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Interspecific variation in thermal denaturation of proteins in the congeneric mussels *Mytilus trossulus* and *M. galloprovincialis*: evidence from the heat-shock response and protein ubiquitination

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Abstract Individuals of two species of blue mussels, *Mytilus trossulus* (Gould, 1850) and *M. galloprovincialis* (Lamarck, 1819), that have different latitudinal distributions, were collected from two locations on the Pacific coast of the USA where their distributions do not overlap. To determine if the congeners were differentially sensitive to thermal stress, we first held individuals of each species at 13 °C for 8 wk and then examined three biochemical indices of thermal damage to cellular proteins: relative levels of the stress protein hsp70, quantities of ubiquitin conjugates and the induction of stress-protein synthesis. The results provide evidence that the northern species, *M. trossulus*, was more thermally sensitive than the southern species, *M. galloprovincialis*. Relative levels of hsp70 and amounts of ubiquitin conjugates were higher in gill tissue from *M. trossulus* than in gill from *M. galloprovincialis*, which suggests that *M. trossulus* was more susceptible to reversible and irreversible protein damage, respectively, than *M. galloprovincialis*. In addition, the patterns of stress-protein expression as measured by in vitro radiolabeling experiments using isolated gill tissue, were significantly different, as follows: (1) the threshold induction temperatures for hsp70 synthesis were 23 and 25 °C for *M. trossulus* and *M. galloprovincialis*, respectively; (2) the overall intensity of synthesis and induction was greater in *M. galloprovincialis* than in *M. trossulus*, particularly at the higher incubation temperatures of 28 and 30 °C; (3) *M. galloprovincialis* expressed a 30 kdalton, stress protein that was not induced in the northern species, *M. trossulus*. Thus, after an 8 wk exposure to a common temperature, the two *edulis*-like mussel congeners appeared to be physiologically distinct with respect to thermal damage to proteins. Due to the energetic cost that is probably associated with environmentally-induced protein damage and maintaining pools of stress proteins, differential organismal thermotolerances and protein stabilites may contribute to setting species distribution-limits. Our data support conclusions of other workers that *M. trossulus* is a more cold-adapted species than *M. galloprovincialis*.

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Introduction

Mytilus edulis was once considered the only species of blue mussel on the North American Pacific coast (for review see Koehn 1991 and Gosling 1992). However, recent evidence suggests that the species formerly known as *M. edulis* actually comprises two members of the *M. edulis* sibling-species complex, *M. trossulus* and *M. galloprovincialis* (McDonald and Koehn 1988; McDonald et al. 1991; Sarver and Loudenslager 1991; Seed 1992; Inoue et al. 1995). McDonald and Koehn (1988) surveyed allozyme polymorphisms in *edulis*-like mussels, and reported that *M. trossulus* was distributed from Alaska to central California and *M. galloprovincialis* was distributed from central California to Baja California, Mexico, with both species and their hybrids occurring at the region of overlap. The proposed geographical distribution of the mussels has been confirmed by other workers who looked at variation at allozyme loci (Sarver and Loudenslager 1991; Sarver and Foltz 1993). Other studies using molecular techniques either have reported similar results (Rawson and Hilbish 1995) or have identified *M. trossulus* further south than studies using electrophoretic data (Geller et al. 1994).

The distribution of *Mytilus trossulus* and *M. galloprovincialis* correlates strongly with the sea-surface temperature cline along the Pacific coast of North America. For example, the average annual maximum sea-surface temperature in August is 25 °C off Baja California, Mexico, and 10 to 13 °C off mainland Alaska (Sea-Surface Temperature Chart, U.S. Hydrographic Office, Washington, D.C., USA; as cited in Lubchenco et al. 1993). Because the mussels are intertidal, they are subject not only to prevailing seawater temperatures but also to thermal stress during emersion. This latter stress may be especially severe, particularly when low tides occur at midday. However, few data are available to quantify thermal stress for these two congeners. We have shown that heating due to solar irradiation can increase body temperature in *M. trossulus* to > 30 °C during summer low tides in northern Washington State (Hofmann and Somero 1995). Thus, local climatic conditions and seawater temperatures act in combination to establish the thermal conditions experienced by intertidal mussels. Although both species may experience high temperatures during periods of emersion, because *M. galloprovincialis* has the more southern distribution pattern, it probably experiences environmental temperatures that are on average warmer than those of *M. trossulus*. Thus, *M. galloprovincialis* may be better adapted to cope with heat stress than its more northern congener.

Proteins are one of the most thermally-sensitive components of organisms, and species with different average body temperatures commonly have proteins with different thermal stabilites (Somero 1995). In previous studies, we have shown that environmental temperature has an impact on the degree of protein damage in natural populations of the intertidal mussels *Mytilus trossulus* and *M. californianus* (Hofmann and Somero 1995; Roberts 1995). We have measured increases in three specific indices of thermal damage to proteins in mussels that were exposed to elevated environmental temperatures as a result of either seasonal differences or microhabitat differences, such as different heights of occurrence in the intertidal zone. The three biochemical indices – levels of ubiquitinated proteins, endogenous levels of the stress protein hsp70 and the induction temperature of stress protein expression – were chosen because they are direct measurements of thermal damage to cellular proteins (Hofmann and Somero 1995; Somero 1995). However, ubiquitin (Ub) conjugates and stress-protein expression represent different aspects of thermal damage to protein in cells. Increased levels of Ub conjugates indicate elevated levels of *irreversibly* damaged proteins in cells and are known to be induced by heat stress in a variety of cell types (Carlson et al. 1987; Parag et al. 1987; Bond et al. 1988). Ubiquitin is a low molecular weight protein that, when bound to damaged substrate proteins, targets them for degradation by cellular proteases (Rechsteiner 1987; Hershko and Ciechanover 1992; Hochstrasser 1995).

Alternatively, variation in the expression of stress proteins in a cell is indicative of changes in the degree of *reversible* protein damage. The expression and accumulation of heat-shock proteins (hsps) in cells is a basic cellular defense mechanism that is induced during exposure to abnormally high temperatures for that cell type or by exposure to other environmental stressors such as anoxia, transition heavy-metals or ethanol (Hightower 1980; Lindquist 1986; Parsell and Lindquist 1993). A key cellular signal for the induction of stress-protein expression is the presence of denatured or abnormal proteins (Hightower 1980; Goff and Goldberg 1985; Ananthan et al. 1986; Parsell and Lindquist 1993). As members of the family of molecular chaperones (Ellis and van der Vies 1991; Becker and Craig 1994), stress proteins prevent aggregation of unfolded proteins (Beckmann et al. 1990; Frydman et al. 1994; Frydman and Hartl 1994) and, in some cases, re-fold thermally denatured proteins (Langer et al. 1992; Schröder et al. 1993; Parsell et al. 1994). In cultured cells, elevated levels of hsps induce thermotolerance such that cells exposed to an initial mild heat stress are later tolerant to temperatures that were lethal prior to the first heat exposure (Bosch et al. 1988; Mizzen and Welch 1988; Sanchez and Lindquist 1990; Li et al. 1991; Solomon et al. 1991; Parsell et al. 1993). Such a mechanism may operate at the organismal level, especially in eurythermal ectotherms, where elevated cellular levels of hsps may contribute to the development of longer-term thermal tolerance such as seasonal acclimatization (e.g. Gehring and Wehner 1995).

Although the stress response is a regulated gene-expression phenomenon found in all organisms examined, there are inter- and intraspecific variations in the stress response that include changes in the endogenous levels of stress proteins and alterations in the temperature at which stress-protein expression is induced (Dietz and Somero 1992; Somero 1995). Neither of these features is genetically fixed, and both have been shown to be subject to acclimation and acclimatization (Oda et al. 1991; Dietz and Somero 1992; Dietz 1994; Hofmann and Somero 1995; Roberts 1995). Thus, variation in accumulated levels of stress proteins and shifts in threshold induction-temperatures serve as good indicators of differential effects of thermal stress on proteins of a species.

In the current study, we examined whether the mussel congeners *Mytilus trossulus* and *M. galloprovincialis* are physiologically differentiated with respect to temperature and whether adults of the two species have different thermal sensitivities that could contribute to maintaining the observed biogeographical patterning. Our approach was to test the effect of temperature on the mussels using techniques we have successfully employed to study thermal damage to proteins in field populations of intertidal mussels. Comparisons of Ub

conjugate levels and characteristics of the stress response were made after both species had been held under controlled laboratory conditions at 13 °C, a temperature within ecologically relevant ranges for the congeners.

Our results show that *Mytilus trossulus* and *M. galloprovincialis* exhibited different levels of ubiquitinated proteins and amounts of endogenous hsp70, and displayed variation in the induction of stress-protein synthesis. These data provide evidence that the two species are physiologically and biochemically differentiated with respect to temperature. With regard to the recent advances in the understanding of the heat-shock response and the ability of these proteins to confer thermotolerance to cells and organisms (Parsell and Lindquist 1993, 1994; Gehring and Wehner 1995; Hofmann and Somero 1995; Somero 1995), our study provides new insights into the roles stress proteins and thermal damage to proteins may play in setting the thermal tolerance-limits of organisms and, thus, in influencing biogeographic patterns of species' distribution.

Materials and methods

Specimen collection and acclimation conditions

Mussels were obtained from two latitudinally distinct sites where the potential for co-occurence and hybridization of the two congeners was remote (McDonald and Koehn 1988; Sarver and Foltz 1993; Geller et al. 1994). Specimens of *Mytilus trossulus* (Gould, 1850) were collected at Garrison Bay on San Juan Island, Washington (Puget Sound: 48 ° 34′ N; 123 ° 9′ W) from an intertidal location. *M. galloprovincialis* (Lamarck, 1819) were collected from the Scripps Pier at the Scripps Institution of Oceanography in La Jolla, California (32 ° 52′ N; 117 ° 16′ W). Collections were made in June and July 1994. No mortality was observed for either species following collection and transport.

Both species were placed in a temperature-controlled 120-liter seawater aquarium and held at 13 °C for 8 wk. During the temperature exposure period, mussels were fed three times per week with an algal suspension designed to support maintenance nutritional requirements (Algae Preserve Diet "B"; Coast Seafoods Company, Bellevue, Washington). No mortality was observed during the course of the experiment.

Western-blot analysis of hsp70 levels

Western-blot analysis was used to compare the relative levels of isoforms of hsp70 in gill tissue from *Mytilus trossulus* and *M. galloprovincialis* following the acclimation period. Prior to electrophoresis, protein determinations were made on the gill extracts using a Coomassie Plus protein assay (Pierce Chemical Co.). Equal amounts of gill protein (10 µg per lane) in SDS (sodium dodecyl sulfate) sample buffer were then separated on a 7% SDS-polyacrylamide gel. The separated proteins were then transferred to nitrocellulose membrane (MSI Nitrobind; 0.45 µm) via semi-dry electrophoretic transfer at 115 mA for 1.5 h, using a transfer buffer composed of 192 m*M* glycine, 24 m*M* Tris base and 20% methanol. The immunodetection was performed using an enhanced chemiluminescense protocol (Lindquist personal communication) described in Hofmann and Somero (1995). The antibody used for the analyses was an anti-hsp70 rat monoclonal antibody (7.10) that recognizes both cognate and heat-inducible forms of hsp70 in a wide variety of eucaryotes (Kurtz et al. 1986). When screened against proteins from mytilid gill, the antibody typically detects a range of 3 to 5 different isoforms after separation by one-dimensional electrophoresis (Hofmann and Somero 1995; Roberts 1995).

Incubation procedures for radiolabeling gill proteins using ^{35}S amino acids

For the induction experiments, gill tissue from *Mytilus trossulus* ($n = 4$) and *M. galloprovincialis* ($n = 5$) was radiolabeled in vitro to test for the induction of hsps over a range of temperatures. In order to avoid heat-shocking the tissue prior to induction experiments, gill lamellae were dissected from mussels underwater in trays containing 13 °C seawater, and individual lamellae were cut into sections of equal size (≅ 100 mg). Individual gill pieces were transferred, one each, into seven microcentrifuge tubes with 500 µl of artificial seawater containing 10 m*M* glucose that had been pre-equilibrated to the required incubation temperature. The temperature range used to test for hsp induction was as follows: 13 ° (control), 17, 20, 23, 25, 28 and 30 °C. After addition of 50 to 60 µCi of ^{35}S-labeled methionine/cysteine amino acid mix (Trans-35 metabolic labeling reagent; ICN Biomedicals), gill pieces were incubated for 2 h in temperature-controlled water baths, with aeration every 20 min.

At the end of the incubation period, the tissue pieces were washed twice with 0.5 ml of 4 °C non-radioactive incubation medium, and homogenized with a teflon pellet pestle in 300 µl of SDS homogenization medium (32 m*M* Tris-HCl, 2% SDS, 1 m*M* phenylmethylsulfonylfluoride (PMSF), pH 6.8). Following homogenization, the gill homogenate was boiled for 5 min, centrifuged for 15 min at 16 000 × g, and the resulting supernatant was removed and stored at − 20 °C for SDS-polyacrylamide electrophoresis.

Electrophoresis, fluorography and analysis of protein-expression patterns

Protein-expression patterns in gill tissue were examined using SDS-polyacrylamide separation of radiolabeled proteins followed by fluorography. Extracts of gill proteins in SDS sample buffer (62.5 m*M* Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.01% bromphenol blue) were loaded to equivalent amount of radioactivity per lane (6×10^5 cpm) and subjected to electrophoresis on a 10% polyacrylamide gel according to the methods of Laemmli (1970). After electrophoresis, the gels were treated with an autoradiographic enhancer (EN^{3}HANCE; NEN) according to the manufacturer's instructions. Fluor-treated gels were dried at 60 °C for 2.5 h and then exposed to X-ray film (Kodak X-OMAT AR5) for 15 h at − 70 °C. Densitometric analysis of protein bands was performed using a Molecular Dynamics scanning densitometer and ImageQuant software (Molecular Dynamics).

Relative levels of hsp70 synthesis were calculated by first standardizing the intensity of absorbance of the hsp70 bands to the intensity of a 42 kdalton non-heat-inducible protein for each sample. Since any elaboration of hsp synthesis at higher temperatures could be argued to be the result of a Q_{10} effect, standardization of the amount of hsp synthesized with a non-heat inducible protein accounts for the effects of temperature on the rate of synthesis of proteins. Finally, the standardized intensity of the hsp70 band was expressed relative to the amount of hsp70 synthesis in the 13 °C control incubations.

Quantitative immunochemical assay for ubiquitin conjugates

The amount of ubiquitinated protein in gill tissue was quantified using a solid-phase immunochemical assay as described by Hofmann and Somero (1995), which is a modified procedure from that developed by Haas and Bright (1985). A ubiquitin-conjugate standard curve was generated using Ub-conjugate standards prepared as described by Haas and Bright. The standard curve ranged from 0 to 1.0 pmol of Ub conjugates. Gill was homogenized in a sample buffer containing 50 m*M* Tris-HCl, pH 7.6, 20 m*M* leupeptin, 1 m*M* PMSF, 5 m*M* iodoacetamide and 2 m*M* EDTA, diluted to 40 µg protein ml^{-1} and then serially diluted in 150 m*M* NaCl so that the signal generated during the immunochemical assay was within the linear range of the X-ray film and within the range of the Ub-conjugate standard curve. Triplicate 100 µl volumes of diluted samples and standards were applied to a pre-hydrated nitrocellulose membrane (0.2 µm; Schleicher and Schuell) using a dot-blot vacuum-apparatus.

Following sample application via gravity flow, the blot was washed three times with 500 µl per well of Tris-buffered saline (25 mM Tris-HCl, pH 7.5, 150 m*M* NaCl) and immunostained as described by Haas and Bright. A rabbit polyclonal anti-Ub-conjugate antibody (provided by Dr. A.L. Haas) was used to detect Ub conjugates. Final detection was conducted using ^{125}I-Protein A (at 2×10^5 to 2.5×10^5 counts min^{-1}). Protein A is a bacterial cell-wall protein that has a high affinity for immunoglobulins from a variety of animals, including rabbit IgG. Dot blots were exposed to X-ray film (Kodak X-OMAT AR) at −70 °C for 24 to 72 h, developed, and densitomentrically scanned at 540 nm on a Micro Tek plate reader.

Determination of radiolabeled amino acid incorporation: TCA precipitation assay

Total radioactivity incorporated into protein during in vitro incubations for the induction experiments was determined using trichloroacetic acid (TCA) precipitation of proteins onto glass-fiber filters. Aliquots (5 µl) of the radioactive gill extracts from the induction experiments were applied to 2.5 cm Whatman GF-C glass-fiber filters and allowed to air-dry. The dried filters were washed in excess volumes of ice-cold 10% TCA followed by two washes in 5% TCA at room temperature. Filters were rinsed in 95% ethanol, dried at room temperature, and counted in a scintillation cocktail. Incorporation values were calculated by expressing total counts of radioactivity as a function of total gill protein. Protein determinations were made using a Coomassie Plus protein assay (Pierce Chemical Co.).

Results

Endogenous levels of hsp70 in mussels held at 13 °C for 8 wk

Using western-blotting techniques, we measured the relative endogenous levels of hsp 70 in gill tissue from specimens of *Mytilus trossulus* and *M. galloprovincialis* following an 8 wk exposure period at 13 °C. As shown in a representative ECL western blot in Fig. 1, the anti-hsp70 monoclonal antibody detected 2 to 3 different forms of hsp70 in gill from both mussels. For quantification purposes, these isoforms have been organized into two groups according to size, a low molecular weight group containing protein ranging from 66 to 68 kdaltons and a high molecular weight group of proteins in the range of 70 to 73 kdaltons (Fig. 1).

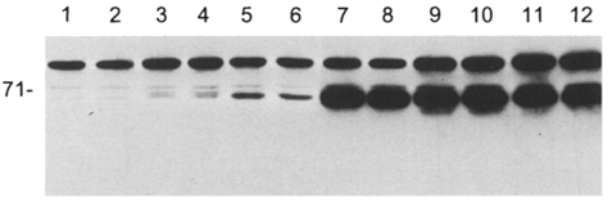

Fig. 1 *Mytilus galloprovincialis* (*Lanes 1 to 6*) and *M. trossulus* (*Lanes 7 to 12*). Western-blot analysis of endogenous levels of isoforms of stress protein hsp70 in gills held at 13 °C for 8 wk. Lanes contain equivalent amounts of protein (10 µg) from gill extracts prepared from three individuals of each species, and show endogenous hsp70 levels for three *M. galloprovincialis* individuals (*Lanes 1 to 6*) and three *M. trossulus* individuals (*Lanes 7 to 12*). Each gill sample was run in duplicate in two consecutive lanes. Immunodetection was performed using rat monoclonal anti-hsp70 antibody and enhanced chemiluminescence protocol. Following application of ECL reagents, blot was exposed to X-ray film for 60 s. The 71 kdalton protein molecular weight standard was generated using Kaleidoscope Pre-stained Standards (BioRad)

After the 8 wk exposure period at 13 °C, gill tissue from the northern species, *Mytilus trossulus*, contained total levels of hsp70 isoforms that were over three-fold greater than those measured in *M. galloprovincialis* (Fig. 2). Gill from *M. trossulus* contained significantly higher levels of the 66 kdalton hsp70 isoforms than did gill from *M. galloprovincialis* (Fig. 2). Levels of the 70 kdalton isoforms were also greater in gill from *M. trossulus* but the difference was not statistically significant (Fig. 2).

Comparison of stress-protein expression in isolated gill samples after 8 wk temperature exposure

Induction temperatures for stress-protein expression were examined in *Mytilus trossulus* and *M. galloprovincialis* after the mussels had been held at 13 °C for 8 wk. The representative fluorograms in Fig. 3 show the patterns of protein synthesis in gill during in vitro radiolabeling incubations over a range of temperatures. As shown in Fig. 3, a high degree of consistency was observed among the specimens within a species in terms of (1) the size classes of proteins synthesized constitutively, (2) the relative band intensity of these constitutively synthesized proteins, which represents the efficiency of incorporation of ^{35}S-radiolabeled amino acid into protein, and (3) the induction of specific bands in response to heat stress. The general expression pattern in both mussel species revealed two classes of proteins: cellular proteins that were synthesized at all temperatures (e.g. a prominently labeled 46 kdalton protein) and a set of stress proteins that were induced at the higher temperatures (Fig. 3). Although isolated gill from both species synthesized a 70 kdalton-class stress

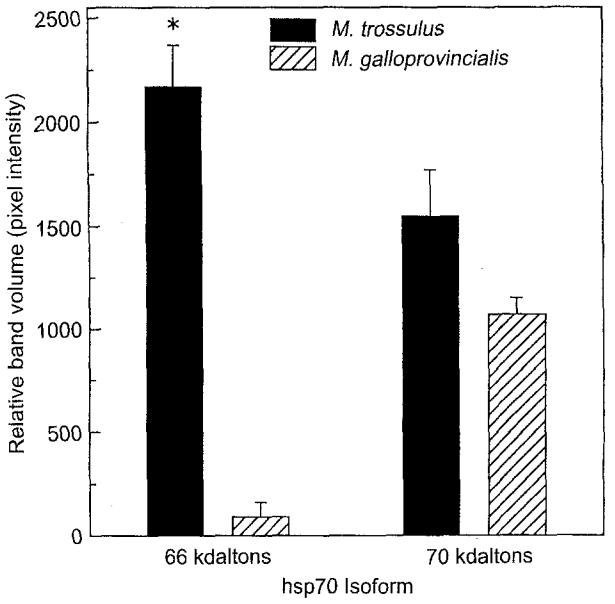

Fig. 2 *Mytilus trossulus* and *M. galloprovincialis*. Relative levels of hsp70 isoforms in gill tissue following exposure to 13 °C for 8 wk. Data are relative intensities of hsp70 isoforms determined from ECL western blots. Band intensities are expressed as pixel intensities measured by scanning laser densitometer. Because all samples were analyzed on a single western blot, no standardization of band intensities was required. Each column represents mean ± SEM (*bars*) of five mussels. *Significantly different from 66 kdalton hsp70 isoforms measured in *M. galloprovincialis* (unpaired *t*-test; $P < 0.05$)

protein, *M. galloprovincialis* also expressed a low molecular weight heat-shock protein of ≃30 kdaltons that was not synthesized in gill from *M. trossulus* (Fig. 3).

The temperature profiles for hsp70 synthesis in isolated gill tissue from *Mytilus trossulus* and *M. galloprovincialis* are shown in Fig. 4. The data were first standardized using the intensity of an internal control protein band (42 kdaltons), and then expressed relative to the amount of synthesis performed by the 13°C control-sample of gill tissue. Thus, as shown in Fig. 4, the 13°C sample value is equivalent to 1, and hsp70 synthesis for all other temperatures is expressed relative to this value.

A strong induction of hsp70 synthesis was first observed at 25 °C in gill from *Mytilus galloprovincialis* (Figs. 3b, 4). The synthesis of hsp70 relative to the 42 kdalton protein was even higher at 28 °C, and synthesis was still evident at 30 °C (Figs. 3b, 4). By comparison, isolated gill from *M. trossulus* exhibited a dampened induction profile (Fig. 4). The synthesis of hsp70 was first observed at 23 °C in *M. trossulus* (Fig. 3a). However, the intensity of the hsp70 band was much fainter than that observed in gill from *M. galloprovincialis* (Fig. 3) and the relative levels of hsp70 synthesis did not vary with temperature (Fig. 4). At the higher temperatures of 28 and 30 °C, the overall incorporation of radiolabeled amino acid into protein was somewhat lower in *M. trossulus* than in *M. galloprovincialis*. Visual inspection of the fluorograms in Fig. 3 shows a qualitative difference, where by the lanes containing the *M. trossulus* 28 and 30 °C samples display bands of reduced intensity compared to the lanes containing *M. galloprovincialis* samples. This observation was supported using TCA-precipitation assays to measure the amount of radioactive protein in the gill extracts. At 28 and 30 °C, *M. galloprovincialis* gill tissue incorporated 34 191 ± 7 857 and 22 057 ± 1 384 counts μg^{-1} protein (mean ± SEM; $n = 5$) into protein, respectively, compared to 21 098 ± 5 404 and 15 388 ± 3 328 counts μg^{-1} protein (mean ± SEM; $n = 4$) by gill tissue from *M. trossulus*. Although these data were not significantly different, they do suggest lower levels of incorporation by gill from *M. trossulus* at high temperatures.

Ubiquitin conjugate levels in gill tissue of mussels after 8 wk temperature exposure

Ubiquitin-conjugate levels in the gill tissue of *Mytilus trossulus* and *M. galloprovincialis* were measured using a Ub-conjugate standard curve ranging from 0.1 to 1.0 pmol Ub conjugates (data not shown). Dot-blotting of serially diluted samples showed that gill from *M. trossulus* contained significantly higher quantities of Ub conjugates than gill from *M. galloprovincialis* ($P \leq 0.05$; Student's *t*-test) following the 8 wk period at 13°C. Specifically, *M. trossulus* gill contained 0.105 ± 0.0102 pmol Ub conjugates μg^{-1} protein (mean ± SEM; $n = 5$), and *M. galloprovincialis* gill contained 0.067 ± 0.0035 pmol Ub conjugates μg^{-1} protein (mean ± SEM; $n = 5$). These measurements suggest that the 13 °C temperature regime for 8 wk resulted in a greater degree of irreversible protein damage in the northern species than in the southern species.

Discussion

Within the marine environment, there are numerous examples of species replacements along latitudinal gradients that are accompanied by differences in environmental temperature (Vernberg 1962; Fields et al. 1993). The present study was designed to examine potential physiological and biochemical underpinnings of the latitudinal separation of the *edulis*-like mussel congeners found on the Pacific coast of North America. Specifically, is the more northern-occurring species, *Mytilus trossulus*, more impacted by high temperature stress than its southern-occurring congener, *M. galloprovincialis*? The physiological determinants of the distribution patterns of the Pacific coast *edulis*-like mussels have not been thoroughly examined, but a few studies suggest that both adults and larval stages of the mussels of the *M. edulis* sister-species complex may have different physiological responses to temperature.

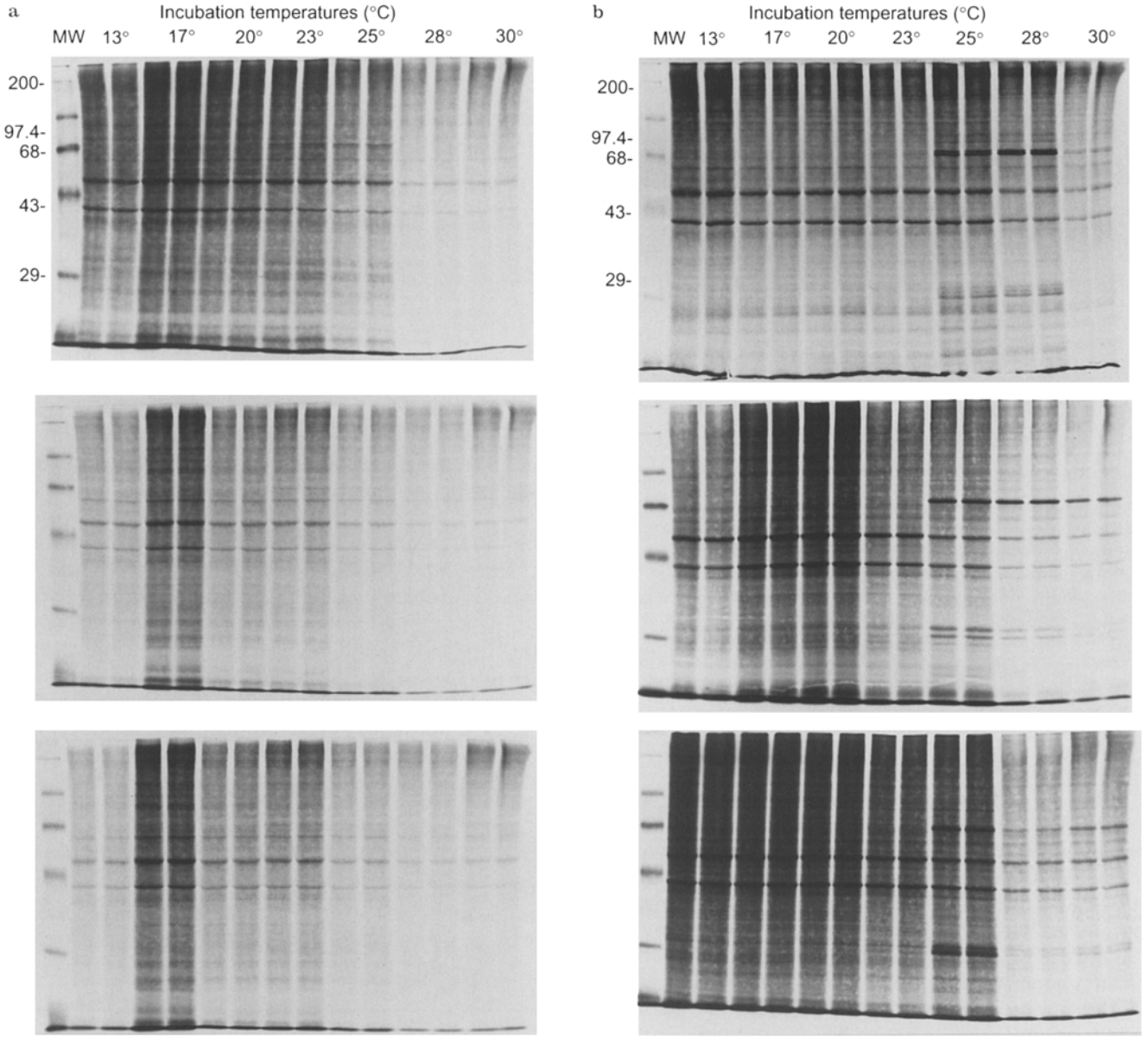

Fig. 3 *Mytilus trossulus* and *M. galloprovincialis*. Heat-shock protein-induction profiles in gills following exposure to 13 °C for 8 wk. Fluorograms from induction experiments on isolated gill tissue from three individuals of *M. trossulus* (**a**) and *M. galloprovincialis* (**b**) are shown, with ^{14}C-labeled protein molecular weight markers (*MW*) in kdaltons and gill samples incubated at temperatures shown. Extracts of gill, radiolabeled with ^{35}S methionine/cysteine amino-acid mixture (Trans-35; ICN Biomedicals) containing 600 000 cpm, were loaded in duplicate lanes and then electrophoresed on 10% polyacrylamide gel. Fluorograms shown were exposed to X-ray film for 15 h at −70 °C

Hilbish et al. (1994) have shown that adult *M. galloprovincialis* exhibited higher feeding and metabolic rates than *M. edulis* when held at 23 °C. Geller et al. (1994) have reported the occurrence of *M. galloprovincialis* larvae at latitudes where adults are not found, which suggests that thermal tolerances of the larvae of these congeners differ.

Interspecific comparisons of ectothermic species from different latitudes have shown variations in protein stability and function correlated with average habitat temperature (Graves and Somero 1982; Swezey and Somero 1982; McFall-Ngai and Horwitz 1990; Dahlhoff and Somero 1993; Lin and Somero 1995). In the current study, our approach was to determine whether differences in the latitudinal distributions of mussels were correlated with interspecific differences in thermal damage to proteins as indexed by the heat-shock response and formation of Ub conjugates. Three significant findings suggest that the *edulis*-like mussel congeners have different thermal sensitivites and that *Mytilus trossulus* is more sensitive to heat stress than *M. galloprovincialis*.

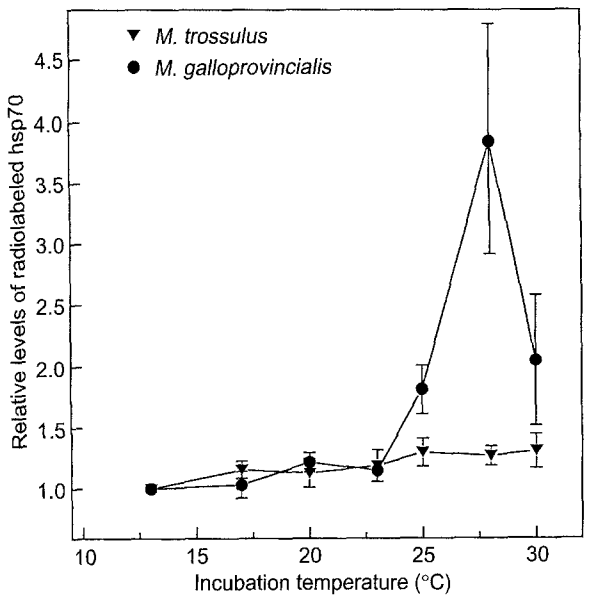

Fig. 4 *Mytilus trossulus* and *M. galloprovincialis*. Relative amounts of radioactivity incorporated into 70 kdalton band during induction experiments on isolated gill. Fluorograms were densitometrically scanned and intensities of 70 kdalton band were standardized with intensity of 42 kdalton internal comparison band. This value was then expressed relative to level of incorporation in 13 °C control incubation, such that value 1.0 represents equality with control incorporation levels after 2 h radiolabeling period. Each data point and bars represent mean ± SEM for $n = 4$ *M. trossulus* individuals and $n = 5$ *M. galloprovincialis* individuals

First, we measured significantly higher levels of hsp70 isoforms in gill from *Mytilus trossulus* as compared to *M. galloprovincialis* after the 8 wk exposure period (Fig. 2). The endogenous levels of the 66 kdalton hsp70 isoform was particularly enhanced in gill from *M. trossulus* relative to gill from *M. galloprovincialis* (Figs. 1, 2). The three-fold higher standing stocks of hsp70 isoforms in gill of *M. trossulus* suggest that this tissue was subject to greater degrees of protein denaturation at 13 °C than gill from the southern species, *M. galloprovincialis*.

It is important to note that the levels of hsp 70 (and ubiquitinated proteins; see below) that were observed after the 8 wk exposure period were a consequence of the thermal exposure to 13 °C in the laboratory. This conclusion is based on the following consideration. The half-lives of hsps in invertebrates and other organisms are short, typically on the order of 1 to 2d (Landry et al. 1982; Chen et al. 1990). Therefore, hsps turn over about as rapidly as other cellular proteins whose average half-lives have been shown to be ≃3 d (Beynon and Bond 1986). Thus, the levels of hsps measured at the end of the 8 wk exposure period are almost certainly due entirely to hsps newly synthesized during this period. The design of this experiment was to conduct an in vivo protein-denaturation experiment in which the two species were held under identical conditions and the resulting effects of this thermal stress on protein denaturation were quantified. An 8 wk exposure period was used to ensure that any hsp70 proteins present at the start of the experiment would be degraded before the hsp70 pools were measured at the end of the thermal exposure period.

Evidence from studies on other ectotherms is consistent with the differential effects of laboratory acclimation on hsp70 standing-stocks in the *edulis*-like mussels. Although a precise mechanism of regulation of hsp70 synthesis is unknown at this time, data from both laboratory and field studies indicate that (1) cellular concentrations of hsps vary with the thermal history of an organism, and (2) there are inter- and intraspecific differences in hsp standing-stocks (Somero 1995). In the laboratory, endogenous levels of hsp70 vary with temperature acclimation in ectothermic animals (Dietz and Somero 1992; Sanders et al. 1992; Wehner and Gehring 1995). Sanders et al. (1992) measured a 4- to 10-fold increase in hsp70 concentrations in *Mytilus edulis* (presumably *M. galloprovincialis*) during an 8 wk acclimation at 27 °C. Standing-stock levels of hsps have also been shown to be subject to seasonal acclimatization in the intertidal mussels *M. trossulus* (Hofmann and Somero 1995) and *M. californianus* (Roberts 1995), a eurythermal estuarine fish *Gillichthys mirabilis* (Dietz and Somero 1992), and freshwater teleosts (Fader et al. 1994). For natural populations of *M. trossulus*, the standing stock of hsp70 in the gill was significantly higher in summer-collected mussels than in winter-collected mussels (Hofmann and Somero 1995). However, at this time, more studies are required to determine whether species adapted to different temperatures have correspondingly different levels of heat-shock proteins.

At the biochemical level, one interpretation of the interspecific differences in hsp70 levels is that gill cells in *Mytilus trossulus* possess proteins that have intrinsically lower thermal stabilities than those of *M. galloprovincialis*. A strong positive correlation between protein thermal stability and evolutionary adaptation temperature has been shown for structural proteins and enzymes in a variety of ectothermic species (see Somero 1995 for review). Based upon these studies, *M. galloprovincialis* would be predicted to have a protein pool with a higher degree of structural stability than *M. trossulus*.

A second major finding of this study provides additional evidence that *Mytilus trossulus* and *M. galloprovincialis* have protein pools with different thermal stabilities and, additionally, shows that the congeners may face different energy costs in coping with heat stress. Elevated quantities of Ub conjugates in gill from *M. trossulus* compared to levels in gill from *M. galloprovincialis* suggest that the northern species had a higher degree of Ub-mediated protein degradation after an 8 wk period at 13 °C (see third subsection of Results), consistent with the hypothesis of lower protein stability in the more northern species. Increased

protein turnover as a result of heat stress could add appreciably to an organism's energy costs (see end of "Discussion").

As in the case of hsp70, the turnover of ubiquitinated proteins is rapid enough to ensure that any ubiquitinated proteins present at the start of the experiment would be degraded long before the end of the 8 wk exposure period. We have found that levels of ubiquitinated proteins vary significantly during the course of the day, in conjunction with tidal cycle effects (Hofmann and Somero 1996). Thus, levels of ubiquitinated proteins rise rapidly after reimmersion at high tide, and decrease to pre-emersion levels within several hours. The rapidity of the turnover of ubiquitinated proteins ensures that the amounts of ubiquitin conjugates present at the end of the 8 wk experiment were the consequence of thermal exposure during this period.

The basic understanding of the role of Ub in protein degradation during heat stress has come from studies of cultured cells. For example, in chicken fibroblasts, concentrations of Ub conjugates increase dramatically, from 3.2 to 7.8 pmol per 10^6 cells, after heat treatment at 45 °C for 60 min, and protein turnover increases once these cells are in recovery (Bond et al. 1988). Our studies are the first to use Ub conjugates as in index of thermal damage in situ (Hofmann and Somero 1995). We have asked whether mussels in nature experience variations in protein degradation based upon temperature changes in their environment, i.e. on a seasonal basis or as a function of microhabitat. Results from field studies conducted in the San Juan Archipelago (Puget Sound, USA) on *Mytilus trossulus* have shown that gill from summer-collected mussels have six-fold higher concentrations of Ub conjugates than gill from winter-collected mussels (Hofmann and Somero 1995). Mussel body-temperatures in summer ranged from 10 to 35 °C during emersion at low tide, whereas winter body-temperatures were on average between 5 and 10 °C. In addition, data from field populations of the rocky outer-coast mussel *M. californianus* show that Ub-conjugate concentrations correlated with tidal height; mussels collected from higher sites that receive more aerial exposure have elevated levels of Ub conjugates (Hofmann unpublished observation). We conclude that levels of ubiquinated proteins are strongly dependent on thermal exposure, and that interspecific differences in protein thermal stability and intraspecific differences in microhabitat conditions influence protein turnover costs.

As the third finding, in vitro radiolabeling experiments showed that gill from *Mytilus trossulus* and *M. galloprovincialis* varied in the patterns of stress-protein expression over a temperature range from 13 to 30°C (Figs. 3,4). *M. galloprovincialis* gill consistently displayed initial synthesis of hsp70 at 25 °C, strong induction at 28 °C, and continued expression of hsp70 at 30 °C (Fig. 3b). In contrast, isolated gill from *M. trossulus* displayed a much reduced stress-protein induction response (Fig. 3a). Hsp70 synthesis was detectable at 23 °C in all specimens examined, but the amount of synthesis and the preferential synthesis over other normal cellular proteins, was never as strong as in gill from *M. galloprovincialis* (see Fig. 3).

Similar to standing-stock concentrations of stress proteins, the induction temperature for stress-protein expression is not a genetically fixed trait for a species and has been shown to be subject to acclimation and acclimatization (Somero 1995). There are relatively few studies that have examined the induction patterns of hsps in natural populations of organisms (Dietz and Somero 1992; Dietz 1994; Sharp et al. 1994; Roberts 1995). Nonetheless, evidence from these organismal studies shows that as an animal is cold-acclimated/acclimatized, the induction temperature for stress protein expression decreases and, during warm-acclimation/acclimatization, the induction temperature increases. Thus, temperature acclimation/acclimatization effectively alters the set-point of the cellular thermometer and changes the temperature required to induce the synthesis of stress proteins.

In this study, the nature of stress-protein induction and the expression patterns for *Mytilus trossulus* and *M. galloprovincialis* were different after both species were given the same thermal exposure. First, the hsp70-induction profile was significantly dampened in *M. trossulus* compared to *M. galloprovincialis* (Fig. 4). The common exposure temperature was at opposite extremes of the habitat temperature range for each species. Exposure to 13 °C is probably in the upper range of seawater temperatures that the northern species, *M. trossulus*, would experience, and is in the lower range of seawater temperatures for the southern species, *M. galloprovincialis*. Elevated levels of endogenous hsp70 in *M. trossulus* gill tissue directly suggested that the 8 wk exposure to 13 °C represented a significant, albeit sublethal heat stress for the northern species. This may have contributed to the dampened expression of hsp70 during the induction experiments (see below). A reduced ability to activate the stress response has been observed in organisms exposed to chronic heat-stress conditions (e.g. Howarth 1991). However, despite the 8 wk period at a relatively warm temperature, the northern species did not induce stress-protein synthesis at a higher temperature than *M. galloprovincialis*. Second, *M. galloprovincialis* synthesized a 30 kdalton protein not observed in gill from *M. trossulus* following the 8 wk exposure period (Fig. 3). It should be noted that *M. trossulus* may be capable of expressing the 30 kdalton protein under different thermal conditions and our results should not be interpreted as representing the absolute hsp-expression pattern for this species. However, for the purposes of our study, this was a significant observation: the two species displayed different hsp-expression patterns under the temperature regime used in the experiment. In the literature, studies of

differential stress-protein expression in other closely related species have shown that the more thermotolerant organism tends to synthesize a greater variety of stress proteins. In two species of the fresh water cnidarian *Hydra*, the more thermotolerant species, *H. attenuata*, synthesizes hsp60, whereas the more thermosensitive species, *H. oligoctis*, does not (Bosch et al. 1988). Limpets of the genus *Collisella* show a similar pattern, where by *C. scabra*, which is found high in the intertidal zone and is exposed to greater degrees of heat stress, expresses more isoforms of hsp70 and hsp60 than does *C. pelta*, a more thermosensitive species that occurs lower in the intertidal (Sanders et al. 1991).

The reduced intensity of synthesis of hsp70 in gill from *Mytilus trossulus* compared to *M. galloprovincialis* was a striking difference in the protein-synthesis patterns in the induction experiments. One hypothesis that might account for the dampening of hsp70 induction is the "cellular thermometer" model for the regulation of stress-protein expression (Craig and Gross 1991). According to this model, an hsp such as hsp70 negatively regulates heat-shock gene expression via a negative feedback loop, and essentially controls its own expression. Under normal cellular conditions, hsp70 is thought to bind to the heat-shock factor (HSF) and prevent this transcriptional factor from activating heat-shock gene expression (Morimoto 1993; Morimoto et al. 1994a, b). During heat shock, hsp70 releases HSF and binds to the increasing number of denatured and misfolded proteins in the cell. Released HSF then activates heat-shock gene transcription and increases the pool of free hsp70, which at some point will reach a threshold high enough to re-sequester HSF into hsp70-HSF complexes and feedback to terminate its own expression. Taken together, the events above describe a mechanism where by hsp70 controls the transcription of heat-shock genes and couples the expression of stress proteins to the functional state of the cell and its requirements for molecular chaperones to deal with protein damage. Thus, cumulative heat-shock events will increase pools of free hsp70 in cells and, hypothetically, alter the nature of the stress response and the temperature required for induction of stress-protein expression (Dietz and Somero 1992; Morimoto 1993). In the light of the model, the elevated levels of hsp70 in *M. trossulus* gill cells may account for the reduced intensity of hsp70 expression in response to in vitro heat shock.

In conclusion, we have shown that *Mytilus trossulus* and *M. galloprovincialis* vary significantly in stress-protein expression and Ub-mediated protein degradation when given a common temperature exposure. In our studies of intertidal marine invertebrates, we have hypothesized that environmentally-induced protein damage may play a role in setting species' distribution limits by impacting organismal energy budgets (Hofmann and Somero 1995). In heat-stressed organisms, cellular energy demands may increase dramatically due to the energy requirements associated with replacing damaged proteins and maintaining stocks of stress proteins. Exaggerated energy costs induced by environmental heat stress would negatively impact a species' energy budget and result in fitness consequences because of reduced energy available for growth and reproduction (Krebs and Loeschcke 1994a, b). As a consequence, thermal denaturation and the associated costs of restoring the native protein pool could be an important component of the cost of thermal-habitat selection (Hawkins 1991; Huey 1991) and therefore contribute to setting species' distribution patterns. The results of our investigation provide evidence that environmentally-induced protein damage and the related energetic and fitness consequences may contribute to the observed geographical separation of adult forms of the congeneric mussels *M. trossulus* and *M. galloprovincialis*.

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