels of hsp70 led to the predicted regulatory changes in human cells lines (Baler *et al.*, 1992; Mosser *et al.*, 1993). Hsp70 levels could be altered by (i) pre-

treating cells with a heat shock, (ii) lowering the amount of nascent polypeptides, which resulted in an increased level of free hsp70 that was available, (iii) microinjection of anti-hsp70 antibodies or (iv) co-transfection of cell lines with additional copies of hsp70 (Baler *et al.*, 1992; Mosser *et al.*, 1993). Increasing levels of hsp70 consistently led to a delayed or attenuated stress response. Association of hsp70 with HSF1 was shown to block DNA-binding by HSF1 during recovery (Abravaya *et al.*, 1992), but may be insufficient to repress the onset of the stress response under *in vivo* conditions (Rabindran *et al.*, 1994).

Mutational studies provided evidence for the involvement of other hsp's in regulating the heat shock response, e. g. some of hsp70's co-chaperones (hsp40 and hsp10) (Craig and Gross, 1991). Hsp90, which modulates the activity of various signal transducing kinases and transcription factors in connection with a multiprotein complex (Buchner, 1999; Mayer and Bukau, 1999; Pratt, 1998), has been indicated to negatively regulate the activation of the heat shock response by interacting with the inactive monomeric and the active trimeric forms of HSF1 (Ali *et al.*, 1998; Duina *et al.*, 1998; Zou *et al.*, 1998). Additionally, HSF1 can form a homotrimer and bind to the HSE independently of any extrinsic factors, suggesting that the transcription factor possesses major intrinsic regulatory properties (Larson *et al.*, 1995; Sarge *et al.*, 1993; Zhong *et al.*, 1998; Zuo *et al.*, 1995). However, the temperature at which a specific HSF1 induces *hsp* gene expression is not absolute and has been shown to change if expressed in transgenic cells of a different species or if examined in cells within an organism that maintain a different body

temperature, e.g. testis cells in mammals (Clos *et al.*, 1993; Sarge, 1995). Thus, the induction and attenuation of the heat shock response are controlled, depending on the stage of the activation cascade, by HSF1 itself, a multiprotein complex that includes HSF1 and at least hsp90, hsp70 and hsp40, a heat shock factor binding protein 1 (HSPB1) and potentially additional proteins that as yet not defined (Satyal *et al.*, 1998; for review see Morimoto, 1998). In general, all of the known extrinsic factors, e. g. mostly hsp's, are repressors of the heat shock response.

The role of hsp's in regulating the variation of induction temperature of the heat shock response during field-acclimatization or laboratory-acclimation within a species or in setting differences in induction temperature or acclimatory plasticity of the stress response among species is still unclear. In brain tissue, induction of hsp90 changed from 28°C in winter- to 32°C in summer-acclimatized goby fishes of the genus *Gillichthys* (Dietz and Somero, 1992). Summer-acclimatized specimens also showed higher endogenous levels of hsp90 than winter-acclimatized fishes. Although the two species studied, *G. seta* and *G. mirabilis*, differ slightly in thermotolerance, they did not differ in the properties of their heat shock responses. Laboratory-acclimation of *G. mirabilis* to 10°, 20° and 30°C shifted induction of hsp90 and hsp70 towards higher temperatures in gill and liver tissue, but endogenous levels of hsp's were not reported (Dietz, 1994). The temperature of induction for hsp70 shifted towards higher temperatures in summer- in comparison to winter-acclimatized mussels of the species *Mytilus californianus* (Roberts *et al.*, 1997). Specimens collected from high and low sites in the intertidal

did not differ significantly in their response. Endogenous levels of hsp70 in summer-acclimatized mussels, however, were higher only in the low molecular mass hsp70 isoform that was detected by a hsp70 antibody, and only in mussels from sites high but not low in the intertidal. A high molecular mass hsp70 isoform was actually higher in winter mussels from sites low in the intertidal. Interestingly, mussels acclimated in the laboratory over a wide range of temperatures showed only minor changes in induction temperature and no changes in endogenous levels of the two hsp70 isoforms that were detected. Together, these studies suggest that the proposed cellular thermometer may also operate under natural conditions. However, the evidence is not always consistent and further studies are required to substantiate and specify the regulatory role of endogenous levels of different hsp70 isoforms in setting patterns of acclimation-induced intraspecific variation in induction of the stress response. The role of hsp70 in regulating patterns of interspecific variation in induction is completely unknown.

Here I examined the regulatory properties of the heat shock response in several snail species of the genus *Tegula* that occupy widely differing thermal niches along the transition from the subtidal to the intertidal zone. By comparing acclimation-induced changes among congeneric species I addressed the following questions: How do closely related species that differ widely in thermotolerance vary in their endogenous levels of hsp70 isoforms? Is interspecific variation in endogenous levels of hsp70 subject to acclimation under laboratory conditions? Do hsp70 isoforms differ in their role in regulating the induction of the stress response?

These data directly relate to a study that provided a comprehensive analysis of acclimatory plasticity of the heat shock response in several *Tegula* congeners (Tomanek and Somero, 1999; Chapter 2). Additionally, I examine how acclimation conditions that vary in temperatures elicit changes in endogenous levels of hsp70 isoforms: First, I followed changes that are induced by varying laboratory-acclimation temperatures within the thermal range that *Tegula* congeners commonly experience under natural conditions and that are slightly below temperatures that induce the heat shock response (13° → 23°C). These temperature changes may occur due to oceanic upwelling events and El Niño episodes. Second, I acclimated specimens to thermal conditions that exposed snails to a daily heat shock (18° → 28°C) and thus attempted to replicate thermal conditions that are commonly experienced by snails in the intertidal zone. These results elucidate how differing thermal regimens change endogenous levels of hsp70 isoforms in congeneric species that occupy widely differing thermal habitats.

Materials and Methods

Organisms, distribution patterns, and collection sites

The three *Tegula* congeners differ in their biogeographic patterns and vertical distributions in the intertidal as follows (see Chapter 2, Figs. 2.1A and B): *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°00'N) and from Sonoma County, California (38°17'N), to the Channel Islands, respectively (Riedman *et al.*, 1981; Watanabe, 1984; Abbott and Haderlie, 1980). *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°00'N) (Riedman *et al.*, 1981; Abbott and Haderlie, 1980).

Specimens of both 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 sizes of specimens were similar among all three species.

Thermal acclimation and experimental design

Experiment 1: The effect of field-acclimatization and laboratory-acclimation under constant temperature conditions. Specimens of all three *Tegula* species were collected for acclimation in mid-July 1997 and either immediately dissected (field-acclimatized control group) or kept in temperature-controlled (13°, 18° and 23°C) circulating seawater (SW) aquaria for 30 to 34 d (n = 5 for each species). Specimens were kept constantly immersed and fed regularly with freshly collected giant kelp (*Macrocystis pyrifera*; experiments 1 - 3).

Experiment 2: The effect of laboratory-acclimation under varying thermal conditions below temperatures that induce heat shock (13° → 23°C). Snails were kept in temperature-controlled SW aquaria at 13°C and transferred to 23°C for 12 h

every day (D1), every other day (D3) or every fifth day (D5) for 30 to 32 days. Specimens (n = 5 for each species) of all treatment groups were dissected 24 h after the last exposure to 23°C.

Experiment 3: Effects of laboratory-acclimation under temperature conditions that induce heat shock (18° → 28°C). Snails were kept in temperature-controlled SW aquaria at 18°C. Every day specimens of *T. brunnea* and *T. funebralis* were exposed to 28°C for 2.5 h and subsequently recovered at 18°C at a specific fixed time that did not correspond with the tidal rhythm. Snails were collected for dissection (n = 5 for each species) after 4 h of recovery (18°C) on day 0 (control), 1, 3, 5, 8, 11, 15, 22 and 29.

Tissue preparation

I dissected gill tissue under conditions that do not induce heat shock (13°C) and immediately placed it in 200 μl (*T. funebralis*, 15.0 to 25.0 mg wet weight) or 300 μl (*T. brunnea* and *T. montereyi*, 30.0 to 45.0 mg wet weight) 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. No

proteolytic activity was detected when I compared samples from this homogenization procedure with protein samples that were not boiled but prepared on ice under otherwise identical conditions (data not shown). 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). Subsequently, proteins were transferred for 1 h 15 min at 80 V (BioRad Protean apparatus) onto nitrocellulose membranes (Nitrobind, Schleicher and Schuell) that were soaked for at least 2 h in transfer buffer (0.025 M Tris-base, 0.193 M glycine, 20% methanol (v/v), pH 8.3 at 20°C). Membranes were dried on tissue paper overnight.

Nitrocellulose membranes were blocked with blocking buffer (0.025 M Tris-Cl, pH 7.5, 0.15 M 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; 0.025 M Tris-Cl, pH 7.5 at 20°C, 0.15 M NaCl), and then incubated with a solution of a monoclonal rat antibody (IgG) against hsp70 (clone 7.10; Affinity BioReagent MA3-001; 1 : 2 500 dilution of hsp70 antibody in buffer A (BA): TBS, 2.5% (w/v) bovine serum albumin , 0.02% (w/v) Thimerosol) for 1 h. 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. I then incubated the membrane 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 finally TBS again. Finally I incubated membranes with a horseradish-peroxidase Protein A solution (1 : 5 000 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 I exposed membranes onto pre-flashed Hyperfilm (Amersham) for 5, 10 and 20 sec 10, 30 and 50 min after ECL treatment to obtain various exposures that were in the linear range of detection.

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

I 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 band intensities of two hsp70 isoforms, one with a molecular mass of about 72 kDa (hsp72), the other of about 74 kDa (hsp74). To account for variation among gels I express band intensities relative to a known amount (80 ng) of a bovine heat shock cognate 70 standard (StressGen SPP-750), which I loaded on each gel.

Differences in endogenous levels of hsp70 isoforms were tested among species within treatments and among treatments within species after a two-way analysis of variance (ANOVA) using the Tukey-test for a *post hoc* pairwise comparison (SYSTAT software, Systat, Inc.). For the two-way ANOVA, species and treatment were used as independent categorical variables, while endogenous levels of hsp70 isoforms were used as the dependent variable. Differences are described as statistically significant on a p-level ≤ 0.05. However, due to the low number of samples ($n = 5$ or 4) of the comparisons presented here, I also indicate p-levels ≥ 0.05 but ≤ 0.10.

Results

The effect of field-acclimatization and laboratory-acclimation under constant temperature conditions

Snails of all three *Tegula* congeners were collected from the field in July 1997 (summer-acclimatized control group) and subsequently acclimated to 13°, 18° and 23°C for 30 to 34 days. Using a hsp70 antibody I detected low (hsp72) and high molecular mass (hsp74) isoforms of hsp70 (Fig. 4.1.). Endogenous levels of both isoforms varied among species that experienced a common acclimation temperature and among acclimation temperatures within a species, e. g., compare intensities of hsp72 with increasing acclimation temperature in *T. brunnea* and *T. montereyi* (Fig. 4.1).

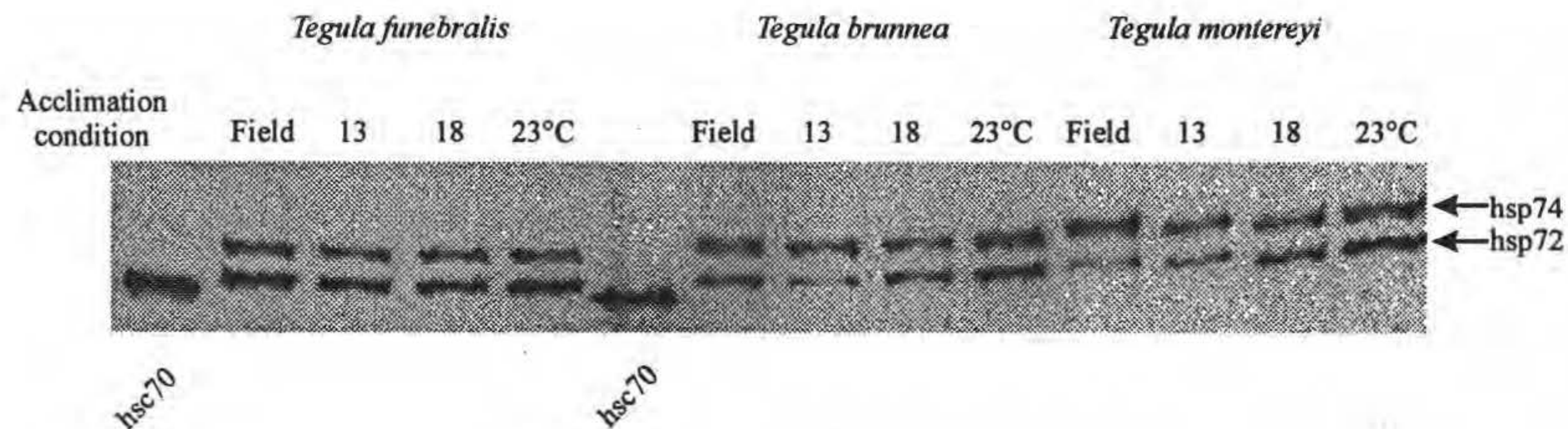
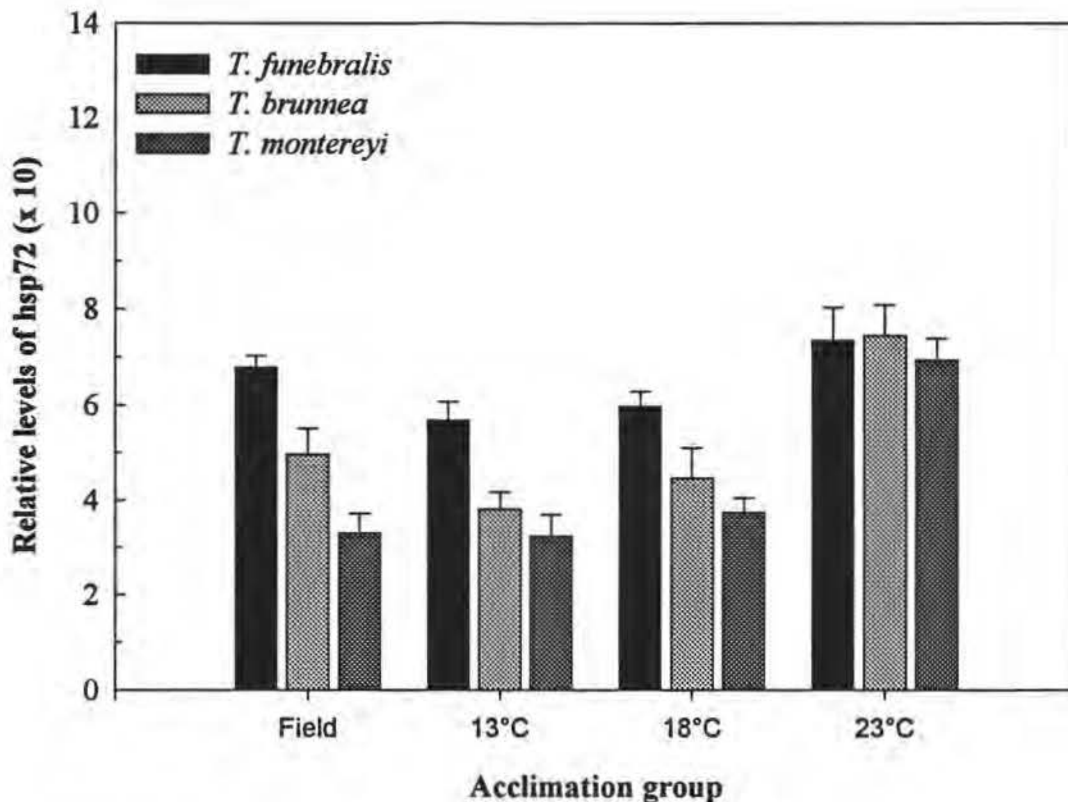


Figure 4.1. Immunoblot analysis (western) of endogenous levels of two hsp70 isoforms (hsp72, hsp74) in three *Tegula* congeners following field-acclimatization (July 1997) and laboratory-acclimation at 13°, 18° and 23°C for 30 to 34 days. Lanes were loaded with 5 μg of protein of gill tissue from a single individual or 80 ng of a bovine heat shock cognate (hsc70). Hyperfilm was exposed for 10 s following detection with the ECL method (see text for further details).



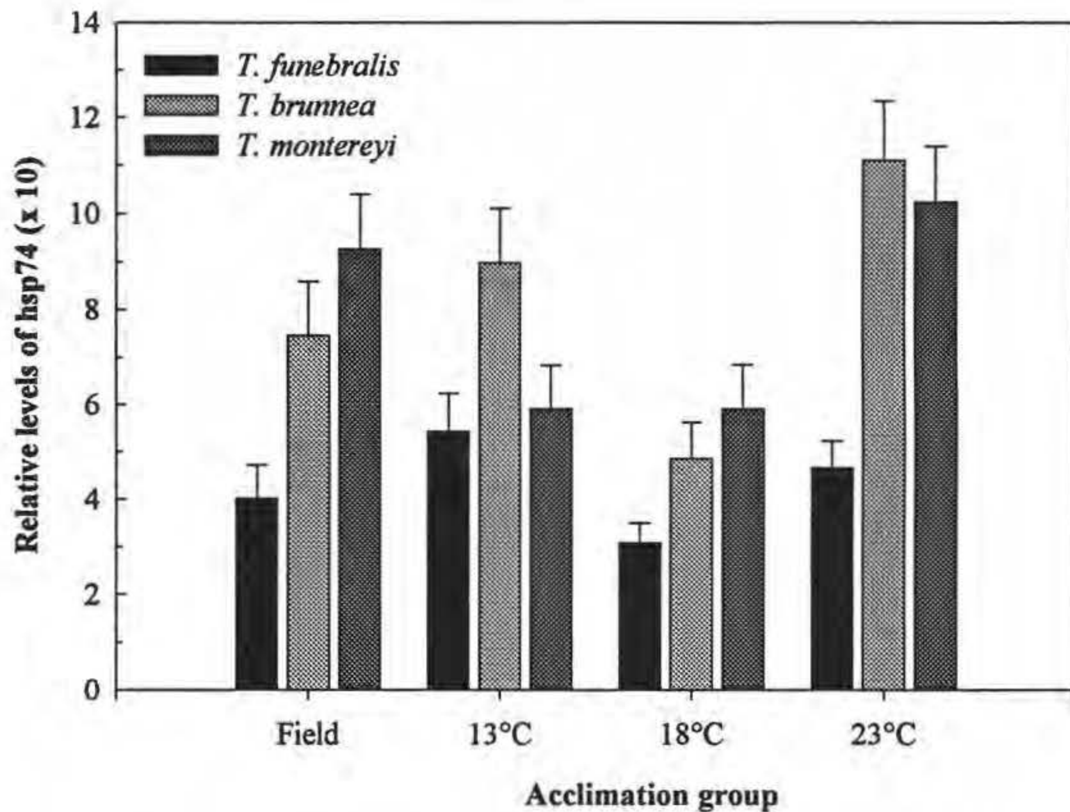
T. funebralis N.S. N.S. N.S. Ref.

T. brunnea XX XX XX Ref.

T. montereyi XX XX XX Ref.

Ref. N.S. XX Ref. N.S. XX Ref. N.S. XX Ref. N.S. N.S.

Figure 4.2. Endogenous levels of hsp72 in three *Tegula* congeners field-acclimatized (July 1997) and laboratory-acclimated to 13°, 18° and 23°C for 30 to 34 days. Long lines indicate pairwise comparisons within species among treatments; short lines indicate comparisons within treatments among species. Ref. refers to the group that all other groups along a line are compared to. P-values: XX $\leq$ 0.05; N.S. = non significant. Data are means ± 1 S.E.M. (n = 5 for all data points).



T. funebralis	N.S.	N.S.	N.S.	Ref.
T. brunnea	N.S.	N.S.	XX	Ref.
T. montereyi	N.S.	X	X	Ref.
	Ref. N.S. XX	Ref. N.S. N.S.	Ref. N.S. N.S.	Ref. XX XX

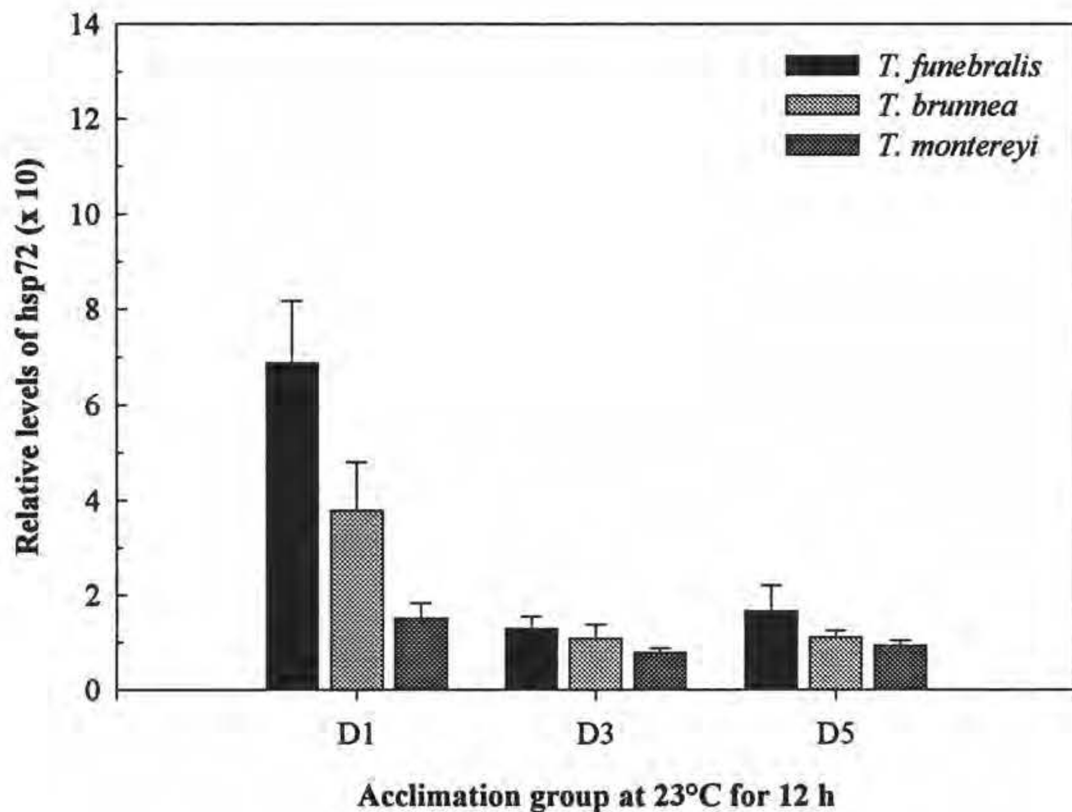
Figure 4.3. Endogenous levels of hsp74 in three *Tegula* congeners field-acclimatized (July 1997) and laboratory-acclimated to 13°, 18° and 23°C for 30 to 34 days. Long lines indicate pairwise comparisons within species among treatments; short lines indicate comparisons within treatments among species. Ref. refers to the group that all other groups along a line are compared to. P-values: XX $\leq$ 0.05; X $\geq$ 0.05 but $\leq$ 0.10; N.S. = non significant. Data are means ± 1 S.E.M. (n = 5 for all data points).

Both low-intertidal to subtidal species, *T. brunnea* and *T. montereyi*, showed lower endogenous levels of hsp72 in summer field-acclimatized, 13° and 18°C-acclimated specimens than the low- to mid-intertidal *T. funebralis* (Fig. 4.2.). In contrast, 23°C-acclimated specimens of all three species contained equal levels of hsp72. However, interspecific differences within treatment groups differed significantly only between *T. funebralis* and *T. montereyi* (in all but the 23°C treatment). Intraspecific comparisons show that endogenous levels were significantly higher at 23°C than in all other treatments in *T. brunnea* and *T. montereyi*, but not in *T. funebralis*.

In contrast to hsp72, endogenous levels of hsp74 were generally higher in *T. brunnea* and *T. montereyi* in comparison to *T. funebralis* (Fig. 4.3.). However, these differences were only significant for the 23°C-acclimated specimens. Intraspecific differences across the various acclimation temperatures were only apparent between the 18° and 23°C treatments and only for *T. brunnea* and *T. montereyi*.

The effect of laboratory-acclimation under varying thermal conditions (13° → 23°C)

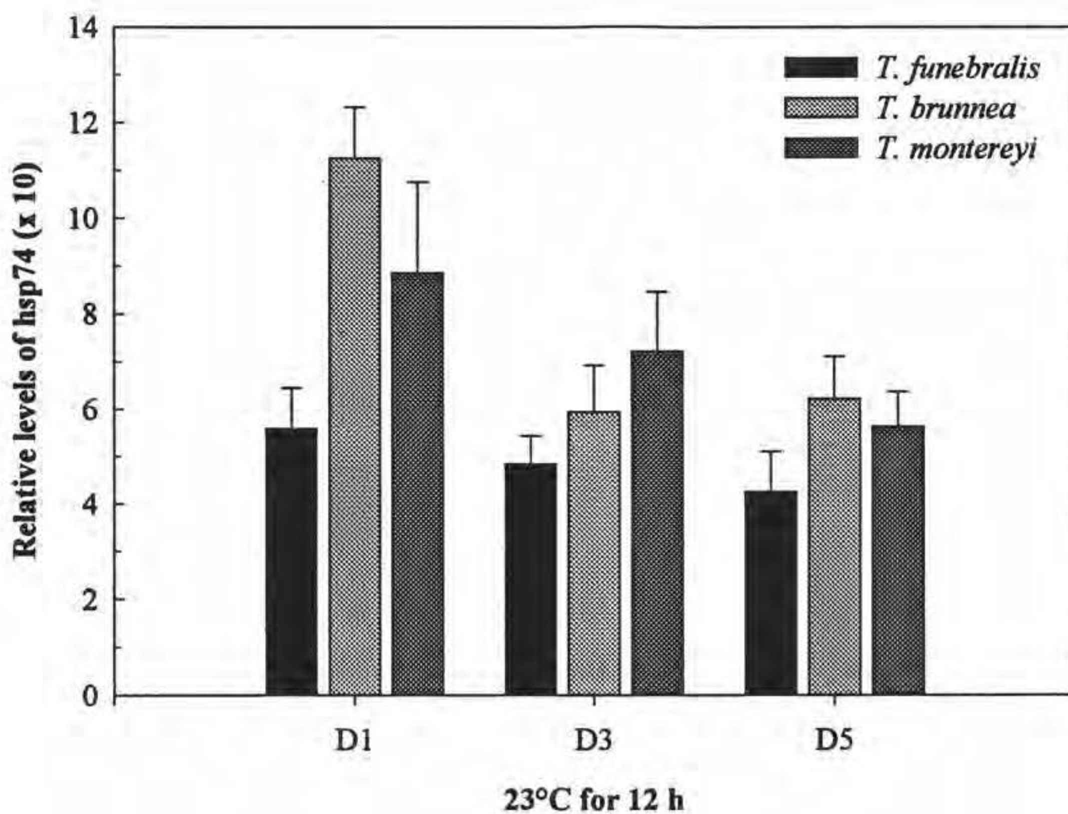
To test the effect that varying temperature regimens may have on endogenous levels of both hsp70 isoforms, I exposed 13°C-acclimated snails to 23°C for 12 h either every (D1), every other (D3) or every fifth day (D5). These temperatures are within the range that is experienced by snails of all three *Tegula* species under natural conditions. However, 23°C represents an extreme



T. funebralis	Ref.	XX	XX
T. brunnea	Ref.	X	X
T. montereyi	Ref.	N.S.	N.S.

Ref. XX XX   Ref. N.S. N.S.   Ref. N.S. N.S.

Figure 4.4. Endogenous levels of hsp72 in three *Tegula* congeners acclimated to 13°C and exposed to 23° for 12 h every (D1), every other (D3) and every fifth day (D5) for 30 to 32 days. Long lines indicate pairwise comparisons within species among treatments; short lines indicate comparisons within treatments among species. Reference: Ref.. P-values: XX ≤ 0.05; X ≥ 0.05 but ≤ 0.10; N.S. = non significant. Data are means ± 1 S.E.M. (n = 5 for all data points except for *T. b.* and *T. m.* in D1 (n = 4)).



T. funebralis Ref. N.S. N.S.

T. brunnea Ref. XX XX

T. montereyi Ref. N.S. N.S.

Ref. XX N.S. Ref. N.S. N.S. Ref. N.S. N.S.

Figure 4.5. Endogenous levels of hsp74 in three *Tegula* congeners acclimated to 13°C and exposed to 23° for 12 h every (D1), every other (D3) and every fifth day (D5) for 30 to 32 days. Long lines indicate pairwise comparisons within species among treatments; short lines indicate comparisons within treatments among species. Reference: Ref.. P-values: XX < 0.05; X > 0.05 but < 0.10; N.S. = non significant. Data are means ± 1 S.E.M. (n = 5 for all data points except for *T. b.* and *T. m.* in D1 (n = 4)).

acclimation temperature for *T. brunnea* and *T. montereyi* and is close to conditions that induce heat shock (24°C) in 13°C-acclimated specimens of both species (Tomanek and Somero, 1999).

Endogenous levels of hsp72 were higher for the D1 than for the D3 and D5 treatments, especially for *T. funebralis* and *T. brunnea* (Fig. 4.4.). Interspecific differences in hsp72 levels were significantly higher in *T. funebralis* than in the two subtidal species, *T. brunnea* and *T. montereyi*, when snails experienced the 23°C exposure daily (D1), but not if they experienced it less frequently (D3 and D5; Fig. 4.4.). Intraspecifically, a less frequent exposure led to significantly lower hsp72 levels in *T. funebralis* and *T. brunnea*, but not in *T. montereyi*.

Endogenous levels of hsp74 showed few inter- and interspecific differences under varying acclimation conditions, although, they tended to be higher in *T. brunnea* and *T. montereyi* than in *T. funebralis* (Fig. 4.5.).

In summary, endogenous levels of hsp72 displayed more variation under varying temperature conditions than hsp74. Surprisingly, when 23°C exposures were experienced every other (D3) and every fifth day (D5), levels of hsp72 were lower in all species in comparison to experiment 1 when I acclimated snails under constant thermal conditions (even in comparison to 13°C-acclimated snails; Fig. 4.2).

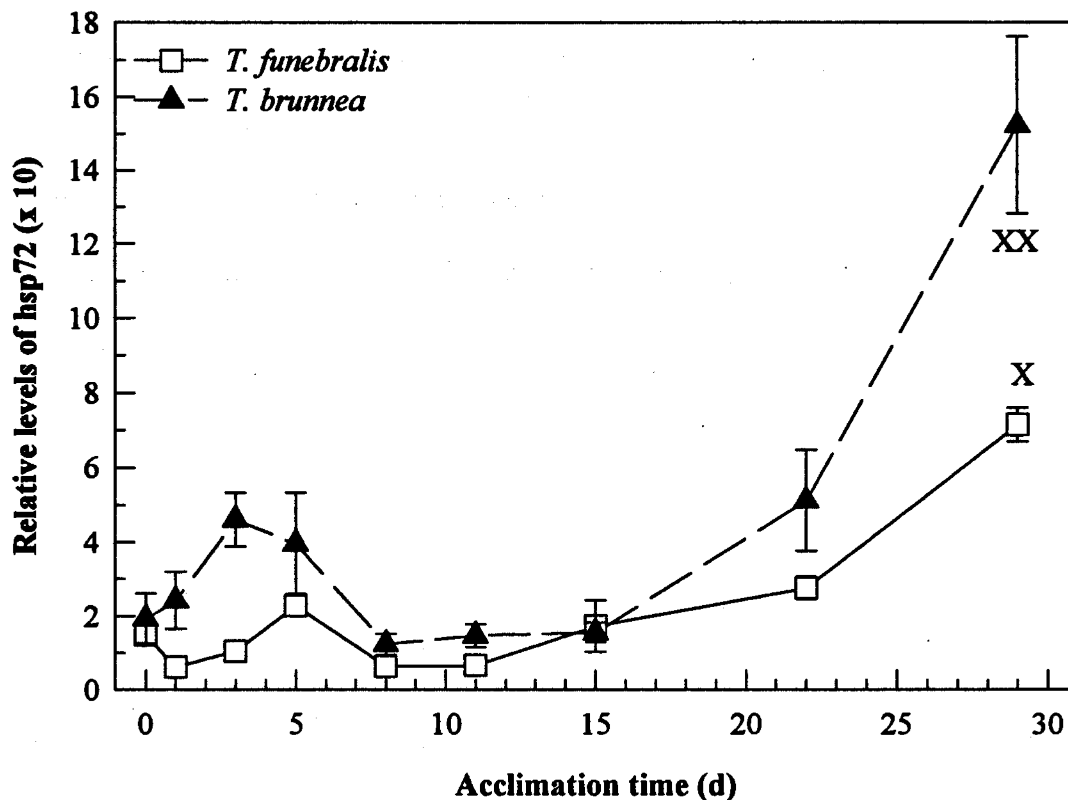


Figure 4.6. Endogenous levels of hsp72 in *T. funebralis* and *T. brunnea* acclimated to 18°C and exposed daily to 28°C for 2.5 h over a 29 day period. Hsp72 levels were only significantly higher in comparison to the control group (0 time point) on day 29: XX ≤ 0.05; X ≥ 0.05 but ≤ 0.10. There were no significant interspecific differences. Data are means ± 1 S.E.M. (n = 5 for all data points except for *T. funebralis* day 5 (n = 4)).

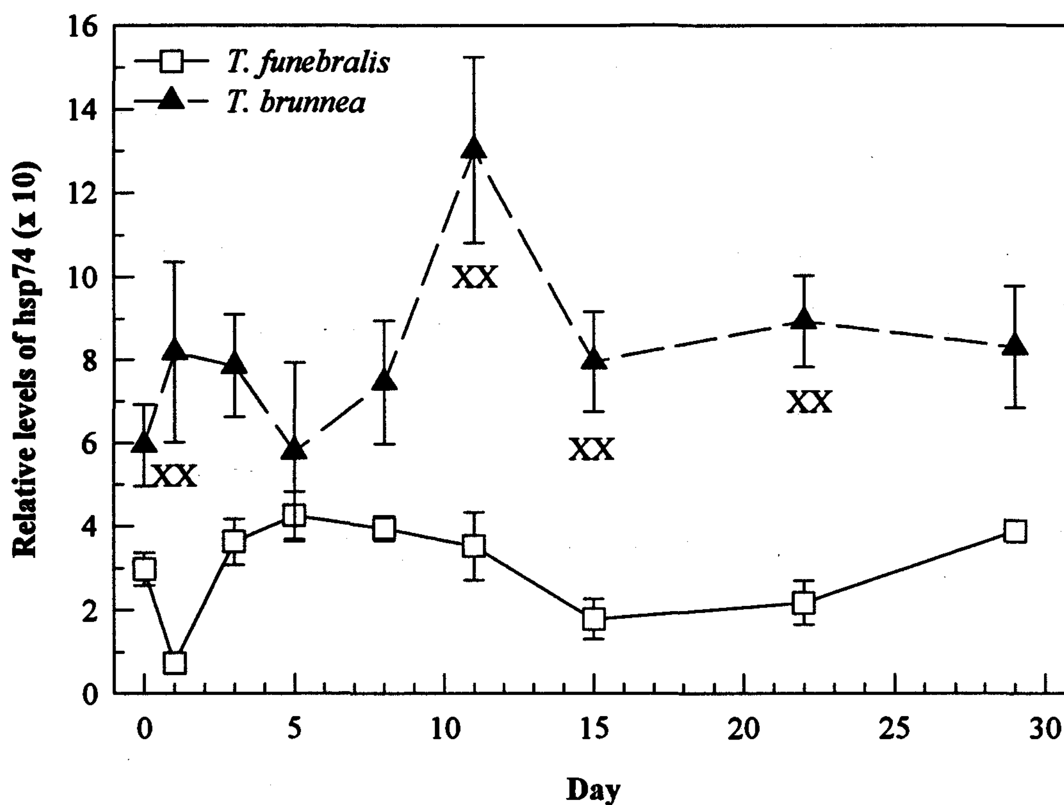


Figure 4.7. Endogenous levels of hsp74 in *T. funebralis* and *T. brunnea* acclimated to 18°C and exposed daily to 28°C for 2.5 h over a 29 day period. P-values for between species comparisons given are indicated: XX $\leq$ 0.05. There was only one significant intraspecific difference (day 11 for *T. brunnea*, not shown). Data are means ± 1 S.E.M. (n = 5 for all data points except for *T. funebralis* day 5 (n = 4)).

Effects of laboratory-acclimation under conditions that induce heat shock

During emersion, intertidal specimens of *T. funebralis* commonly experience temperatures in the high 20°C range, and such temperatures can induce the heat shock response (Tomanek and Somero, 1999). To evaluate the effects such thermal exposures may have on the endogenous levels of the two hsp70 isoforms, I exposed snails acclimated at 18°C to daily treatment at 28°C for 2.5 h at a specific set time that did not correspond with the tidal rhythm. Thus, in contrast to the previous experiments 1 and 2, *T. funebralis* and *T. brunnea* experienced thermal conditions that induced the activation of the heat shock response. I quantified endogenous levels of both isoforms on day 0 (control), 1, 3, 5, 8, 11, 15, 22 and 29.

Endogenous levels of hsp72 responded with a slight (but not significant) increase during the first five days, decreased to control levels after 8 days and subsequently increased again after day 22 to the highest levels during the course of acclimation (Fig. 4.6). Although the two-way analysis of variance indicated a significant time, species and interaction effect, the Tukey pairwise comparison only indicated significant differences in hsp72 levels between control and day 29 samples (for both species). In *T. brunnea*, levels of hsp72 were slightly higher, but not always significantly, than in *T. funebralis*, very much in contrast to the former two experiments when snails did not get exposed conditions that induce heat shock (Fig. 4.2. and 4.4). Relative to experiment 1, in which I acclimated snails under constant temperature conditions, hsp72 levels were relatively low (Fig. 4.2).

During the 29 days of acclimation, hsp74 levels did not change significantly in either species, with only one exception (day 11 in *T. brunnea*; Fig. 4.7.). However, in contrast to hsp72, interspecific differences in hsp74 were significant on several of the sample days, but especially after the first 28°C exposure and then from day 11 to day 22. These differences paralleled results from the two former experiments (Fig. 4.3. and 4.5).

Discussion

The regulatory role of hsp70 isoforms under constant temperature conditions

Previously, I showed *via* metabolic labeling with ^{35}S-amino acids how the induction of the heat shock response varies in field-acclimatized and laboratory-acclimated specimens of several *Tegula* congeners (Tomanek and Somero, 1999; Chapter 2). In general, induction of hsp70 synthesis, the most strongly-expressed hsp in *Tegula* species, occurred at lower temperatures in the low-intertidal to subtidal *T. brunnea* and *T. montereyi* than in the mid- to low-intertidal *T. funebralis*, and induction shifted to higher temperatures with increasing acclimation temperature in the former two species only. These acclimatory changes in induction temperature of hsp70 were completed with a shift in acclimation temperature from 13° to 23°. Thus, I hypothesized that endogenous levels of hsp70 isoforms in their role as repressors of the heat shock response could regulate these interspecific and acclimation-induced intraspecific differences in induction temperature.

Patterns of acclimatory changes in the low molecular mass hsp70 isoform, hsp72, did correspond closely with the negative regulatory role of hsp70 that is predicted by the cellular thermometer model (Craig and Gross, 1991; Morimoto, 1998). Higher induction temperatures in *T. funebralis* in comparison to *T. brunnea* and *T. montereyi*, at lower acclimation temperature, 13° and 18° (Tomanek and Somero, 1999; Chapter 2), correlated with higher endogenous levels of hsp72 in *T. funebralis*, suggesting that higher levels of hsp72 do indeed repress the induction of the stress response in *Tegula*. Furthermore, within species, higher acclimation temperatures led to increased levels of hsp72 in *T. brunnea* and *T. montereyi*, which was correlated with the predicted shift of induction towards higher temperatures (Tomanek and Somero, 1999).

Endogenous levels of the high molecular mass hsp70 isoform, hsp74, were highest at the highest acclimation temperature in *T. brunnea* and *T. montereyi* and thus may additionally contribute to a shift in induction towards higher temperatures with increasing acclimation temperature. Although not consistently, hsp74 levels were generally lower in *T. funebralis* than in *T. brunnea* and *T. montereyi*. The finding that much fewer changes in induction temperature were paralleled by changes in endogenous levels of hsp74 may indicate that it plays a rather minor role in regulating the transcriptional activation of the heat shock response.

Therefore, it appears that hsp72, rather than hsp74, functions as a repressor of the induction of the heat shock response. In light of the cellular thermometer model, acclimatory patterns of hsp72 expression seem to regulate not only

interspecific, but also acclimation-induced intraspecific differences in induction temperature.

The regulatory role of hsp70 isoforms under varying temperature conditions

Temperature conditions vary greatly in the intertidal zone under natural conditions on a daily basis and can activate the heat shock response (Tomanek and Somero, 1999). Seawater temperatures can vary due to seasonal changes and El Niño episodes. By acclimating *Tegula* species under varying temperatures that resembled these natural conditions, I was able to follow how such conditions affect endogenous levels of the two hsp70 isoforms. However, I do not know how induction temperatures change under these acclimatory conditions. First, I exposed snails to varying thermal conditions that were close to, but slightly below, temperatures that induce the heat shock response in 13°C-acclimated snails (13° → 23°C). Second, I subjected snails daily to conditions that induce heat shock (18° → 28°C), not unlike those experienced by snails in the intertidal zone.

Thermal conditions (13° → 23°C) that changed every 12 h every (D1), every other (D3) or every fifth day (D5) affected levels of hsp72 more than of hsp74 (Fig. 4.4 and 4.5). Specifically, endogenous levels of hsp72 changed to a greater extent between D1, D3 and D5 in *T. funebralis* than in *T. brunnea*, and *T. brunnea* showed greater changes than *T. montereyi*. These results demonstrate again that hsp72 reflects temperature variation better than hsp74. However, some of the results are difficult to interpret. Why are endogenous levels of hsp72 lower

in snails that experience a 23° exposure every third or fifth day than in snails that experience constantly 13°C? Endogenous levels of hsp's are apparently regulated by other factors than the sum of heat shock-inducing body temperatures. It should be remarked that these experiments are the first attempt to test how thermal regimen that vary the acclimation temperature daily regulate endogenous levels of hsp's. Such information is important for predicting how well hsp's actually indicate stress under natural thermal conditions that are almost never constant.

In my third experiment, *T. funebralis* and *T. brunnea* experienced heat shock-inducing temperatures daily, but were given the opportunity to recover at a non-heat shock-inducing temperature, similar to the naturally occurring thermal conditions that are common for snails and mussels in the intertidal zone (Hofmann and Somero, 1996b; Tomanek and Somero, 1999). I observed interspecific differences only for hsp74 (Fig. 4.7). Hsp72 levels were only elevated significantly at day 29 during the time course of acclimation (Fig. 4.6.). Although there were no statistically significant interspecific differences in hsp72 between *T. funebralis* and *T. brunnea*, it was somewhat surprising that levels of hsp72 were almost consistently higher in the latter species. This may suggest that heat shock-inducing conditions induce a relatively stronger response in *T. brunnea* than in *T. funebralis*.

In general, in *Tegula* congeners hsp72, rather than hsp74, seems to correlate with interspecific and acclimation-induced intraspecific variation in the induction temperature of the stress response. The results presented in this study support a negative regulatory role for hsp72 as predicted by the model of the cellular

thermometer. As one of the main, however, probably not the sole, repressors of the stress response in *Tegula*, hsp72 does not only play a regulatory role for acclimatory-induced plasticity, but also seems to set interspecific differences in the induction of the stress response, a completely novel finding.

My interpretation of the findings presented here in regard to the regulatory role of hsp70 isoforms are supported by several studies of intertidal mussels of the genus *Mytilus* that applied the same hsp70 antibody (Hofmann and Somero, 1995; Roberts *et al.*, 1997). The study by Roberts *et al.* (1997) reported induction temperatures of the stress response as well as endogenous levels of two hsp70 isoforms in field-acclimatized and laboratory-acclimated intertidal mussels of *Mytilus californianus*. In *M. californianus* the low (LMM) rather than the high molecular mass (HMM) hsp70 isoform showed endogenous levels that corresponded to shifts in induction of the stress response under field conditions, similar to my findings. However, I did not establish the homology between the LMM hsp70 isoforms in *Mytilus* and *Tegula*. The LMM hsp70 isoform also reflected thermal changes better in *M. trossulus* (Hofmann and Somero, 1995).

In conclusion, this and previous studies indicate that the model of the cellular thermometer, as it has been proposed to operate in *Escherichia coli*, *Saccharomyces cerevisae* and mammalian cell lines (Craig and Gross, 1991; Morimoto, 1998), seems to exist also in marine invertebrates (Hofmann and Somero, 1995; Roberts *et al.*, 1997). The role of hsp70 as a repressor of the induction of the stress response has now been confirmed under conditions of field-

acclimatization (Roberts *et al.*, 1997) and laboratory-acclimation (this study). However, this role is only specific to a subset of the number of hsp70 isoforms that are present in the organisms so far studied. There is evidence that the cellular thermometer may not only be set by temperature alone, as some of the predicted outcomes could not be observed following thermal acclimation under laboratory conditions (Roberts *et al.*, 1997). It therefore has to be shown if the acclimatory patterns that I found to exist in *Tegula* congeners between induction temperatures of the stress response and endogenous levels of the hsp70 isoforms can also be found under natural conditions. My findings indicate that endogenous levels of hsp72 may set interspecific differences in induction temperatures of the stress response that can be minimized but not completely eliminated by acclimation. Thus, changes in endogenous levels of hsp70 may be limited genetically and may thereby determine interspecific differences among congeners that vary widely in thermotolerance (Tomanek and Somero, 1999).

Chapter 5

Variable Patterns of Protein Thermal Stabilities in Several Marine Snails (Genus *Tegula*) from Different Thermal Habitats

Abstract

Protein thermal stabilities of orthologous homologs, quantified as loss of enzymatic activity following incubation at denaturing temperatures, often positively correlate with species' adaptation temperatures. Here I tested if a correlation between the thermal stabilities of five metabolic enzymes (cytosolic malate dehydrogenase (cMDH), pyruvate kinase, aldolase, glutamate dehydrogenase, and aspartate aminotransferase) and adaptation temperature exists in three *Tegula* species that inhabit widely differing thermal niches. Thermal stabilities of cMDH and pyruvate kinase were lower in the two stenothermal low-intertidal to subtidal species, *T. brunnea* and *T. montereyi*, than in their eurythermal mid- to low-intertidal congener *T. funebralis*. Aldolase did not display any interspecific differences. Thermal stabilities of glutamate dehydrogenase and aspartate aminotransferase were lower in *T. funebralis* than in *T. brunnea* and *T. montereyi*. Protein thermal stability, thus, does not correlate consistently with adaptation temperature among *Tegula* congeners that differ widely in thermotolerance. Comparisons of the loss of cMDH activity following incubation of tissue homogenates (*in vitro*) or intact tissue (*in situ* conditions) to various

denaturing temperatures showed that detection of interspecific differences in cMDH stability depended greatly on the conditions under which the enzyme is incubated. Although the loss of enzymatic activity is a "macroscopic" measurement of thermal denaturation and thus can not indicate what structural "microscopic" changes cause activity loss, the results presented in this study raise a caveat about the generalization that protein thermal stabilities are correlated with adaptation temperature. Furthermore, the results emphasize the need to consider protein stability under *in situ* as well as *in vitro* conditions.

Introduction

Temperature greatly affects higher levels of protein structure, e. g. the secondary, tertiary and quaternary structure, because a protein's native conformation is highly dependent on weak (non-covalent) molecular interactions: Hydrogen bonds, and ionic, van der Waals' and hydrophobic interactions, that are highly temperature sensitive (Hochachka and Somero, 1984; Somero, 1995; 1997; Stryer, 1995). The effect temperature has on these various interactions and on the greater thermodynamic unit, e.g. the cytoplasm, surrounding a protein leads to a fine balance between stabilizing and de-stabilizing forces. This balance results in a small net stabilization free energy that is equivalent to only a few non-covalent bonds and leads to marginal stability of higher order structure (Jaenicke, 1991). The marginal stabilities of proteins limit the temperature range over which a given protein can function (Somero, 1995; 1997).

One key property of proteins, thermal stability, is often found to correlate positively with organisms' adaptation temperatures in orthologous homologs, proteins encoded by the same gene locus in different species (Somero, 1995; Somero, 1997). Studies in which species from distant taxonomic groups were compared found a positive correlation between stability and adaptation temperature for collagen (Bailey, 1968), pyruvate kinase (Low and Somero, 1976), myofibrillar ATPases (Johnston and Walesby, 1977), actin (Swezey and Somero, 1982), trypsin (Genicot *et al.*, 1988), eye lens crystallins (McFall Ngai and Horwitz, 1990) and A_4-lactate dehydrogenase (A_4-LDH; Somero, 1997). Even studies that compared protein thermal stabilities among closely related taxonomic groups that inhabit different thermal environments, e. g., cytosolic malate dehydrogenase in congeneric species of abalone (genus *Haliotis*; Dahlhoff and Somero, 1993), detected a positive correlation with adaptation temperature. However, A_4-LDHs of congeneric barracuda fishes (genus *Sphyraena*; (Holland *et al.*, 1997), gobiid (Fields and Somero, 1997) and notothenioid teleosts (Fields and Somero, 1998) as well as B_4-LDH allozymes of different populations of the fish *Fundulus heteroclitus* (Place and Powers, 1984) failed to display a positive correlation between stability and adaptation temperature. Although stabilities were compared for a number of proteins, most studies did not extend their inter- or conspecific comparison beyond a single protein. Thus, we do not know if protein thermal stabilities consistently differ among species that differ widely in thermotolerance.

Interspecific differences in protein thermal stabilities may set interspecific variation in the induction temperature of the heat shock response, the enhanced synthesis of heat shock proteins, which is thought to be activated by protein denaturation (Parsell and Lindquist, 1993; 1994; Satyal and Morimoto, 1998).

Here I hypothesized that protein thermal stabilities may be generally higher in the eurythermal mid- to low-intertidal *T. funebralis* in comparison to the stenothermal low-intertidal to subtidal *T. brunnea* and *T. montereyi*, all temperate species that are common along the Pacific coast of California and differ widely in thermotolerance (Hellberg, 1998; Tomanek and Somero, 1999). To test this hypothesis, I compared the thermal stabilities of five enzymes in foot tissue: cytosolic malate dehydrogenase (cMDH), pyruvate kinase, aldolase, glutamate dehydrogenase and aspartate aminotransferase.

Additionally, I investigated how interspecific differences in thermal stabilities may depend on experimental conditions. Commonly, the greatest accuracy in detecting differences in thermal stability is ascribed to protein samples that are highly purified. In most comparisons I incubated tissue homogenates or *in vitro* preparations that essentially contain all the cellular molecules, only ten-fold diluted. However, preparations of tissue homogenates do not reflect protein thermal stability under *in vivo* conditions. Under *in vivo* conditions proteins may be fully or partly protected from thermal denaturation due to the stabilizing effects of high intracellular protein concentrations, compatible osmolytes, temperature-dependent pH effects, or the protective activity of molecular chaperones (Parsell

and Lindquist, 1993; 1994; Somero, 1997; Somero and Yancey, 1997). To test the effect the cellular environment may have on protecting proteins from thermal denaturation, I measured thermal stabilities of one enzyme, cMDH, under *in vitro* and *in situ* conditions. Under *in situ* conditions I used intact foot tissue in thermal exposures.

The results of this study suggest that protein thermal stabilities, at least when measured by loss of activity, and adaptation temperature do not consistently correlate positively in congeners of *Tegula*. Furthermore, proteins may be better protected from temperature stress by their cellular environment, and interspecific differences in thermal stabilities may change when compared under differing (*in vitro* and *in situ*) conditions.

Materials and Methods

Organisms, distribution and collection site

For species distribution see Chapter 2. Specimens of *T. funebralis*, *T. brunnea* and *T. montereyi* were collected at Hopkins Marine Station of Stanford University in Pacific Grove, California, USA (36°36'N, 121°54'W) and, if not immediately sacrificed, kept at 10 – 12°C in a seawater tank and fed weekly with freshly collected giant kelp (*Macrocystes pyrifera*). I used exclusively large adults in all experiments, and selected similar-sized specimens for all species.

Tissue preparation and conditions of incubation

Foot tissue was dissected for three specimens of each species separately on ice (4°C) and homogenized in 5 volumes of homogenization buffer (20 mM imidazole-Cl, pH 7.0 at 20°C, 1 mM EDTA, 5 mM DTT (EDTA and DTT were omitted from preparations for cMDH) using a tissue homogenizer (Janke and Kunkel Ultra-Turrax T25) at 4°C to reduce proteolytic activity. The homogenate was subsequently centrifuged at 15 800 g at 4°C for 30 min. Enzyme activity was adjusted to 0.5 units/min. Homogenates were kept on ice at all times except during incubation. I incubated 200 µl aliquots of homogenate for various times, at various temperatures in 1.5 ml microcentrifuge tubes. Incubation temperatures differed among enzymes and were selected according to the degree that they distinguished thermal stabilities between species. For *in situ* preparations I dissected foot tissue and kept half of the tissue in homogenization buffer on ice (0 time point) and incubated the other half at elevated temperature. Following incubation I prepared tissue homogenate of the foot tissue as described above.

Enzyme assay conditions

In all assays I quantified enzymatic activity by measuring the change in absorbance at 340 nm that is associated with the conversion of the reduced form of nicotinamide adenine dinucleotide (NADH) to the oxidized form (NAD^+) either directly or indirectly by coupling the formation of the product to another enzyme reaction that included NADH (or NAD^+) as a cofactor. Change in absorbance was

measured with a spectrophotometer (Perkin-Elmer Lambda 3B) at a wavelength of 340 nm at 20.0°C for all assays.

Activity measurements of cMDH: Tissue homogenates were assayed for activity in assay buffer (80 mM imidazole-Cl, pH 7.0 at 20°C, 100 mM KCl, 0.15 mM NADH, 0.2 mM oxaloacetate) by following the decrease in absorbance due to the oxidation of NADH to NAD^+ at 20°C following incubation to 35°, 40° and 45°C for various times.

Pyruvate kinase: The formation of pyruvate by pyruvate kinase was quantified by coupling the reaction to the lactate dehydrogenase reaction in which pyruvate is converted to lactate, a reaction that oxidizes NADH to NAD^+ using the following assay buffer: 80 mM Tris-Cl, pH 7.5 at 20.0°C, 100 mM KCl, 10 mM $MgSO_4$, 1.0 mM phosphoenolpyruvate, 5 mM ADP, 0.15 mM NADH, 0.1 mM fructose-1,6-bisphosphate and 15 U/ml lactate dehydrogenase.

Aldolase: The reaction of aldolase cleaves fructose-1,6-bisphosphate, a C6-carbohydrate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate, two C3-molecules. By converting glyceraldehyde 3-phosphate to 1,2-bisphosphoglycerate through the reaction of α–glycerolphosphate dehydrogenase I quantified the activity *via* the reduction of NAD^+ to NADH (and an increase in absorbance at 340 nm) using the following assay buffer: 100 mM Tris-Cl, pH 7.6 at 25°C, 0.15 mM NADH, 3 mM fructose-1,6-bisphosphate, 50 U/ml triose phosphate isomerase and 5 U/ml α–glycerolphosphate dehydrogenase (Churchill and Livingstone, 1989).

Glutamate dehydrogenase: The enzyme catalyzes the oxidative deanimation of glutamate to α-ketoglutarate, ammonium ion and NAD^+, a reaction I followed directly by measuring the decrease in absorbance at 340 nm. Assay buffer: 50 mM imidazole-Cl, pH 7.0 at 20.0°C, 250 mM ammonium acetate, 0.1 mM EDTA, 0.1 mM NADH, 1 mM ADP and 14 mM α-ketoglutarate (Mommsen *et al.*, 1980).

Aspartate aminotransferase: The enzyme catalyzes the amino group transfer from alanine to α-ketoglutarate whereby it produces oxaloacetate and glutamate. Oxaloacetate can be converted to malate *via* the malate dehydrogenase reaction, which oxidizes NADH to NAD^+. Assay buffer: 50 mM imidazole-Cl, pH 7.0 at 25.0°C, 0.15 mM NADH, 40 mM aspartate, 7 mM α-ketoglutaric acid, 0.025 mM pyridoxal phosphate and excess malate dehydrogenase (Mommsen *et al.*, 1980).

Statistical analysis

Differences in thermal stability among species were tested for their statistical significance by a repeated measures analysis of variance that included all data points (ANOVA; Systat 6.0; Systat, Inc.). The estimated model contained species as the independent variable (between-subjects factor) and six (or less in case of *in situ* preparations) repeated measurements of residual activity (time; within-subjects factor) as the dependent variable. *In vitro* and *in situ* incubations were compared in the case of cMDH by including treatment as an additional independent variable in the repeated measures ANOVA. Differences were assigned to be statistically significant with a p-value of 0.05 or smaller.

Results

Thermal stabilities of cytosolic malate dehydrogenase (cMDH) in vitro and in situ

Generally, species order of cMDH thermal stability correlated positively with species' thermotolerance for both *in vitro* and *in situ* measurements, with the eurythermal species *T. funebralis* displaying the most stable cMDH (Figs. 5.1A – C). Losses of enzymatic activity over time *in vitro* were significantly different among species at all three incubation temperatures: 35.0°, 40.0° and 45.0°C. In contrast, thermal stabilities of *in situ* incubations only differed between species at 35.0°C, but not at higher temperatures. Loss of activity was generally faster *in vitro* in comparison to *in situ* incubations. The *in vitro* and *in situ* thermal stabilities differed from each other at 40.0° and 45.0°C, but not at 35.0°C.

Thermal stabilities of pyruvate kinase, aldolase, glutamate dehydrogenase and aspartate aminotransferase

Foot tissue homogenates (*in vitro* preparation) displayed higher thermal stabilities of pyruvate kinase in *T. funebralis* than in *T. brunnea* and *T. montereyi* at 47.5° and 50.0°C, but not at 45.0°C (Figs. 5.2A - C). Thus this series of exposures illustrates that the detection of interspecific differences in thermal stability is temperature dependent.

Loss of aldolase activity following incubation at denaturing temperatures (55.0° and 57.5°C) did not show any interspecific differences (Figs. 5.3A and B).

Aspartate aminotransferase (Fig. 5.4) and glutamate dehydrogenase (Fig. 5.5), two enzymes of amino acid metabolism, showed interspecific differences at

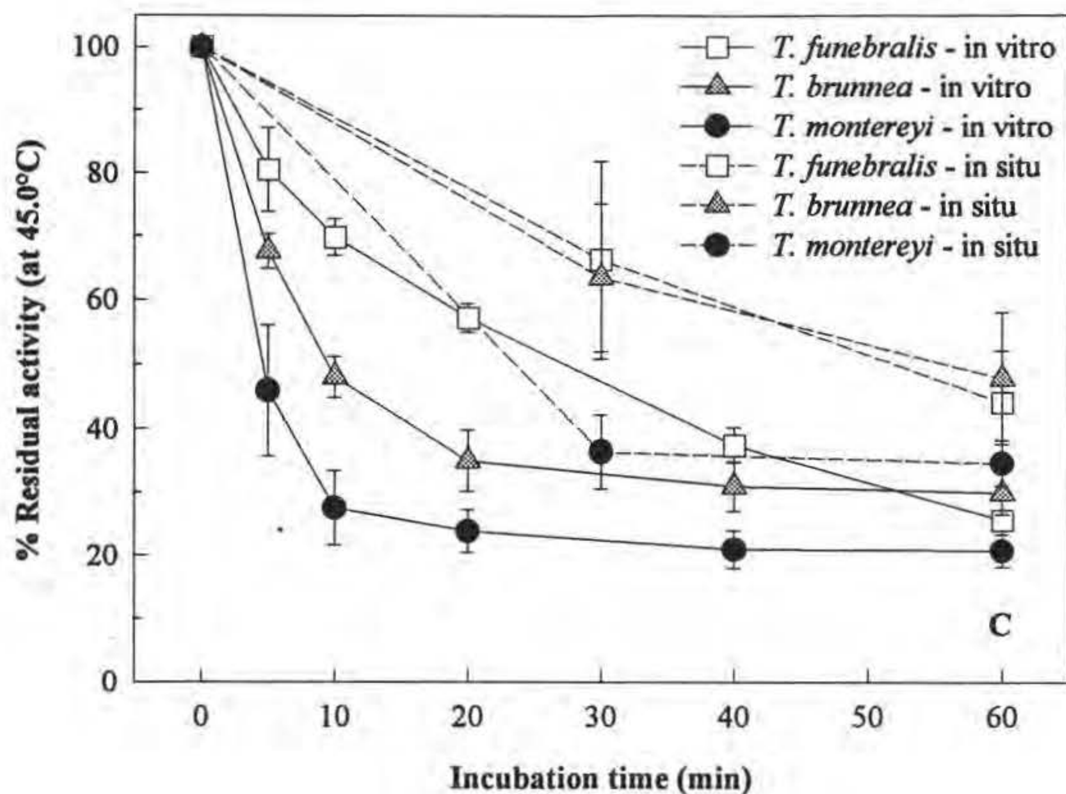
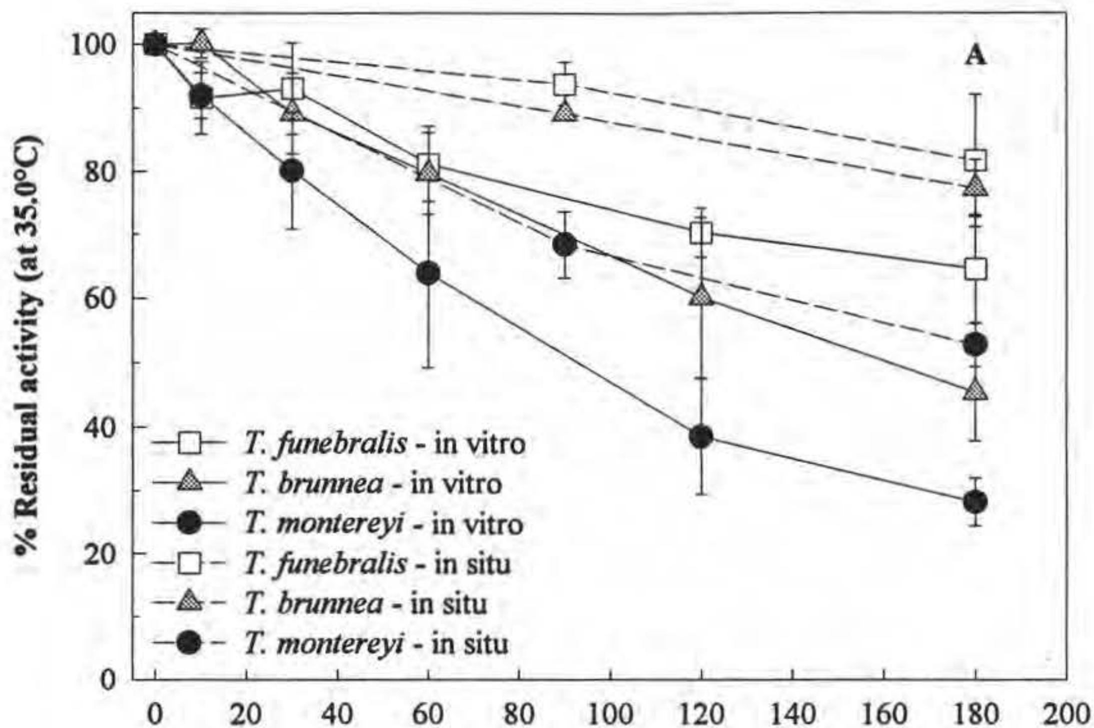


Figure 5.1. Loss of activity of cytosolic malate dehydrogenase with incubation time at various temperatures (A: 35.0°C, B: 40.0°C and C: 45.0°C) for *in vitro* (solid line) and *in situ* (dashed line) treatments. Values represent means ± 1 S.E.M. (n = 3 for all data points except for *T. brunnea* and *T. funebralis* for the *in situ* treatment at 40.0°C n = 7).



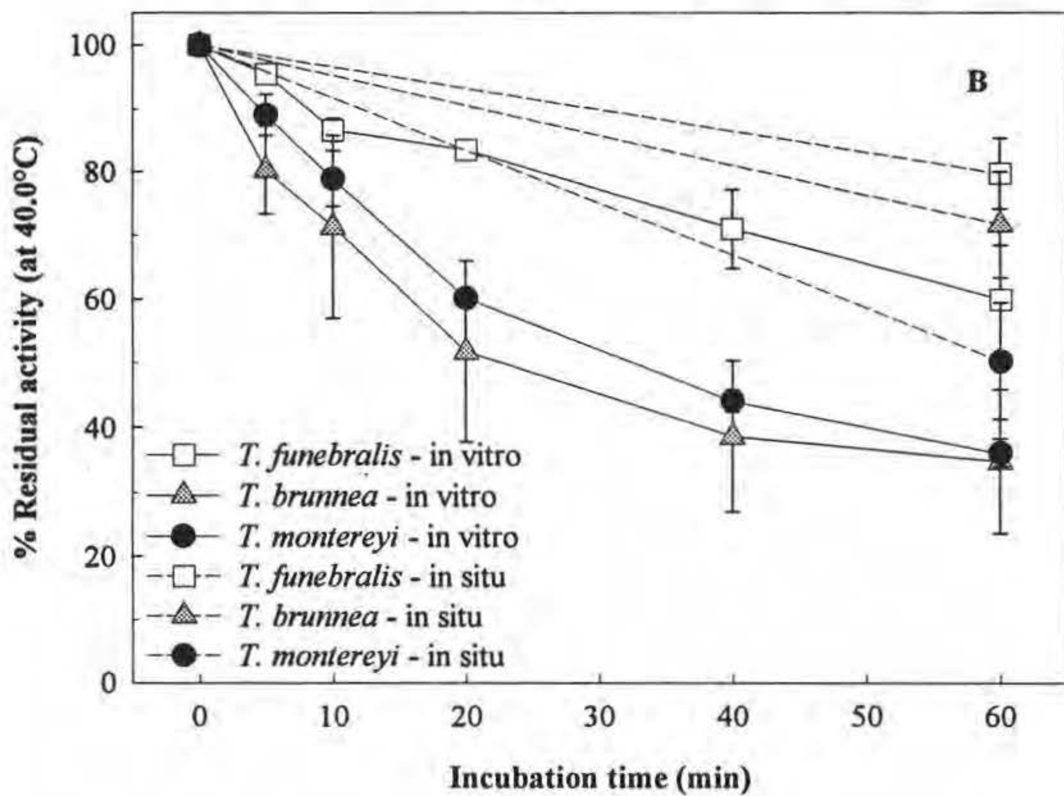


Figure 5.1 (continued)

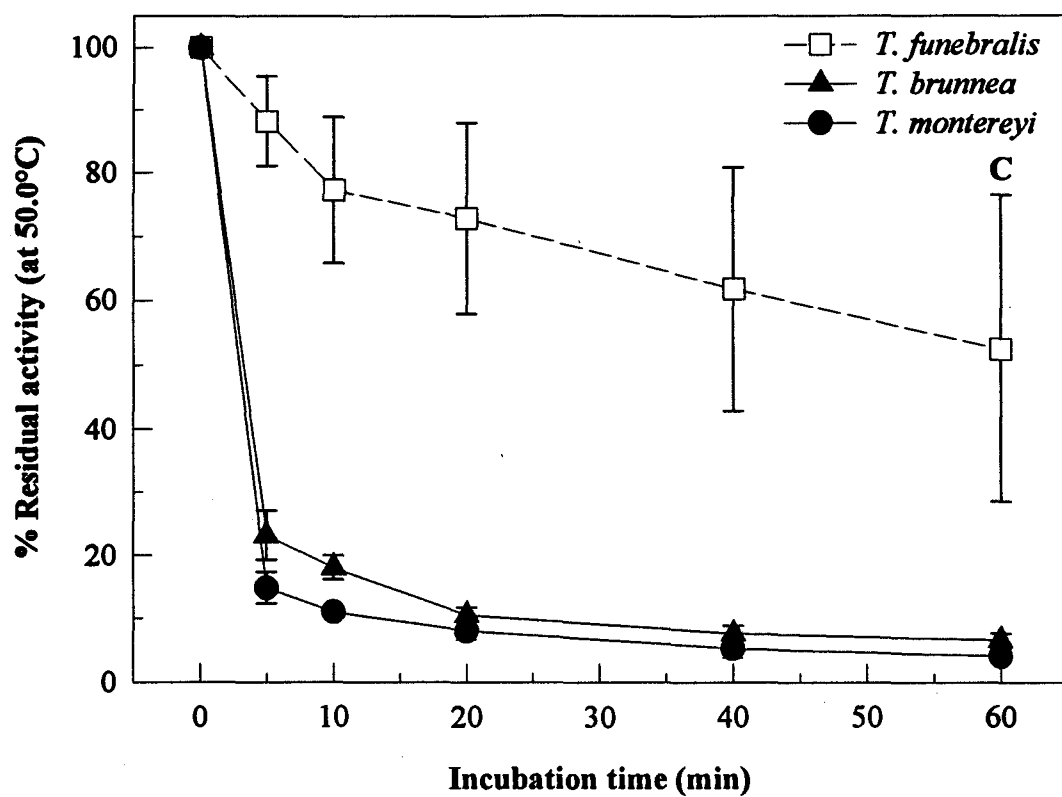
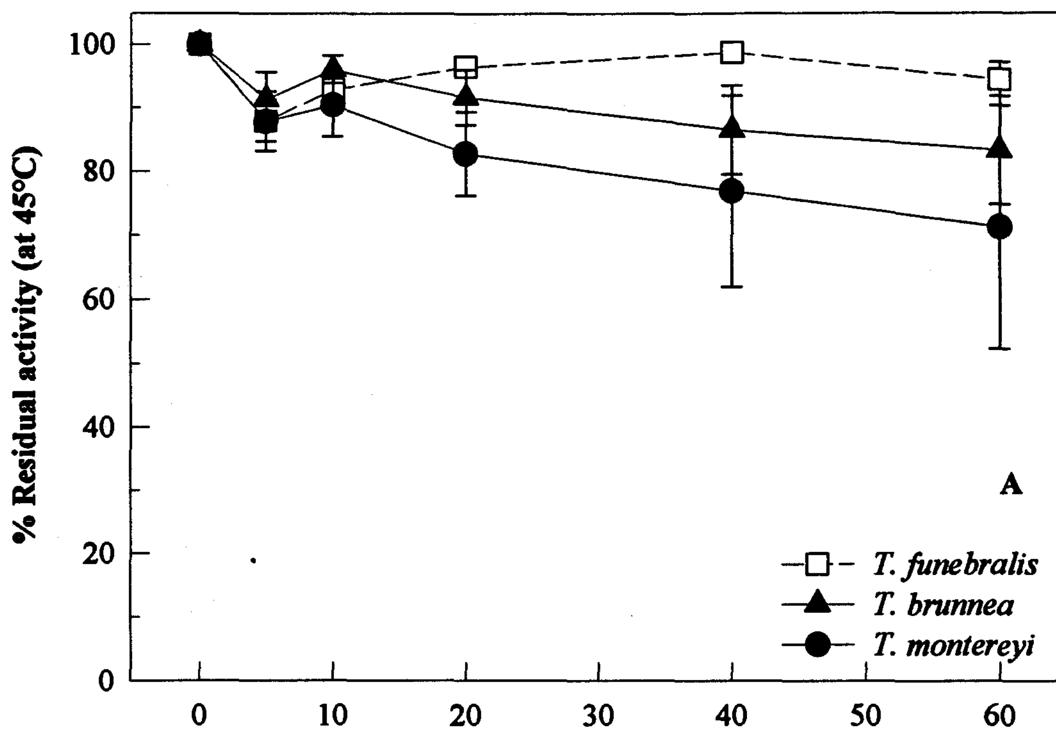
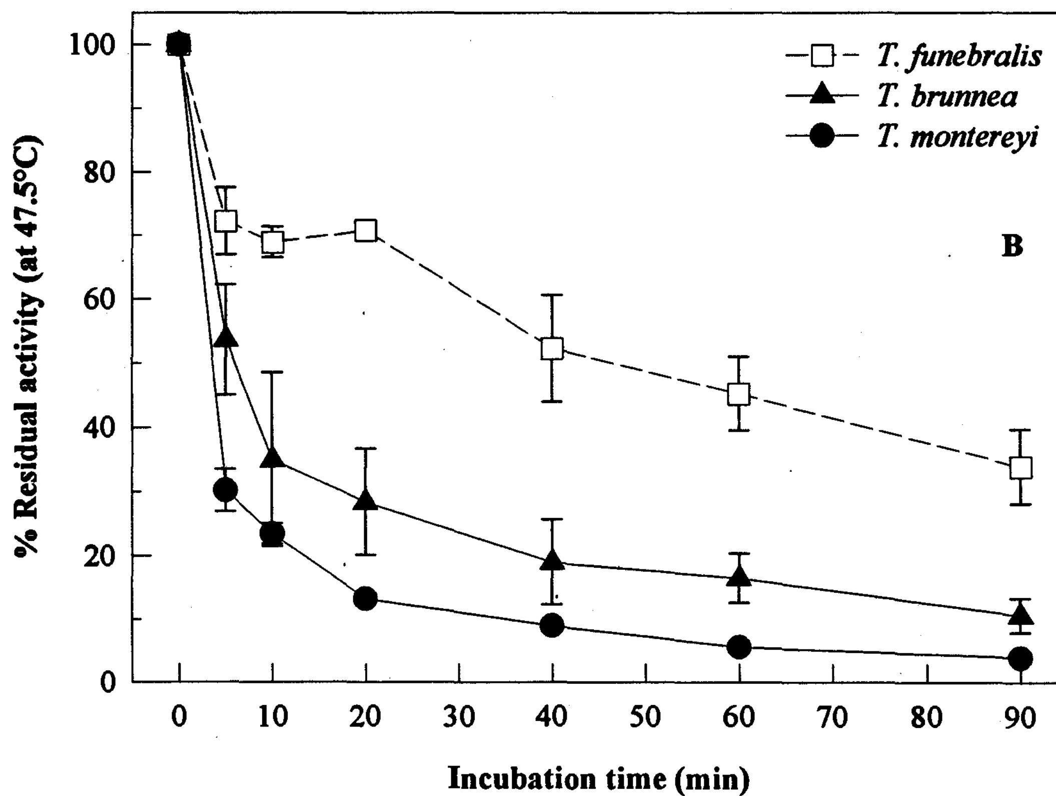


Figure 5.2. Loss of activity of pyruvate kinase with incubation time at various temperatures (A: 45.0°C, B: 47.5°C and C: 50.0°C) for *in vitro* treatments. Data represent means ± 1 S.E.M. (n = 3 for all data points).





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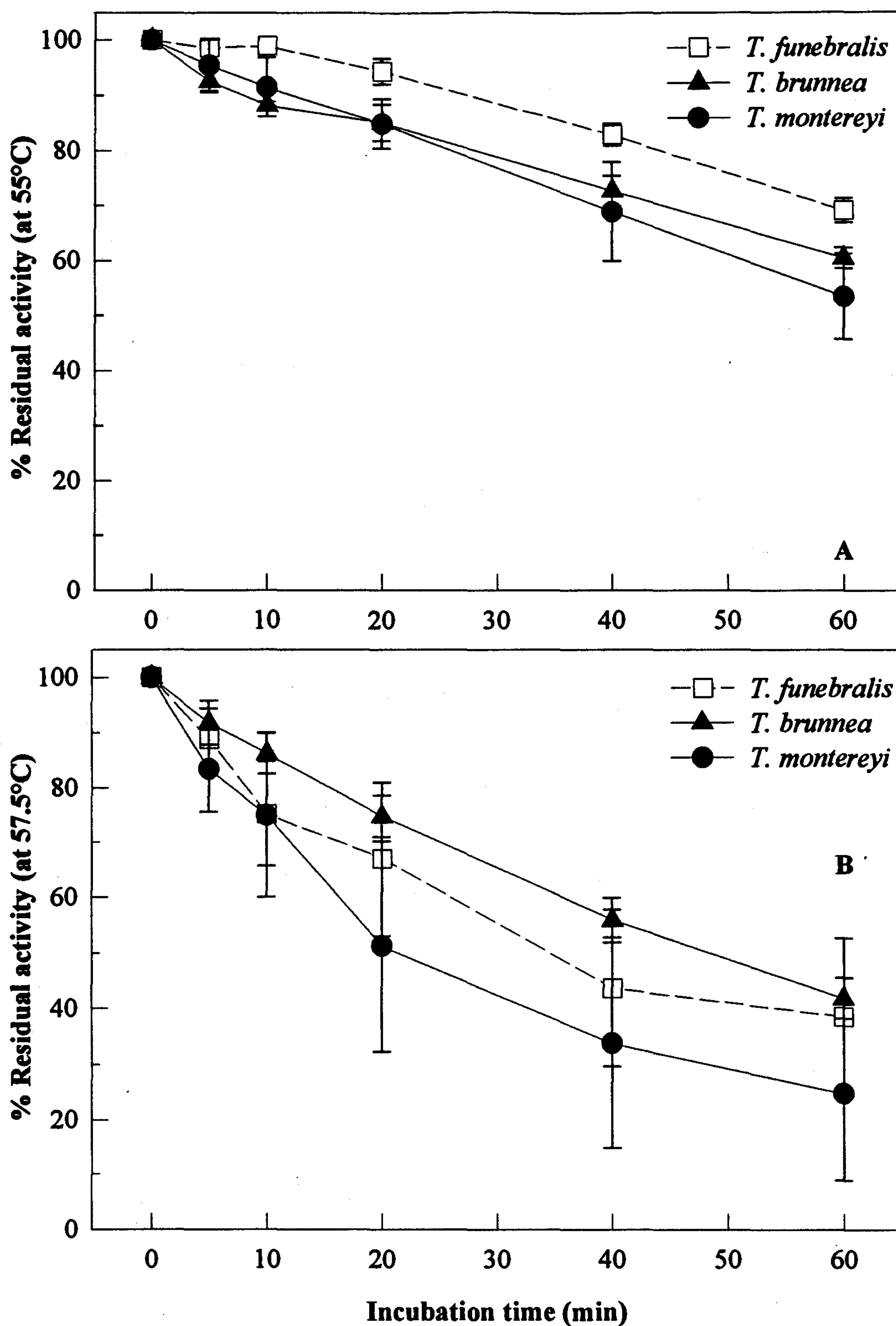

Figure 5.3. Loss of activity of aldolase with incubation time at various temperatures (A: 55.0°C and B: 57.5°C) for *in vitro* treatments in three *Tegula* congeners. Data represent means ± 1 S.E.M. (n = 3 for all data points).

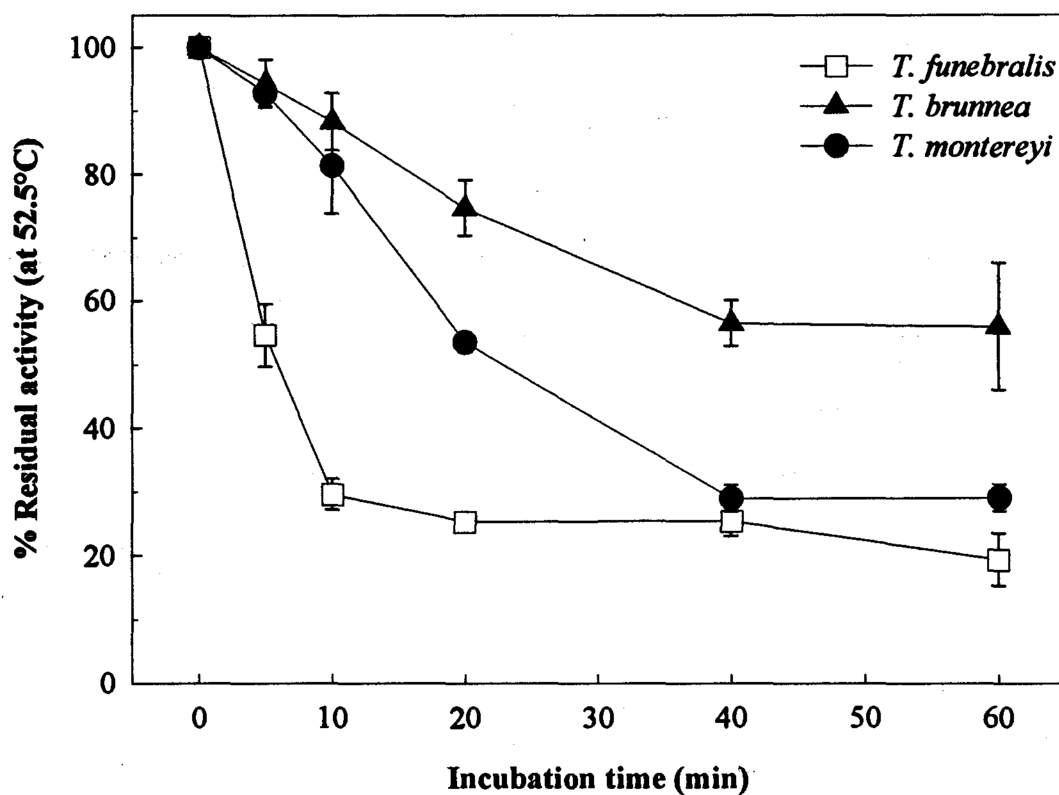

Figure 5.4. Loss of activity of aspartate aminotransferase with incubation time at 52.5°C for *in vitro* treatments in three *Tegula* congeners. Data represent means ± 1 S. E.M. (n = 3 for all data points).

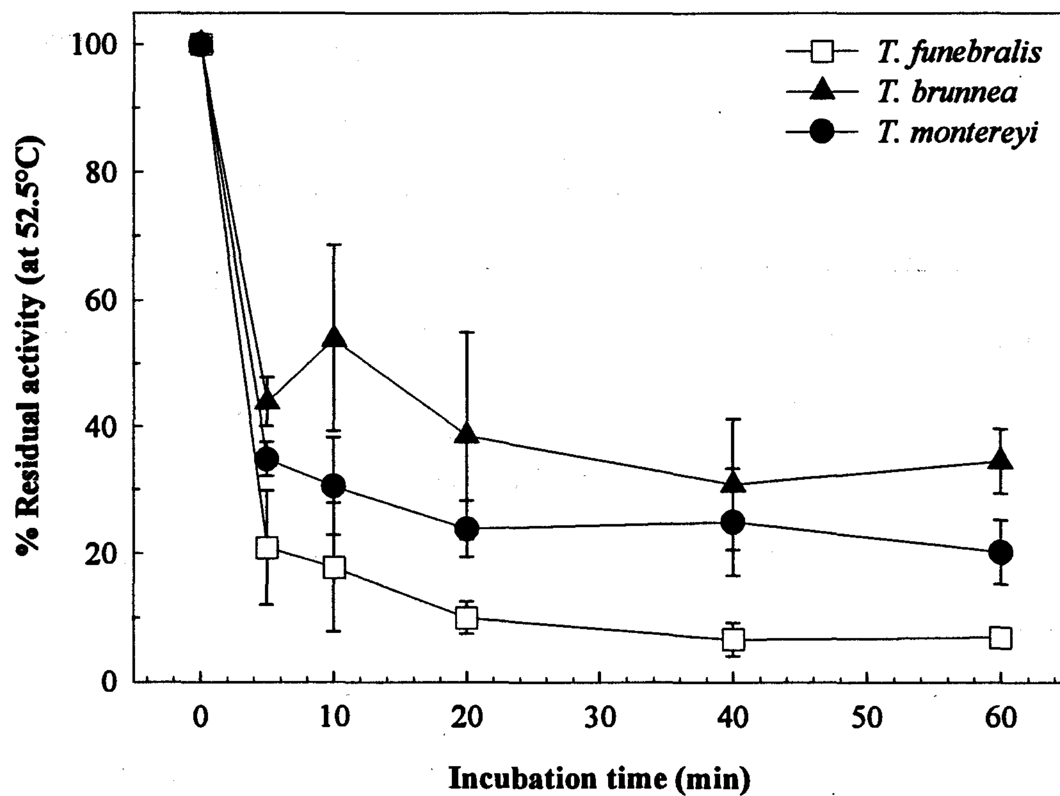

Figure 5.5. Loss of activity of glutamate dehydrogenase with incubation time at 47.5°C for *in vitro* treatments in three *Tegula* congeners. Data represent means ± 1 S.E.M. (n = 3 for all data points).

52.5°C. However, for both enzymes, thermal stabilities were lower in *T. funebralis* than in *T. brunnea* and *T. montereyi*.

Discussion

The comparison of thermal stabilities of five metabolic enzymes, measured as residual activity after incubation at denaturing temperatures, of three congeners of *Tegula* that differ widely in thermotolerance showed no consistent correlation between enzyme structural stability and the species' thermal niche. Detection of interspecific differences in thermal stability of cMDH greatly depended on the experimental conditions under which the protein was exposed to the incubation temperature, either *in vitro* and *in situ* conditions.

Although protein thermal stabilities are often positively correlated with adaptation temperature, several exceptions have been found in which such a correlation could not be established (for review see Somero, 1995, 1997). Most orthologous homologs for which a positive correlation was found were compared among distantly related species that differ widely in thermotolerance, e. g. between the desert iguana *Dipsosaurus dorsalis* and the Antarctic fish *Pagothenia borchgrevinki* in case of actin (Swezey and Somero, 1982). But positive correlations between stability and adaptation temperature were also found among closely related species, e. g. cMDH stability among abalone congeners (genus *Haliotis*) that differ in their thermal niches (Dahlhoff and Somero, 1993). On the other hand, cases for which no positive correlation could be established, e.g. for A_4-

lactate dehydrogenases of several species of Antarctic and South American notothenioid teleosts (Fields and Somero, 1998) and B_4-LDH allozymes between northern and southern populations of the fish *Fundulus heteroclitus* (Place and Powers, 1984) only compared confamilial, congeneric or conspecific organisms. The pattern found for *Tegula* congeners suggests that protein thermal stabilities do not correlate consistently with a species' thermal niche in closely related species. However, so far there has been no experimental evidence presented that would suggest that this pattern is also true for orthologous homologs from species that are more distantly related. Thermal stabilities may thus correlate more generally with a species' adaptation temperature among more distantly related than closely related species. This hypothesis is supported by the observation that when thermal stabilities are found to correlate positively with adaptation temperature they tend to do so independently of the phylogenetic relationship between orthologous homologs, e. g., actin thermal stabilities are similar in the desert iguana *D. dorsails*, the rabbit and the chicken (Swezey and Somero, 1982).

I hypothesized that interspecific differences in induction temperature of the heat shock responses in the three *Tegula* congeners, which tended to correlate positively with species' thermotolerances (Tomanek and Somero, 1999), may be in large determined by protein thermal stabilities. The pattern of thermal stabilities that is found in this study does not seem to support this hypothesis. However, thermal stabilities of enzymes were measured in foot tissue and induction temperatures of the heat shock response were established for gill tissue. Tissue-

specific differences in induction temperature have been found to exist (Clos *et al.*, 1993; Sarge *et al.*, 1995; Sarge *et al.*, 1993). This and the observation that other proteins, e.g., cytoskeletal proteins, are among the first to be damaged by thermal stress, raise doubts if the findings presented here provide a well-founded basis to reject a correlation between thermal stability and the induction temperature of the heat shock response (Parsell and Lindquist, 1994; Welch and Suhan, 1985).

Additionally, it is not known which structural "microscopic" changes lead to the loss of enzymatic activity. The loss of activity may reflect processes other than the thermal denaturation of an enzyme, e.g., protein-protein interactions due to interactions of hydrophobic side chains that are being exposed due to increased thermal motion may also lead to a decrease in activity. Thus it is difficult to infer what physical changes take place when enzymatic activity decreased and if the loss of activity is actually a measurement of protein stability *per se*.

The comparison of thermal stabilities of cMDH under *in vitro* and *in situ* conditions raises an important caveat regarding the effect sample (or protein) preparation have on detecting interspecific differences. First, *in situ* conditions resulted in increased thermal stabilities in comparison to *in vitro* conditions at 40° and 45°C, but not at 35°C. Thus, at least cMDH is better protected from thermal denaturation at higher temperatures in its "native" cellular environment than in a highly diluted buffer system. Although the mechanisms of these protective effects were not subject of this study, I would assume that the protective role of high intracellular protein concentrations, compatible osmolytes, pH-temperature

interactions and the chaperoning effects of heat shock proteins may be responsible for the increased thermal protection (Somero, 1997; Somero and Yancey, 1997). Second, differences among species that existed under *in vitro* conditions at 40.0° and 45.0°C disappeared under *in situ* conditions (Fig. 5.1B and C). The existence of interspecific differences in thermal stability among orthologous homologs, therefore, depends on the preparation of the protein. As an extension of these results, it seems questionable if interspecific differences in thermal stabilities that were measured with purified protein samples may be found or relevant under physiological (or *in situ*) conditions.

In conclusion, protein thermal stabilities are not consistently correlated with adaptation temperature among closely related species, even if they differ widely in thermotolerance. Interspecific differences in thermal stabilities are dependent on the preparation of the protein and thus, if possible, should be shown to exist under native cellular (*in situ*) conditions.

Chapter 6

Summary

The main objective of this thesis has been to elucidate the adaptive importance of a biochemical process, the heat shock response, in determining thermotolerance and biogeographic distribution patterns in marine snails of the genus *Tegula*. The expression of hsp's is, *a priori*, a biochemical process that represents a likely candidate to play a significant role in setting distribution limits due to its importance in conferring tolerance to extreme thermal exposures on the organismal level. The species of the genus *Tegula*, with their widely differing vertical and biogeographic distribution patterns and their genetic similarity, provided an ideal comparative system to study the phenotypic variation of features of the heat shock response. By characterizing the thermal niche that the various species occupy, I was able to address how genetically fixed and acclimation-induced variation in the stress responses may contribute to setting biogeographic patterns. A comparison of protein thermal stabilities and endogenous levels of two hsp70 isoforms that, according to the model of the cellular thermometer, are hypothesized to play a regulatory role in the transcriptional activation of the stress response, showed how genetically fixed and acclimation-induced variation may be controlled among *Tegula* congeners that differ widely in thermotolerance.

Features of the heat shock response that differ phenotypically among species and change with acclimation to various temperatures can be characterized

by acclimating species to controlled thermal conditions in the laboratory. I acclimated four congeneric species that differ widely in thermotolerance: Two species are found in the mid- and low-intertidal, *T. funebralis* and *T. rugosa*, and two in the low-intertidal and subtidal, *T. brunnea* and *T. montereyi*. Snails that inhabit the mid- to low-intertidal are frequently emersed (air-exposed), and thus attain higher absolute body temperatures and experience much greater changes in temperatures than snails that inhabit the low-intertidal to subtidal zone. By acclimating *Tegula* snails to various temperatures for over four weeks, I was able to distinguish how species differ after they experienced a common acclimation temperature (phenotypic variation that is likely to be genetically fixed) and how these interspecific differences of hsp expression change with acclimation within a species (acclimation-induced variation). Interspecific differences indicate that the temperature of the onset (T_{on}), the maximal level (T_{peak}) and the inactivation of synthesis of hsp70 and all other proteins (T_{off}) correlate with a species' thermal niche. T_{on} and T_{peak}, unlike T_{off} of hsp70, increased with increasing acclimation temperature. T_{peak} of hsp70 increased with increasing acclimation temperature in all three species, but T_{on} of hsp70 only increased in the two stenothermal species *T. brunnea* and *T. montereyi*.

Acclimatory patterns in three other hsp's, hsp38, hsp77 and hsp90 differ in some aspects from patterns described for hsp70. Expression of these hsp's differs interspecifically and these differences can change with acclimation, however,

interspecific differences in T_{off} are similar in all hsp's and stay unchanged with increasing acclimation temperature.

In light of the differing characteristics of the species' thermal niches, interspecific differences in T_{off} that were not subject to acclimatory changes indicate that T_{off} may set limits to thermotolerance and may limit *T. brunnea* and *T. montereyi* from inhabiting the mid-intertidal zone. In turn, for *T. funebralis* and *T. rugosa*, T_{off} seems to be close to the highest temperatures that these species are likely to experience in the mid- to low-intertidal zone. Both species therefore live close to their thermal limits at which an important biochemical process as the heat shock response can function. Furthermore, the frequency with which both species are likely to activate the energetically costly heat shock response indicates that the thermal exposures that are typically experienced in the intertidal zone may require a substantial amount of energy. This energy requirement may necessitate changes in how energy sources are allocated. Both findings, that the energetically costly heat shock response is frequently induced and that T_{off} is close to the highest body temperatures that *T. funebralis* and *T. rugosa* experience, suggest that snails that inhabit the thermally more variable mid-intertidal are more likely to be affected by global warming than their subtidal congeners.

The process of heat-inactivation of hsp (and protein) synthesis (T_{off}) was further characterized by following the time course of the heat shock response after a 30°C exposure over a 50 h time period, an exposure that was ultimately lethal for *T. brunnea*, but not for *T. funebralis*. Differences in onset, duration and intensity of

the response indicated that a 30°C exposure, which is frequently experienced by *T. funebralis*, but never by *T. brunnea*, represents a much more severe stress for the latter species. Furthermore, I showed that the rate with which the exposure temperature is reached is likely to determine the severity of the stress, probably by thermally damaging cellular processes that precede the successful translation of proteins, e. g. transcription and translation of *hsp* mRNA.

Following acclimation to a common temperature, species differed in T_{on} of hsp70 at lower but not at the highest acclimation temperatures. Comparisons of endogenous levels of two hsp70 isoforms showed that the genetically fixed differences between species and acclimation-induced differences observed within species may be regulated according to the model of the cellular thermometer. Importantly, interspecific differences in T_{on} seem likely to be set by differing endogenous levels of hsp72, a low molecular mass hsp70 isoform. However, protein thermal stabilities, which I hypothesized to also play an important role in setting interspecific differences, do not consistently correlate with adaptation temperature.

Although additional hsp's may also contribute to the regulation of interspecific differences in T_{on}, these results indicate that endogenous levels of hsp70 isoforms may be setting interspecific differences that cannot be overcome by acclimatory changes.

This study therefore suggests that the features of the heat shock response that I have found to differ among *Tegula* congeners that occupy widely varying

thermal habitats are of great importance in setting limits to thermotolerance and distribution ranges. These limits are partly set by genetically fixed properties (range of change in T_{on} and T_{peak}, T_{off}). Differences that were observed to exist between species and changed due to acclimation within species seem to be set by features that are part of a cascade of events that activate the transcription of hsp's according to the model of the cellular thermometer.

This study may represent the strongest evidence yet for the contribution of a biochemical process in setting limits to thermotolerance and distribution. Although biochemical processes are unlikely to be the sole determinants of environmental temperature ranges, the results presented here illustrate how important they can be.

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