

EVIDENCE FOR PROTEIN DAMAGE AT ENVIRONMENTAL TEMPERATURES: SEASONAL CHANGES IN LEVELS OF UBIQUITIN CONJUGATES AND hsp70 IN THE INTERTIDAL MUSSEL *MYTILUS TROSSULUS*

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Summary

We examined the seasonal variation in environmentally induced protein damage in natural populations of the intertidal mussel *Mytilus trossulus*. In order to compare the state of protein pools during seasonal variations in environmental temperature, we used solid-phase immunochemical analysis to quantify ubiquitin conjugate concentrations and relative levels of the stress protein hsp70. The two biochemical indices were selected for their cellular roles in irreversible and reversible protein denaturation, respectively. Proteins that are ubiquitinated are irreversibly damaged and are degraded by intracellular proteases; stress proteins act as molecular chaperones to re-fold thermally denatured proteins and, thus, indicate degrees of reversible protein damage. Comparisons involved mussels collected in February and August from two study sites: an intertidal site which subjected animals to a wide range of body temperatures (from approximately 10 to 35 °C in summer), and a subtidal site where animals remained submerged throughout the tidal cycle. Our results show that quantities of ubiquitin conjugates and hsp70 were greater in gill tissue from summer-collected mussels than in gills of winter-collected

specimens. Ubiquitin conjugate and hsp70 levels were also greater in mussels collected from an intertidal location than in mussels from a submerged population. Our results show that the high summer temperatures normally experienced in the field are sufficient to cause increased denaturation of cellular proteins. Despite increases in the concentrations of heat shock proteins in summer-acclimatized mussels, elevated levels of irreversibly denatured, i.e. ubiquitinated, proteins were still observed, which indicates that the heat shock response may not be able to rescue all heat-damaged proteins. The energy costs associated with replacing heat-damaged proteins and with maintaining the concentrations and activities of heat shock proteins may contribute substantially to cellular energy demands. These increased energy demands may have an impact on the ecological energetic relationships of species, e.g. in the allocations of energy for growth and reproduction, and, as a consequence, may contribute to determining their distribution limits.

Key words: ubiquitin, hsp70, heat stress, protein damage, temperature, *Mytilus trossulus*.

Introduction

Recently, there has been an increased interest in the energetic and evolutionary importance of environmental stress, particularly heat stress (Parsons, 1993; Huey and Kingsolver, 1993; Lenski and Bennett, 1993; Huey, 1991; Huey and Bennett, 1990; Hoffmann and Parsons, 1991). It is acknowledged that temperature stress has an impact on organismal function and energetics, but the components that make up the metabolic cost of heat stress have not been clearly identified (Hawkins, 1991). A potentially significant and largely unexplored source of the cost of thermal damage is irreversible protein denaturation. Surprisingly, there is little information as to whether thermal denaturation of proteins takes place in organisms in the field during sublethal heat stress, i.e. under thermal conditions that may be extreme but are not uncharacteristic of the thermal habitat of an organism.

Several lines of evidence point to an important role for protein degradation due to thermal stress. First, proteins are relatively thermolabile because the weak interactions that stabilize the higher levels of protein structure that are necessary for protein function are strongly perturbed by temperature changes (Jaenicke, 1991; Somero, 1995). Net free energies of stabilization for most globular proteins are only approximately 21×10^{-3} – 84×10^{-3} J mol⁻¹, i.e. of the order of a few noncovalent bonds (Dill, 1990; Jaenicke, 1991). Many protein domains are thought to melt and cause subsequent protein unfolding at temperatures that are within physiological ranges (Parsell and Lindquist, 1993). Second, the thermostability of proteins is positively correlated with species' adaptation temperatures (Jaenicke, 1991; Somero, 1995), which indicates that each species possesses cellular proteins whose structures

are adapted to a particular temperature range. Any large departures from this thermal habitat range could result in denaturation. Finally, given that the predicted cost of protein synthesis in ectotherms such as mussels (Hawkins, 1985) and fish (Houlihan, 1991) ranges from 18 to 26 % of total metabolic heat losses, the energy required to replace damaged proteins may contribute greatly to the cost of thermal stress.

The present study examines the relationship between environmental temperature and protein damage and investigates whether the temperatures that an ectothermic animal experiences in its natural habitat are sufficient to result in protein damage. Our approach was to measure two biochemical indicators of damage to proteins under natural temperature conditions in a eurythermal intertidal mollusc, *Mytilus trossulus*. The two indices were selected on the basis of their known roles in the processes of irreversible and reversible denaturation of proteins (Parsell and Lindquist, 1993). First, we measured levels of ubiquitin conjugates in tissues from animals under a variety of environmental temperature regimes as a direct molecular means to assay for thermally induced irreversible protein denaturation. Ubiquitin, a small protein of 76 residues that is found in all eukaryotic cells, is ligated to damaged cellular proteins in an ATP-dependent fashion and targets these proteins for degradation by cytoplasmic non-lysosomal proteases (for reviews, see Rechsteiner, 1987; Finley and Chau, 1991; Hershko and Ciechanover, 1992). The free form of ubiquitin is heat-inducible (Bond and Schlesinger, 1985; Bond *et al.* 1988), and increased levels of ubiquitin conjugates have been measured in a variety of cells exposed to heat shock under laboratory conditions (Carlson *et al.* 1987; Parag *et al.* 1987; Bond *et al.* 1988). To our knowledge, ubiquitin conjugates have never been used as a biomarker for protein damage in an environmental context, and the present study thus represents the first examination of ubiquitin conjugates in an animal during thermal stress in the field.

Second, we used western blot analysis to quantify the relative amounts of the stress protein hsp70 in tissue samples. Stress proteins are heat-inducible proteins found in all species. Their synthesis is either induced or enhanced when organisms are exposed to temperatures several degrees above optimal body temperature (Lindquist, 1986). Furthermore, the presence of abnormal and denatured proteins is a key cellular signal for hsp induction (Ananthan *et al.* 1986; Parsell and Lindquist, 1993). Evidence suggests that hsps act as chaperones in the cell to re-fold and essentially 'rescue' proteins damaged by thermal denaturation (Skowrya *et al.* 1990; Schröder *et al.* 1993; Beckmann *et al.* 1990; Ellis and van der Vies, 1991; Gething and Sambrook, 1992; Craig, 1993; Becker and Craig, 1994). Although increased concentrations of hsps are indicative of thermal stress, they are not of themselves indicative of increased amounts of irreversible denaturation. Thus, elevated levels of hsps may simply reflect effective rescue of heat-damaged proteins, rather than increases in irreversible protein denaturation.

An excellent study system in which to examine questions of environmentally induced protein damage is the sessile

invertebrates of the rocky intertidal zone. For these species, thermal stress can be well characterized and will vary with location, season and tidal stage (Newell, 1979). We focused on populations of *Mytilus trossulus* located on San Juan Island in Puget Sound, Washington. *M. trossulus* is an *edulis*-like mussel found along the Pacific coast of North America from central California to Alaska (McDonald and Koehn, 1988) and is common in protected waters and on the outer coast of Washington and Oregon (Suchanek, 1978). Several features make mussels a particularly suitable organism for studies of heat stress during tidal emersion. Mussels are generally found in exposed locations on rock reefs in the intertidal zone and lack any locomotory capabilities to seek shelter in crevices. This relatively sessile lifestyle, combined with a dark absorbent shell, makes mussels likely to experience maximal thermal stress in the intertidal zone. The field site in the San Juan Islands was chosen because the meteorological and tidal characteristics of that area increase the chance that intertidal mussels will experience heat stress during low tide. Low tides in summer occur in the middle of the day, subjecting the exposed intertidal mussels to wide ranges of body temperature. Because the San Juan Archipelago is in the rain shadow of the Olympic Mountains, San Juan Island tends to be drier and have more sunny days than the outer coast of Washington or Oregon (Dayton, 1971).

Comparisons of two San Juan Island populations of *M. trossulus*, one from an exposed intertidal site and one from a site where animals remained submerged throughout the tidal cycle, reveal that quantities of ubiquitin conjugates and levels of hsp70 varied between collection sites and on a seasonal basis. These results provide the first evidence from field populations that thermal stress enhances rates of protein degradation. The consequences of heat-induced reversible and irreversible denaturation for energy budgets are discussed.

Materials and methods

Collection of field temperature data

The mussel *Mytilus trossulus* (Gould) was studied at two different sites on San Juan Island, Washington (Puget Sound: 48° 34' N; 123° 9' W), British Camp and Argyle Creek. At British Camp, mussel body temperatures were measured in the field using an Omega digital thermometer (HH 82) that recorded from wire thermocouples placed inside the mussel shell. Temperatures were routinely collected from the same five individuals of the same size class (5–6 cm) every 20 min during tidal cycles. Small holes (2.5 mm in diameter) were drilled into the shells in order to allow placement of the thermocouple. Between temperature measurements, the hole was covered by clay to prevent abnormal evaporative water loss from the mussel. At Argyle Creek, the mussels were continually submerged and only water temperature at the time of collection was monitored. Air and water temperatures were recorded using a dry bulb thermometer.

Collection and preparation of gill tissue samples

Whole gill lamellae were dissected from mussels in the field

and immediately frozen on an aluminum block chilled on dry ice. Samples were transported back to the laboratory on dry ice and stored at -70°C . In preparation for electrophoresis and solid-phase immunoassays, 100–200 mg of tissue sample was homogenized in 4 volumes (w/v) of a buffer containing 50 mmol l^{-1} Tris-HCl, pH 7.6, 20 mmol l^{-1} leupeptin, 1 mmol l^{-1} phenylmethylsulfonylfluoride (PMSF), 5 mmol l^{-1} iodoacetamide and 2 mmol l^{-1} EDTA (Beers and Callis, 1993). Protease inhibitors (leupeptin and PMSF) were added to prevent degradation of the ubiquitin conjugates during cell disruption. The homogenate was centrifuged at $16\,000g$ for 5 min at 4°C . From the resulting supernatant, a sample was reserved for protein determination (bicinchoninic acid method; Smith *et al.* 1985) and the remaining material was mixed 1:1 (v/v) with an SDS sample buffer (50 mmol l^{-1} Tris-HCl pH 6.8, 10% glycerol, 2% 2-mercaptoethanol) containing 10% SDS, boiled for 5 min and centrifuged at $16\,000g$ for 15 min. The supernatant was removed and stored at -20°C prior to immunochemical analysis.

Solid-phase immunoassay for ubiquitin conjugates

Levels of ubiquitin conjugates in gill tissue were measured using modifications of the solid-phase assay developed by Haas and Bright (1985). Ubiquitin conjugate and free ubiquitin standards were prepared following the methods outlined in Haas and Bright (1985).

In preparation for dot blotting, gill extracts in SDS sample buffer were thawed, boiled for 1 min and diluted to $40\mu\text{g ml}^{-1}$ in saline (150 mmol l^{-1} NaCl). The diluted sample was then serially diluted in saline containing 0.01% SDS so that the signal generated in the immunochemical assay was linear with respect to the amount of protein loaded and within the linear range of the associated ubiquitin conjugate standard curve. Using a dot blot vacuum apparatus (BioRad), triplicate $100\mu\text{l}$ volumes of samples and ubiquitin conjugate standards were applied to a nitrocellulose membrane ($0.2\mu\text{m}$, Schleicher and Schuell) that had been prehydrated for 4 h in distilled water. Sample volumes were allowed to pass through the nitrocellulose *via* gravity flow and then washed three times with $500\mu\text{l}$ of Tris-buffered saline (TBS; 25 mmol l^{-1} Tris-HCl, pH 7.5, 150 mmol l^{-1} NaCl) under gentle vacuum. After washing, the membrane was gently blotted dry and fixed at 70°C for 15 min. Following sample application, nitrocellulose membranes were immunostained as described in Haas and Bright (1985), with the exception that the immunostained blots were not heat-fixed prior to autoradiography. Ubiquitin conjugates were detected using a polyclonal rabbit anti-ubiquitin conjugate antibody (provided by Dr Arthur L. Haas). After the final incubation in ^{125}I -labelled Protein A ($3.37\text{--}4.22\text{ kBq ml}^{-1}$), the blots were exposed to X-ray film (Kodak X-OMAT AR) at -70°C for an empirically determined time (usually 24–72 h), developed and densitometrically scanned at 540 nm on a MicroTek plate reader.

Western blot analysis of ubiquitin conjugates

Immunoblotting of ubiquitin conjugates was performed using the methods of Haas and Bright (1985) with the

following exceptions. For SDS-polyacrylamide gel electrophoresis, equal amounts of protein ($20\mu\text{g}$) in SDS sample buffer (62.5 mmol l^{-1} Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.00125% Bromophenol Blue) were electrophoresed on a 7.5% SDS-polyacrylamide gel using the methods described by Laemmli (1970). Electrophoretic transfer was performed using a semi-dry transfer apparatus at 115 mA for 1 h using a transfer buffer composed of 25 mmol l^{-1} Tris base, 192 mmol l^{-1} glycine and 20% methanol. The final immunostaining step was performed using enhanced chemiluminescence detection (ECL) following the manufacturer's instructions (ECL western blotting detection reagents; Amersham).

Western blot analysis of heat shock protein hsp70 isoform levels in mussel gills

Relative levels of a heat shock protein, hsp70, were determined using western blot analysis of the gill extracts prepared for ubiquitin conjugate dot blots as described above. Equal amounts of protein ($10\mu\text{g}$) in SDS sample buffer were electrophoresed on a 7% SDS-polyacrylamide gel as described above. Following separation, the proteins were electrophoretically transferred onto prehydrated nitrocellulose membranes (MSI NitroBind, $0.45\mu\text{m}$) using a semi-dry transfer method. Transfer was carried out at 115 mA for 1.5 h with a transfer buffer containing 25 mmol l^{-1} Tris base, 192 mmol l^{-1} glycine and 20% methanol. Transfer conditions were optimized to ensure complete transfer of proteins in the 70 kDa region of the gel.

Western blotting was then performed using an enhanced chemiluminescence protocol (S. Lindquist, personal communication). Following transfer, the membrane was blocked overnight with 5% nonfat dry milk in phosphate-buffered saline (PBS; 10 mmol l^{-1} sodium phosphate, 150 mmol l^{-1} NaCl, pH 7.4). After three 5 min washes in PBS containing 0.1% Tween-20, the blot was incubated for 1.5 h in the primary antibody solution composed of a rat anti-hsp70 antibody (hybridoma 7.10 provided by Dr Susan Lindquist) diluted 1:2500 in a solution containing 2% nonfat dry milk in PBS, 20% fetal calf serum, 0.02% thimerosal and 1 mmol l^{-1} PMSF. The blot was washed three times for 10 min with PBS/0.1% Tween-20, incubated for 30 min in bridging antibody (rabbit anti-rat IgG; Vector Laboratories), diluted 1:2000 in blocking solution, and then washed three times for 5 min with PBS/0.1% Tween-20. The presence of the primary antibody was detected by a 1 h incubation in horseradish peroxidase (HRP)-conjugated Protein A (diluted 1:5000 in blocking solution) followed by three 5 min washes in PBS containing 0.3% Tween-20 and three 5 min washes in PBS/0.1% Tween-20. As a final incubation, the blot was incubated in a 1:1000 solution of streptavidin-HRP conjugate in a buffer containing 50 mmol l^{-1} Tris-HCl, pH 7.5, 150 mmol l^{-1} NaCl, 25 mg ml^{-1} bovine serum albumin (BSA) and 0.02% thimerosal. This final step was required in order to visualize the biotinylated protein molecular mass markers (Amersham) that were loaded onto each gel used for western

blotting. The biotinylated standards were loaded in equal quantities on each gel at 1.0 μg total protein. After one 10 min wash in PBS/0.3% Tween-20 and two 10 min washes in PBS/0.1% Tween-20, the western blot was developed using ECL detection following the manufacturer's instructions (Amersham). Following incubation of the blot in the ECL detection reagents, the blot was routinely exposed to pre-flashed X-ray film sensitive to blue light (Hyperfilm-ECL; Amersham) for 5–30 s. The developed film was then densitometrically scanned on an LKB Ultrascan XL laser densitometer.

Results

Mussel body temperatures in the field

One major objective of this study was to document mussel body temperatures during exposure at low tide in both summer and winter, and to correlate these data with biochemical indicators of protein damage. Surprisingly few data are available on the changes in body temperatures that intertidal invertebrates experience during seasonal and tidal cycles. Using a digital thermometer and thermocouples, body temperatures of *M. trossulus* were measured over a tidal cycle on 1 August 1993 (tide = -1.0 at 11:00 h) and 4 February 1994 (tide = 0.3 at 18:11 h). The mussels in this study were located on a rock reef at British Camp, an intertidal site in a sheltered bay on the west side of San Juan Island (Garrison Bay; Puget Sound). The temperatures of five individuals of the same size class (5–6 cm) were taken immediately after the mussels had emerged from the water as the tide receded, and thereafter at 20 min intervals until the animals were again immersed in water. In summer, after 2 h of emersion, body temperatures had increased from the 10 °C ambient water temperature, to over 30 °C (Fig. 1). Tidal emersion lasted 7–8 h at the British Camp site and mean *in situ* mussel temperatures remained elevated at 30 °C or higher until the tide returned (Fig. 1). Peak mussel body temperatures ranged from 25 to 35 °C over 10 days of data collection during the summer. Consequently, the nature of the heat stress for mussels at the intertidal site in the summer was characterized by high body temperatures and prolonged periods at those elevated temperatures. It should be noted that the mussels were never observed to gape during emersion, as other bivalves such as *Mytilus californianus* have been observed to do during aerial exposure (Bayne *et al.* 1976). This observation is in agreement with studies on *M. edulis* (Coleman, 1973; Widdows *et al.* 1979).

In February, seawater temperature in Garrison Bay was 7.4 °C, approximately 3 °C lower than that recorded in August. However, mean mussel body temperatures were much lower in winter than in summer as a result of a combination of lower air temperatures and reduced solar radiation. In February, mussel body temperatures showed only a transient 8 °C elevation over seawater temperature, and this occurred during a brief break in the cloud cover. After this short sunny period, body temperature decreased to a value close to the ambient water temperature (Fig. 1). Measurements on several days in

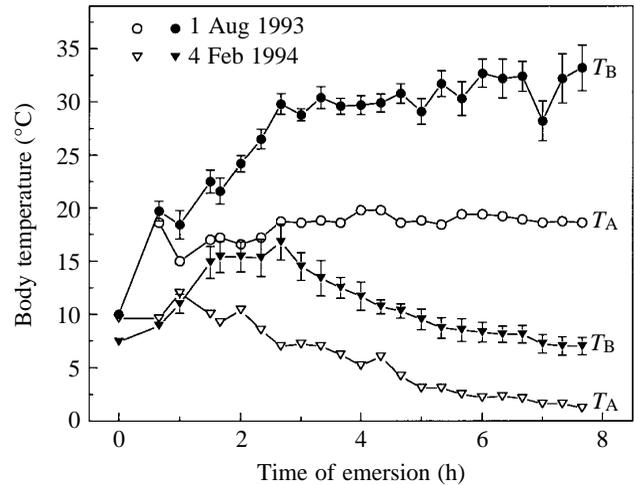


Fig. 1. *In situ* body temperatures of *Mytilus trossulus* recorded during a tidal cycle at British Camp. Measurements of mussel tissue temperature (T_B ; filled symbols) and ambient air temperature (T_A ; open symbols) were taken every 20 min. All temperature data were collected from the same mussels ($N=5$) throughout the period of tidal exposure. For mussel body temperature, points represent the mean \pm s.e.m.; for air temperature, points represent single determinations.

winter showed that peak body temperatures were no higher than 15–17 °C, and peak temperatures were of short duration relative to summer conditions. For most specimens on most days, winter body temperatures ranged only between 5 and 10 °C, compared with a range of 10 to 30–35 °C in summer. At Argyle Creek, mussel body temperatures were assumed to be equal to the seawater temperature at the time of collection. Summer seawater temperatures in the creek ranged from 15 to 18.5 °C and were generally 6–8 °C higher than open-water temperatures (recorded by NOAA temperature gauge on Friday Harbor dock). In February, the temperature of the sea water flowing through Argyle Creek was 5–7 °C, similar to seawater temperatures recorded at British Camp.

Levels of ubiquitin conjugates in mussel gill during summer and winter

In order to confirm the presence of ubiquitin conjugates in mussel gill, tissue extracts were examined using western blotting. The molecular mass distribution of ubiquitin conjugates in mussel gill is shown in Fig. 2. The majority of conjugates appeared in the high molecular mass region of the gel above 100 kDa. This observation is consistent with other studies that have shown a typical high molecular mass 'smear' that indicates that ubiquitin is ligating to a variety of proteins (Carlson *et al.* 1987; Pickart *et al.* 1991). In addition, chains of ubiquitin molecules form on targeted proteins. The length of the chain will influence the apparent molecular mass of ubiquitin conjugates because each ubiquitin moiety adds approximately 8 kDa to the conjugate.

Levels of ubiquitin conjugates were quantified, in pmoles of ubiquitin conjugates per microgram protein, using dot blot immunoassays with an accompanying ubiquitin conjugate

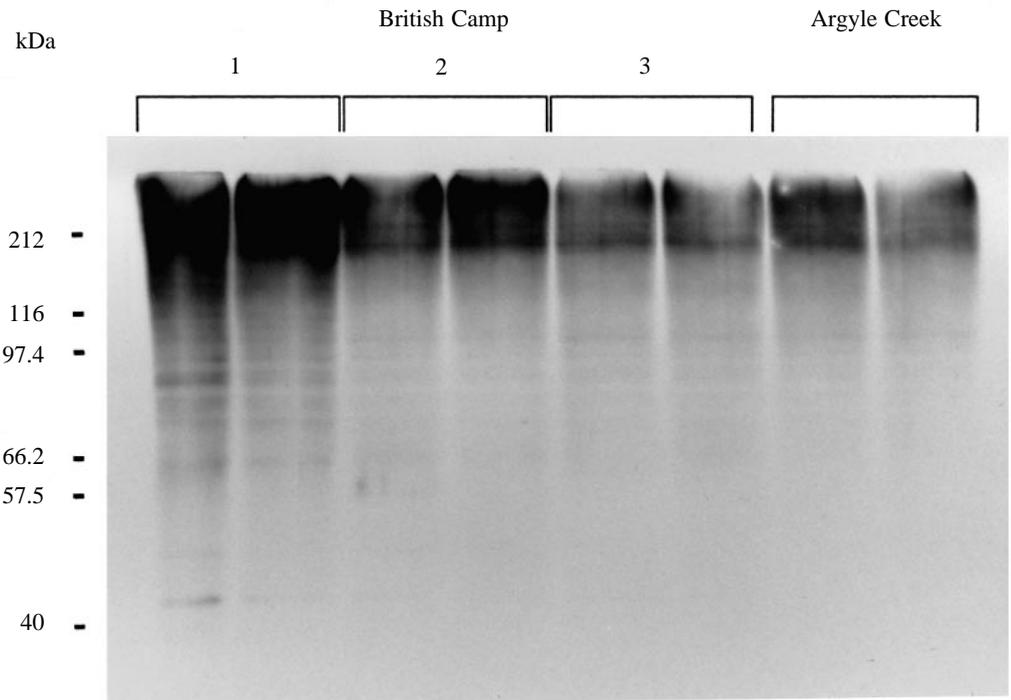


Fig. 2. Western blot analysis of ubiquitin conjugates in gill tissue of the mussel *Mytilus trossulus*. Equivalent amounts of gill protein ($20 \mu\text{g}$) were separated on a 7.5% SDS-polyacrylamide gel, followed by electrotransfer and immunostaining using enhanced chemiluminescence detection. The developed blot was exposed to X-ray film for 30 s. Lanes shown are duplicate samples of three individuals from British Camp and one mussel collected at Argyle Creek.

standard curve (data not shown). Fig. 3 shows the levels of ubiquitin conjugates measured in gill tissue from *M. trossulus* collected in summer and winter at British Camp and Argyle Creek. At British Camp, the amount of ubiquitin conjugates was significantly higher in gills of summer-collected mussels compared with winter-collected mussels (Fig. 3). However, there was no significant change in ubiquitin conjugate levels over the 8 h period of emersion during the tidal cycle in either summer or winter (Fig. 3). Similarly, at Argyle Creek, summer levels of ubiquitin conjugates were higher than winter levels in mussel gill, but the summer levels were not as high as those measured in summer-collected mussel gill from British Camp (Fig. 3). The levels of ubiquitin conjugates in gills of winter-collected mussels did not differ between Argyle Creek and British Camp specimens.

Relative endogenous levels of the stress protein hsp70 in mussel gill

The monoclonal antibody used in the immunoblot analysis recognizes a conserved epitope of the constitutive and heat-inducible forms of hsp70 in a variety of eukaryotic cells (Kurtz *et al.* 1986). Using one-dimensional electrophoresis, up to four different isoforms of hsp70, which ranged in size from 68 to 76 kDa, were detectable in gill tissue of *M. trossulus*. However, not all forms were detectable in all specimens and, therefore, the comparisons of endogenous levels were performed using only the two forms of hsp70 that were always present, the 68 and 76 kDa isoforms. A representative western blot using enhanced chemiluminescence detection is shown in Fig. 4.

The results of quantitative western analyses of the levels of both hsp70 isoforms in the different populations are presented in Fig. 5. Equivalent amounts of gill protein from individual

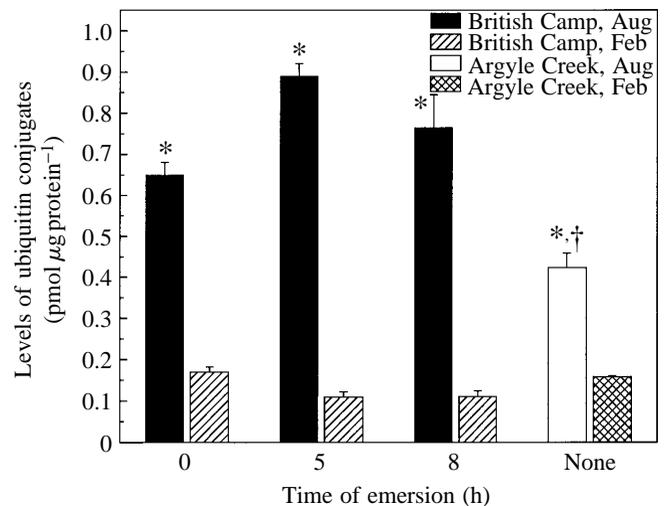


Fig. 3. Comparative levels of ubiquitin conjugates in gill tissue of summer- and winter-acclimatized *Mytilus trossulus*. Levels of ubiquitin conjugates were measured using the solid-phase immunoassay described by Haas and Bright (1985). The values for ubiquitin conjugates were determined using a ubiquitin conjugate standard curve ranging from 0 to 1 pmole of ubiquitin conjugates per dot blot well (data not shown). For this figure, the x-axis represents the absolute emersion time experienced by the mussels. For the collections at British Camp, the time of day varied as a result of seasonal changes in the tidal cycle. Specifically, the August collection was made from 07:00 to 15:00 h and the February collection was taken from 13:00 to 21:00 h. Bars represent the mean + S.E.M. for gill samples from five mussels except for the following: British Camp (August) 0 h, $N=4$; 5 h, $N=3$. *Significantly different from the corresponding winter collection (ANOVA; $P<0.05$); †significantly different from specimens collected at British Camp site (ANOVA; $P<0.05$).

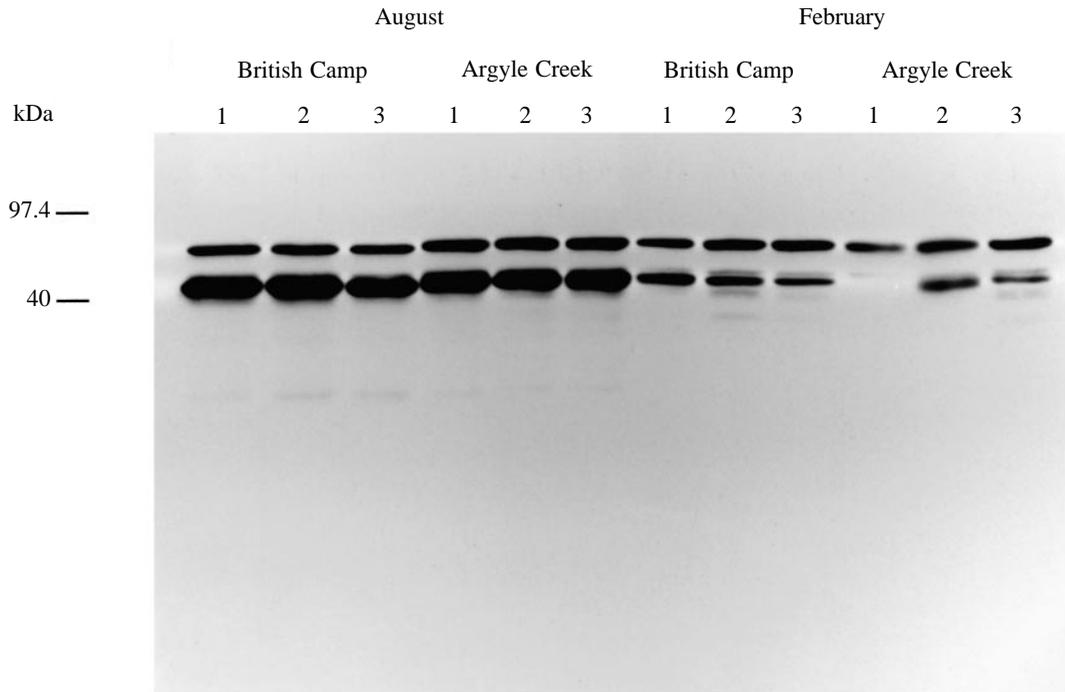


Fig. 4. Western blot analysis of hsp70 isoforms in gill of summer- and winter-acclimatized *Mytilus trossulus*. Lanes contain equivalent amounts of protein (10 μ g) from gill tissue of three individuals collected at the British Camp and Argyle Creek sites in August and February. Immunodetection was performed using the ECL method and the blot was exposed to film for 15 s.

mussels were analyzed and no significant differences in protein content of gill tissue were measured for mussels from British Camp and Argyle Creek as a function of season (data not shown). In order to compare multiple immunoblots and to compensate for variation in the antibody binding during the ECL detection, equivalent amounts of biotinylated protein molecular mass markers were run on each gel. The intensities of the hsp70 bands were compared with the intensities of one of the marker bands. For these analyses, the amount of protein molecular mass standard used and the amount of gill sample applied to the gel were adjusted by serial dilution to ensure that the band intensity was within the linear range of the detection of the X-ray film. The data presented in Fig. 5 are normalized and represent levels of hsp70 isoforms relative to the standard marker band. Note that this normalization method can yield relative concentrations of less than 1 for hsps. A general comparison of the two hsp70 isoforms revealed that the 68 kDa isoform was found in higher levels in gill tissue for all

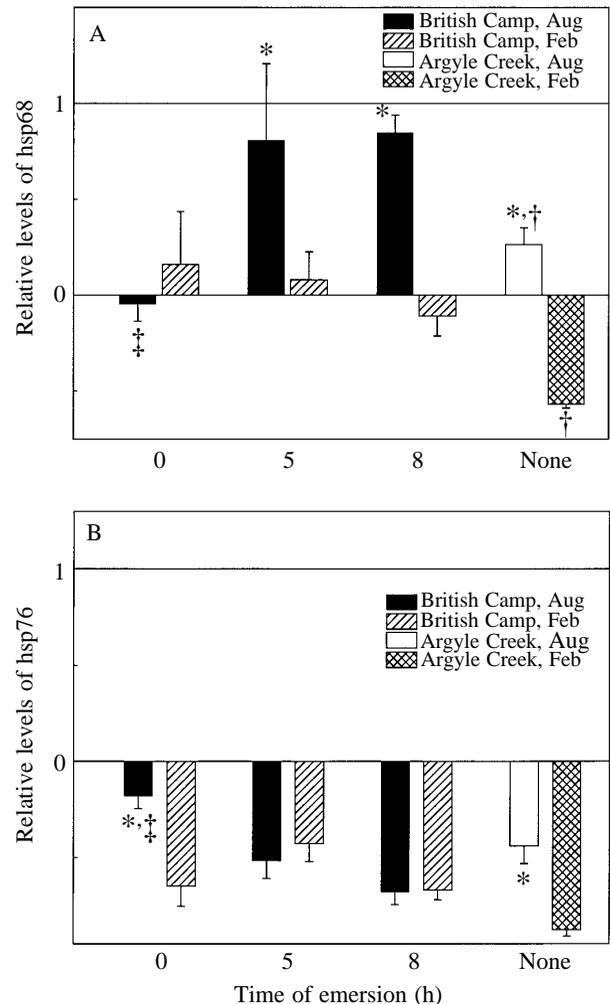


Fig. 5. Western blot analysis of relative levels of the 68 kDa (A) and the 76 kDa (B) isoforms of the stress protein hsp70 detected in gill tissue of *Mytilus trossulus*. The data shown represent intensities of hsp70 isoform bands (measured by a laser densitometer) standardized using the intensity of one biotinylated marker run on each gel. In A and B, the zero point represents unity with the marker band, and the bars show increases or decreases relative to the intensity of the marker band. Each bar represents the mean + S.E.M. for five mussels, except for British Camp (August) where $N=4$ for 0 h and $N=3$ for the 5 h time point. *Significantly different from the corresponding winter collection (ANOVA; $P<0.05$); †significantly different from samples collected at British Camp (ANOVA; $P<0.05$); ‡significantly different from the 5 h and 8 h time points (ANOVA; $P<0.05$).

collections except for the February sampling at Argyle Creek (Fig. 5). In addition, the smaller isoform showed a greater degree of seasonal variation (Fig. 5A). This suggests that the 68 kDa isoform of hsp70 may be a more heat-inducible form than the 76 kDa protein, which exhibited less variation with site and season.

Relative levels of the 68 kDa isoform varied significantly on a seasonal basis in gill from mussels collected at both sites (Fig. 5A). Despite avoiding the stress of tidal emersion, mussels at Argyle Creek were exposed to seawater temperatures as high as 18.5 °C in the summer. This temperature, although much lower than the peak temperatures experienced by the British Camp mussels, appears to be high enough to favor enhanced levels of hsps in this species. Furthermore, in mussels from British Camp, there was an increase in levels of the 68 kDa isoform in gill from 0 to 5 h of emersion (Fig. 5A). This increase may be due to rapid induction of hsp synthesis by the high body temperatures observed under summer conditions. By 5–8 h of emersion, the concentration of the 68 kDa protein reached levels that were significantly higher than those measured in summer-collected Argyle Creek mussels.

Overall, the relative levels of the 76 kDa isoform of hsp70 were considerably lower than those of the 68 kDa isoform (Fig. 5B). During tidal emersion in August, the levels of the 76 kDa protein decreased over 8 h of exposure. In February, the levels remained low and unchanged over the 8 h time course. In mussels collected at Argyle Creek, summer levels of the 76 kDa isoform were not significantly different from those measured in the intertidal population at British Camp. However, the winter levels of the 76 kDa protein were significantly lower than the summer levels in the Argyle Creek mussels.

Discussion

Our results show that two indicators of protein denaturation, ubiquitin conjugate formation and levels of the stress protein hsp70, varied positively with *in situ* body temperatures of the intertidal mussel *Mytilus trossulus* on a seasonal basis and as a function of average habitat temperature at two different study sites. The two biochemical indices used in this study provide information about different aspects of the protein pool. Elevated levels of ubiquitin conjugates indicate that there are enhanced amounts of irreversibly damaged proteins in cells. In contrast, elevated endogenous levels of hsp70 isoforms indicate a greater need for molecular chaperones, a requirement that suggests increased amounts of reversible thermal denaturation. Despite the elevated hsp70 levels in summer-collected mussels, elevated quantities of ubiquitin conjugates in these same specimens show that irreversible denaturation occurred at high environmental temperatures. Taken together, the data suggest that while the heat shock response is enhanced in animals during exposure to high temperatures, the presence of elevated concentrations of molecular chaperones may not be sufficient to prevent irreversible protein denaturation in the face of heat stress.

It should be noted that we did not detect changes in concentrations of ubiquitin conjugates in the mussels sampled during a full tidal cycle at British Camp. The ubiquitin conjugate data suggest that the rate of protein degradation *via* the ubiquitin-mediated proteolytic pathway did not vary diurnally. These data conflict with the time course of ubiquitination observed in studies of the heat shock response in isolated cells. In chicken fibroblasts, heat stress at 45 °C for 60 min resulted in a large and rapid increase of ubiquitin conjugates from 3.2 to 7.8 pmol per 10⁶ cells (Bond *et al.* 1988). Similarly, rat hepatoma cells showed protein degradation and an increase in ubiquitin conjugates after a 2 h heat shock at 43 °C (Parag *et al.* 1987). One explanation for the discrepancy between our results and those of the studies of isolated cells is that our experiments were conducted *in situ* and, therefore, were subject to factors not present in the *in vitro* experiments on isolated cells. During aerial exposure, mussels enter a state of metabolic depression where a variety of mechanisms act to suppress the metabolic rate to only a few per cent of normal levels (de Zwann, 1983; Widdows *et al.* 1979; Widdows and Shick, 1985). Studies of the physiological response of *Mytilus edulis* to aerial exposure have shown that anaerobic ATP production was only about 12% of the aerobic value (Widdows *et al.* 1979). Ubiquitination of proteins may be limited during emersion because a reduced cellular energy charge may not be conducive for the energy-consuming nature of ubiquitin conjugate formation. An important role for *in vivo* levels of adenylates in ubiquitin conjugate formation has been shown in another invertebrate, the crustacean *Artemia franciscana*, where ubiquitin conjugate levels are greatly reduced in anoxic embryos when cellular energy charge is reduced (Anchordoguy and Hand, 1994). Thus, given the likelihood of reduced cellular energy charge in mussels during aerial exposure, protein ubiquitination may be delayed until the mussels are in metabolic recovery following re-immersion. Alternatively, the increased levels of ubiquitin conjugates in summer-acclimatized mussels may also reflect a reduction in the rate of conjugate degradation as an energy-saving strategy. We are currently investigating the kinetics of ubiquitin conjugate formation during recovery from tidal emersion.

Another significant finding of this study was the seasonal differences in endogenous levels of hsp70 isoforms in mussel gill tissue. Concentrations of hsps in ectotherms are known to be subject to acclimation in the laboratory (Sanders *et al.* 1992; Dietz and Somero, 1992). For example, *M. edulis* was acclimated at 27 °C for 8 weeks and concentrations of hsp70 in gill tissue increased 4- to 10-fold over levels in control mussels at 17 °C (Sanders *et al.* 1992). However, our study is one of the few studies to examine the influence of environmental temperature on endogenous levels of stress proteins in animals in nature and to document hsp levels in field-collected specimens. Seasonal changes in another stress protein have been observed in the brain of the eurythermal goby *Gillichthys mirabilis*, where summer-acclimatized fish had higher levels of hsp90 than did brain collected from winter-acclimatized fish (Dietz and Somero, 1992).

Seasonal changes in endogenous concentrations of stress proteins may contribute to the development of seasonal thermotolerance. Several studies have shown a correlation between concentrations of hsp70 and the degree of induced thermotolerance (Bosch *et al.* 1988, 1991; Hahn and Li, 1990; Li *et al.* 1991; Solomon *et al.* 1991; Sanchez and Lindquist, 1990; Sanchez *et al.* 1992; Parsell *et al.* 1993). Seasonal thermotolerance that is conferred by elevated endogenous levels of hsp70 in cells may be particularly important in eurythermal ectotherms such as intertidal invertebrates.

The mechanism by which hsp70 participates in cellular biochemistry to confer thermotolerance to cells in organisms in nature is probably twofold. As mentioned earlier, hsp70 is thought to act as a molecular chaperone to stabilize and re-fold thermally denaturing proteins. Most evidence for this function comes from *in vitro* experiments with DnaK, the hsp70 homologue in *Escherichia coli*. DnaK has been shown to reactivate thermally inactivated RNA polymerase (Skowyra *et al.* 1990), to act in concert with other proteins, DnaJ and GrpE, in a chaperone complex to repair thermally denatured luciferase (Schröder *et al.* 1993) and to prevent protein aggregation *in vitro* (Langer *et al.* 1992). In addition to a presumptive role in re-folding thermally damaged proteins, hsp70 appears to be part of a molecular chaperone complex that binds to polypeptides translating on ribosomes and prevents premature folding and aggregation of nascent polypeptides (Frydman *et al.* 1994). Thus, hsp70 plays a vital role in protein biogenesis under normal cellular conditions. This latter cellular role for hsp70 has potential significance for organisms during growth. Enhanced levels of protein synthetic activity may require greater quantities of hsp70 to chaperone the increased amount of ribosome-bound polypeptides. Such a scenario may apply to *M. trossulus* in the summer, when warmer temperatures could foster higher growth rates. To our knowledge, there are no data that relate growth state, starvation or degree of protein synthesis activity to cellular levels of molecular chaperones.

Our discovery that sublethal heat stress can lead to elevated concentrations of hsp70 and increased amounts of irreversible protein denaturation has implications for ecological energetics and species distribution patterns. The metabolic cost of heat damage to proteins could significantly impact an animal's energy budget. First, the normal housekeeping level of protein degradation is a significant energy cost for cells. For example, in a rat liver cell, the average half-life of a protein is 3 days (Beynon and Bond, 1986). The cost of protein synthesis is estimated to be 18–26 % of the energy budget of an ectothermic organism (Houlihan, 1991; Hawkins, 1985). If environmental conditions increase rates of protein loss, the metabolic costs of replacing proteins will become even higher. Second, the cost of protein replacement is compounded by the energy expended to synthesize and maintain pools of heat shock proteins. Furthermore, the activity of hsp70 and the process of protein re-folding involve a considerable expenditure of cellular energy. For example, in *E. coli*, it is estimated that 100 ATP molecules are required for GroEL, a heat shock protein, to salvage one

rhodanase protein (Martin *et al.* 1991). Finally, should a protein become irreversibly denatured, the degradation of target proteins to peptides and amino acids through the ubiquitin proteolytic pathway involves several ATP-dependent steps associated with ubiquitin activation, ligation to the targeted protein and hydrolysis by cytoplasmic proteases.

In summary, temperature has long been recognized as a key environmental factor that influences habitat selection, especially in ectotherms (Huey, 1991). Our study on intertidal mussels suggests that a part of the consequences of habitat temperature may be the cost of maintaining the integrity of the protein pool, *via* degradation of irreversibly damaged proteins, salvage of thermally denatured proteins by molecular chaperones or *de novo* synthesis of lost proteins. This metabolic cost may be one of the components that, over the short term, influence organismal physiology and determine whether the animal will survive and reproduce in its environment. Such an influence of the cost of protein degradation would then contribute to determining the suitability of an organism's habitat and, therefore, may be a selective force in habitat selection. Given the interaction of environmental stress, metabolic cost and the physiological capacity of an organism to survive in a particular thermal habitat, environmentally induced protein degradation may contribute to determining the distribution patterns of species on microhabitat and latitudinal scales.

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