Patterns of Variation in Levels of Hsp70 in Natural Rocky Shore Populations from Microscales to Mesoscales[1]

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Synopsis.   An important step in connecting the organismal response to thermal stress to patterns of community structure is determining at what scale discernable levels of variation are manifested. The temperature signal to which organisms may potentially respond varies at many spatial scales including microhabitat, tidal height, site and latitude. A number of studies have taken physiological assessment of the heat shock response (HSR) into the intertidal both as a tool for examining the HSR in nature and for examining the utility of HSR molecules as population or community level indicators. Most commonly, immunodetection of the total pool of the Hsp70 family of isoforms is used. Here we present data on levels of Hsp70 in intertidal organisms from microhabitat to the mesoscale. Our data and previously published work show that Hsp70 levels vary at all scales examined, similar to other physical and biological variables of interest. This demonstrates both the potential utility of Hsp70 detection as a molecular tool for field biologists and to the care that must be taken in assessing scale of variation when looking for potential bioindicator molecules.

INTRODUCTION

The heat shock response (HSR) has been studied extensively in the laboratory since its description in 1962 (see Lindquist, 1986). More recently, physiologists have begun examining the function of the HSR in the natural environment and in non-model organisms (see Feder and Hofmann, 1999 for review). Similarly, ecologists have long recognized the important role that temperature plays in structuring intertidal communities (Orton, 1929a, b; Doty, 1946; Hutchins, 1947), and have more recently gained access to improved molecular tools to examine physiological responses to thermal stress. An important step in connecting the organismal response to thermal stress to patterns of community structure is determining at what scale discernable levels of variation are manifested. The temperature signal to which organisms may potentially respond varies at many spatial scales including microhabitat, tidal height, site, and latitude, and many temporal scales, including tidal, daily, and seasonal. A number of studies have taken physiological assessment of the HSR into the intertidal both as a tool for examining the HSR in nature and to examine the utility of HSR molecules, the heat shock proteins, as population or community level bioindicators.

Heat shock proteins (Hsps) are molecular chaperones which, in general, assist in protein folding and the prevention of cytotoxic aggregations resulting from improperly folded proteins adhering to one another (Parsell and Lindquist, 1993; Hartl, 1996; Fink, 1999).

Hsps can be present constitutively within cells and perform many protein folding functions, including both folding of new proteins and re-folding of damaged proteins. In contrast, inducibly expressed proteins are up-regulated in response to protein damage. This damage may occur in response to anything that will cause protein denaturation including thermal stress, osmotic stress, and chemical damage (Lindquist, 1986). On exposed rocky shores, osmotic and chemical stress is minimal, therefore temperature is thought to be the primary cause of the induction of Hsps.

Temperature and desiccation stress are both strongly implicated in regulation of populations and community structure on rocky shores, particularly in setting the upper intertidal height at which organisms can survive (Connell, 1961; Jenik and Lawson, 1967; Paine, 1974). As such, species and individuals surviving at higher tidal heights experience more time out of water and risk exposure to elevated temperatures. Considerable variation can take place at smaller and larger spatial scales as well. At a smaller scale, microhabitat variation, such as the differences in shore slope and aspect, can result in significant temperature variation (Wethey, 1984; Williams and Morritt, 1995; Helmuth and Hofmann, 2001). At the mesoscale (10–100s of kilometers), local climate can vary significantly, causing altered body temperatures on the rocky shore (Helmuth, 1999). On a latitudinal scale, temperature might be expected to decrease with increasing latitude, but the nature of that decrease will depend greatly on the timing of tidal exposure (Denny and Paine, 1998).

Recent physiological work on rocky shore organisms has shown that species living in different tidal zones show functional variation in their HSR (Tomanek and Somero, 1999, 2000). How individuals within species vary in their expression of Hsps and at what scale is also of great importance to ecologists. Given that temperature can vary at spatial scales from cen-

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timeters to 100s of kilometers, it is useful to ask whether physiological indices of thermal stress vary at the same scales. Such data are necessary in order to explore the function of the HSR in the natural environment and to explore the utility of physiological measures as possible bioindicators.

Immunodetection of the total pool of the Hsp70 family of isoforms via western blotting is an increasingly popular method for assessing the HSR of individuals in natural populations. Here we present the results from three studies measuring Hsp70 levels in intertidal organisms as examples and review some published studies of within species variation in Hsp expression done on intertidal organisms.

For the first study, variation of Hsp levels between microhabitats was examined in the New Zealand intertidal limpet, *Cellana ornata*. *C. ornata* inhabits the rocky shores of the South Island of New Zealand. On broad rocky benches of Box Thumb, south of Christchurch, NZ, *C. ornata* was observed to crowd into crevices during low tides, though many individuals remained on broad flat rock surfaces. Since crevices may provide a refuge from thermal stress, collections were made of individuals from both crevices and flat areas of the rock to determine if individuals using crevice habitats had lower levels of Hsp70 than those using flat areas of the rock during low tide.

The two remaining studies were conducted on rocky shores in central Oregon. Previous work has established that important ecological and physiological variation can occur at the "mesoscale" of 10s to 100s of kilometers (Bustamante *et al.*, 1995; Menge *et al.*, 1997*a*, *b*). Sites that may be geographically within a defined terrestrial or oceanographic ecoregion may experience dramatically different oceanic and climatic conditions. For example, Boiler Bay and Strawberry Hill, OR differ in nearshore water column productivity, strength of consumer effects, and community structure (Menge *et al.*, 1997*a*, *b*). The California mussel, *Mytilus californianus*, which dominates the mid intertidal, grew faster at Strawberry Hill than at Boiler Bay (Menge *et al.*, 1994) and had higher protein synthetic capacity (Dahlhoff and Menge, 1996). At Strawberry Hill, whelks had higher body temperatures and higher Hsp70 pools than those at Boiler Bay (Dahlhoff *et al.*, 2001). Air temperatures recorded from data loggers in the low zone indicate Strawberry Hill air temperatures were generally higher than those at Boiler Bay, creating the *a priori* expectation that Strawberry Hill may be a more thermally stressful site than Boiler Bay.

In order to assess variation in Hsp expression within the mussel bed, among tidal heights within the bed, between sites, and between sampling dates, we performed a nested sampling for *M. californianus*, during July and August 1999. In addition to measuring levels of Hsp70 isoforms, the levels of ubiquitin conjugates were also analyzed. Ubiquitin targets irreversibly damaged proteins which are subsequently degraded by the proteasome, whereas inducible forms of Hsp70 target proteins for re-folding and rescue of function (Rechsteiner, 1987; Ciechanover, 1998; Buckley *et al.*, 2001). We analyzed both in order to compare two potential biomarkers of organismal stress (Hofmann and Somero, 1995). Collections were made in July and August, to obtain more than one sample from a warm period of the year. We predicted that Hsp levels would be higher at Strawberry Hill than Boiler Bay, and higher in the upper portion of the mussel bed.

In the last study, collections of the finger limpet, *Lottia digitalis*, were made over 26 mo from the high and mid intertidal at Boiler Bay and Strawberry Hill to compare Hsp70 levels between tidal zones and among seasons. In these collections we predicted that Hsp70 levels would be higher in summer months than in winter and higher in the high intertidal zone than in the mid.

These studies along, with the previous work, elucidate variation at scales of centimeters to 10s of kilometers. Work at the latitudinal scale has not been done for Hsps on rocky shore organisms, although work from terrestrial systems indicate that such variation should exist in these populations as well (see Feder and Hofmann, 1999 for review). We present these data and a brief literature review to describe variation at the between microhabitat, within zone, between zone and between site scale on the rocky shore.

Materials and Methods

New Zealand collections

New Zealand collections were made at "Box Thumb," a broad, rocky bench on the northern side of the Banks Peninsula, near Christchurch, NZ (approx. 43°34′S, 172°48′E). Specimens of the limpet *C. ornata* were collected from open and crevice habitats in the high zone on the wave-protected area of the bench. *C. ornata* were dissected in the field, flash frozen, and stored at −80°C. Tissue was transported to Oregon State University (Corvallis, OR) on dry ice and stored at −80°C prior to analysis.

C. ornata foot tissue was homogenized in 5×50 mM Immidazole (pH 7.2), 2 mM EDTA, 1 mM PMSF. Homogenate was then mixed 1:1 with 64 mM Tris HCl, 4% SDS (pH 6.8). Gel electrophoresis, transfer to nitrocellulose membrane, and western blot analysis was performed as described in Helmuth and Hofmann (2001, after Hofmann and Somero, 1995) for detection of both cognate and inducible forms of Hsp70, except for the following. Each gel also contained a sample of purified Hsc70 (5 μg of bovine Hsc70; Stressgen) as a standard and positive control, and prestained molecular weight markers (kaleidoscope prestained standards, broad range; Biorad). Western blots were developed using SuperSignal chemiluminescent substrate according to manufacturer's instructions (Pierce). The resulting images on X-ray film were densitometrically scanned on a Molecular Dynamics Personal Densitometer. Band intensity was quantified using ImageQuaNT software (Molecular Dynamics). Intensity of sample

bands was divided by intensity of Hsc70 standard to allow comparison between gels.

All variables were log transformed prior to analysis to improve normality. *C. ornata* data were divided into a high molecular weight isoform (HMW), and a low molecular weight isoform (LMW). Data were analyzed via a *t*-test between crevice and flat collections.

Oregon collections

Oregon collections were conducted at two sites, Boiler Bay (44°49′48″N, 124°3′36″W, NAD27), and Strawberry Hill (44°15′N, 124°6′36″W, NAD27). Both consist of broad rocky benches. The mid zone at both sites is dominated by the California mussel, *M. californianus*. The high zone at both sites is dominated by acorn barnacles (*Balanus glandula* and *Chthamalus dalli*) and fucoid algae, with higher cover of algae at Boiler Bay and higher acorn barnacle cover at Strawberry Hill (Menge, 1992; Menge *et al.*, 1994, 1997*a*, *b*).

During July and August 1999 (July 16, both sites; Aug. 25, Boiler Bay; Aug. 26, Strawberry Hill) we performed a nested sampling for *M. californianus*, in order to assess variation in Hsp expression within the mussel bed, among tidal heights within the bed, between sites, and between sampling dates. In moderately wave-exposed areas, three vertical transects were laid down and five mussels collected from ''low-bed,'' ''mid-bed,'' and ''high-bed'' portions of the mussel bed (considered to biotically define the mid-intertidal zone, Menge *et al.*, 1994, 1997*a*, *b*) in each transect. Low-bed and high-bed levels were considered the high and low edge of the mussel bed, respectively. Mid-bed areas were determined by surveying to a height exactly between the high-mid and low-mid collections. Collections were made twice at Boiler Bay and Strawberry Hill in July and August. Specimens were transported live on ice to the laboratory, and dissected immediately. Gill tissue was flash frozen on dry ice and stored at −80°C until processed for analysis.

M. californianus tissue homogenate was prepared according to the methods of Roberts *et al.* (1997). Western blots for detection of Hsp70 levels were conducted as with *C. ornata*.

Ubiquitin conjugate analysis methods were modified from Hofmann and Somero (1995). Homogenates were diluted with phosphate buffered saline (PBS: 10 mmol/l sodium phosphate, 150 mmol/l NaCl, pH 7.4) to a concentration of 1 μg protein/ml, so that the signal generated by the immunochemical assay was within the linear range of the associated ubiquitin conjugate standard curve (ubiquitin conjugate provided by Dr. Arthur L. Haas). Using a dot blot apparatus (Biorad), ubiquitin conjugate standards and samples (in triplicate, 0.1 μg of sample protein) were applied to a nitrocellulose membrane (0.45 micron) that had been pre-hydrated for 1 hr in distilled water. Sample volumes were allowed to pass through the nitrocellulose via gravity flow, then washed twice under gentle vacuum with PBS. After washing, the blots were heat fixed at 70°C for 20 min. Ubiquitin conjugates were detected using a polyclonal rabbit anti-ubiquitin conjugate antibody.

Immunodetection of Ubiquitin conjugates was performed using an enhanced chemiluminescence protocol. Membranes were rehydrated and incubated for 1 hr in blocking solution (5% nonfat dried milk in PBS). Membranes were subsequently incubated in a primary antibody solution composed of polyclonal rabbit anti-ubiquitin conjugate antibody (provided by Dr. Arthur L. Haas) diluted 1:2,000 in blocking solution. After incubation in primary antibody solution, membranes were washed in Tris-buffered saline (TBS: 50 mM Tris-HCL, 150 mM NaCl, pH 7.5) for 5 min, then twice in 0.05% Triton X-100 in TBS, and TBS for 5 min. The presence of primary antibody was detected by a 1 hr incubation in horseradish peroxidase (HRP)-conjugated goat IgG (H+L) anti-rabbit antibody (Biorad; diluted 1:3,000 in blocking solution) and washed as above. Final detection was performed using chemiluminescence (SuperSignal, Pierce) and scanned using a Fluor-S imager (Biorad). Ubiquitin conjugate levels were then determined by comparison to a standard curve based on serial dilutions of a ubiquitin conjugate standard control. Preliminary regression analysis demonstrated a slight but significant relationship between ubiquitin conjugate levels, the hmw isoform of Hsp70 and body size, the range of which was broader than in other collections described (3.8–8.8 cm). In order to account for size variation, for both Hsp and ubiquitin conjugate levels, results were normalized to body weight estimated from rostral-caudal length. Previous collections at both sites had established a length weight relationship of log (weight) = −0.9782 + 2.83582 log (length), with $r^2 = 0.995$, and no differences between sites (unpublished data, P. M. H.). Protein amounts were divided by weight as calculated from the previous equation.

Collections of the finger limpet, *Lottia digitalis*, were made 6 times from July 1996 to September 1998. Due to dangerous wave action on the Oregon coast, collections were not made consistently during the winter. Collections were classified as winter/spring (Dec. to early April) or summer/fall (July to early Sept.). Collections were at low tide and usually within 24 hr of each other when done at separate sites except for summer 96 (6 d separation) and winter 96 (48 H). *L. digitalis* from 0.7–1.3 cm were collected haphazardly from non-crevice, non-mussel bed areas in the mid and high zones. Samples were collected in two ways, either foot tissue was removed in the field and flash frozen on dry ice, or whole limpets were flash frozen on dry ice in the field. In both cases samples were stored at −80°C until processed for analysis.

L. digitalis foot tissue was homogenized in 5 × 50 mM Immidazole (pH 7.2), 2 mM EDTA, 1 mM PMSF. Homogenate was then mixed 1:1 with 64 mM Tris HCl, 4% SDS (pH 6.8). Protein assays and western blots were conducted as with *C. ornata* and *M. californianus*, as described above for Hsp70.
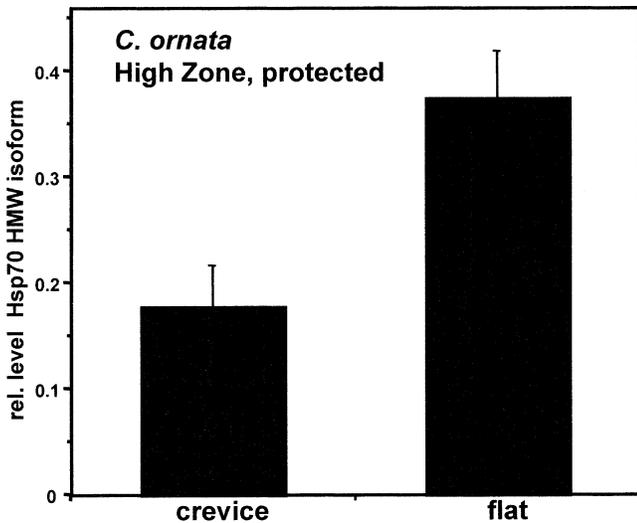
FIG. 1.   Relative level of Hsp70 HMW isoform in *C. ornata* collected from flat and crevice habitats at Box Thumb, New Zealand. Bars indicate standard error.

TABLE 1.   *Analysis of variance of weight normalized levels of the 72 kDa isoform of Hsp70 by site (Boiler Bay and Strawberry Hill), collection (July and August), and tide-level (high-bed, mid-bed, and low-bed). Bold indicates $P \leq 05$. Model $r^2 = 0.620572$.*

Source of variation	df	F ratio	P
Site	1	2.6442	0.1168
Collection	1	18.6382	**0.0002**
Tide-level	2	6.6704	**0.0049**
Site × collection	1	3.3547	0.0792
Site × tide-level	2	8.0327	**0.0021**
Collection × tide-level	2	0.1336	0.8756
Site × collection × tide-level	2	3.5829	**0.0432**
Transect(tide-level, site, coll)	24	2.3577	**0.0012**

fashion as Hsp data. To examine the inter-relationship between the isoforms of Hsp70 and ubiquitin conjugate levels, the 72 kDa and 75 kDa data were regressed against the ubiquitin conjugate data for each collection period, July and August. Visual inspection of residuals was conducted to assess normality of all data.

All variables were log transformed prior to analysis to improve normality, and all analyses were run using JMP software (SAS Institute). *L. digitalis* data were analyzed in an ANOVA with site (Boiler Bay or Strawberry Hill), season (winter/spring and summer/fall), and zone (high or mid) as fixed effects, and log weight-normalized relative amount of Hsp as the dependent variable. Dependent variables for *M. californianus* data were divided into a high molecular weight band (approx. 75 kDa) and a low molecular weight band (approx. 72 kDa, Helmuth and Hofmann, 2001). Neither set of data were normal after transformation. *M. californianus* data were analyzed in an ANOVA with site (Boiler Bay and Strawberry Hill), collection (July and August), and tide-level (high-bed, mid-bed, and low-bed) as fixed effects. Transect nested within zone, site, and collection was treated as a random effect. Ubiquitin conjugate data were analyzed in the same

RESULTS

New Zealand collections

Two bands near 70 kDa were detected and visualized with Hsp70 antibodies on western blots of tissue from *C. ornata*. Higher amounts of the HMW isoform of Hsp70 occurred in *C. ornata* in open flat areas than in limpets in crevices (Fig. 1, *t*-test, $P = 0.0036$). In contrast, amounts of the LMW isoform did not differ between limpets in crevice or open flat microhabitats (*t*-test, $P = 0.7806$).

Oregon collections

In *M. californianus*, the 72 kDa isoform levels showed high amounts of variability between collections, tide-levels, sites, and transects (Fig. 2). Results indicate effects of collection (Table 1, ANOVA, $P = 0.0002$), tide-level ($P = 0.0049$), site × tide-level ($P = 0.0021$), site × tide-level × collection ($P = 0.0432$) and transect ($P = 0.0012$). 72 kDa isoform levels from
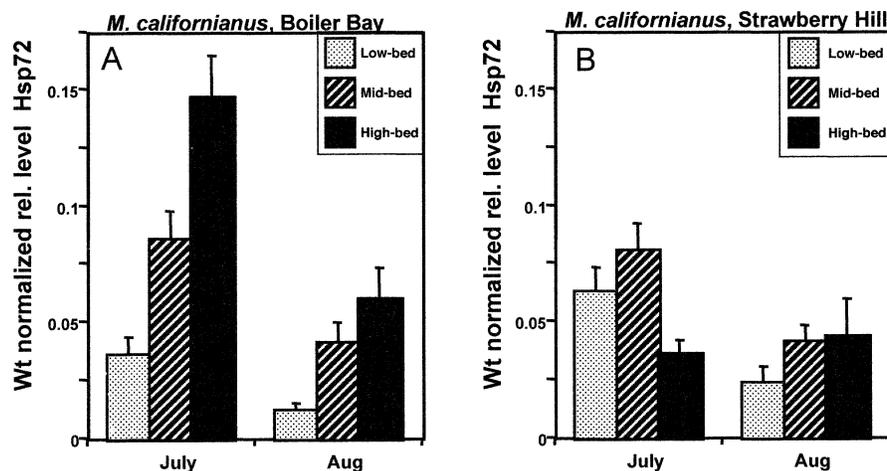

FIG. 2.   Weight-normalized relative level of Hsp72 in *M. californianus* by collection month at (A) Boiler Bay, OR and (B) Strawberry Hill, OR. Bars indicate standard error.

TABLE 2. *Analysis of variance of weight normalized levels of the 75 kDa isoform of Hsp70 by site (Boiler Bay and Strawberry Hill), collection (July and August), and tide-level (high-bed, mid-bed, and low-bed). Bold indicates $P \leq 0.05$. Model $r^2 = 0.565658$.*

Source of variation	df	F ratio	P
Site	1	0.2398	0.6287
Collection	1	13.1186	**0.0013**
Tide-level	2	3.3414	0.0522
Site × collection	1	1.4443	0.241
Site × tide-level	2	0.2257	0.7996
Collection × tide-level	2	1.976	0.1603
Site × collection × tide-level	2	2.6374	0.0919
Transect(tide-level, site, coll)	24	3.1091	**<0.0001**

TABLE 3. *Analysis of variance of weight normalized levels of ubiquitin conjugate by site (Boiler Bay and Strawberry Hill) and tide-level (high-bed, mid-bed, and low-bed) for the July collection. Bold indicates $P \leq 05$. Model $r^2 = 0.630801$.*

Source	df	F ratio	P
Site	1	0.2184	0.6410
Collection	1	8.6599	**0.0038**
Tide-level	2	57.5406	**<0.0001**
Site × collection	1	13.3216	**0.0004**
Site × tide-level	2	11.8750	**0.0001**
Collection × tide-level	2	1.976	0.2616
Site × collection × tide-level	2	22.6366	**<0.0001**
Transect(tide-level, site, coll)	24	5.4926	**<0.0001**

Boiler Bay high-bed in July were higher than those in the mid-bed and low-bed (Tukey-Kramer $P < 0.05$). Similarly, 72 kDa isoform levels from Boiler Bay low-bed in July were lower than those in the mid-bed and high-bed (Fig. 1, Tukey-Kramer $P < 0.05$). Within a site and collection, no other tide-levels were different from any other. The 75 kDa isoform also showed considerable variability with effects of collection (Table 2, Fig. 3, ANOVA, $P = 0.0013$) and transect ($P < 0.0001$).

Ubiquitin conjugate levels varied in a similar fashion as the 72 kDa isoform with significant effects of collection (Table 3, Fig. 4, ANOVA, $P = 0.0038$), tide-level ($P > 0.0001$), site × collection ($P = 0.0004$), site × tide-level ($P = 0.0001$), site × collection × tide-level ($P < 0.0001$), and transect ($P < 0.0001$). Ubiquitin conjugate levels at Boiler Bay high-bed, both July and August, and Strawberry Hill high- and mid-bed in August were significantly higher than other site, collection, tide-level combinations. Levels of the 72 kDa in both July and August were correlated with ubiquitin conjugate levels (regression $r^2 = 0.5983$, $P < 0.0001$ and $r^2 = 0.5570$, $P < 0.0001$, respectively),

indicating that the ubiquitin conjugate levels and relative amounts of 72 kDa isoform give comparable measures of organismal response. The 75 kDa isoform was also significantly correlated with ubiquitin conjugate levels in both July and August, but to a lesser degree ($r^2 = 0.3069$, $P < 0.0001$ and $r^2 = 0.3055$, $P < 0.0001$). Inspection of the residuals in the relative level of 75 kDa isoform vs. ubiquitin conjugate level indicate that 75 kDa levels are higher than predicted by ubiquitin conjugate levels at Boiler Bay mid-bed, and lower than predicted at Strawberry Hill low-bed and high-bed in July. In the August collection, residuals indicate that Strawberry Hill mid-bed and Boiler Bay high-bed levels of the 75 kDa isoform were lower than predicted by ubiquitin conjugate levels.

Hsp70 levels in *L. digitalis* varied with season, site and zone (season × site and season × zone interactions (Fig. 5). Relative levels of Hsp70 varied with site (ANOVA, $P = 0.044$), season ($P < 0.0001$), zone ($P < 0.0001$), site × season ($P = 0.0003$), and season × zone ($P = 0.0063$). Relative Hsp70 levels were highest in summer months, with amounts at Boiler Bay in the summer occurring at higher levels than at Boiler Bay
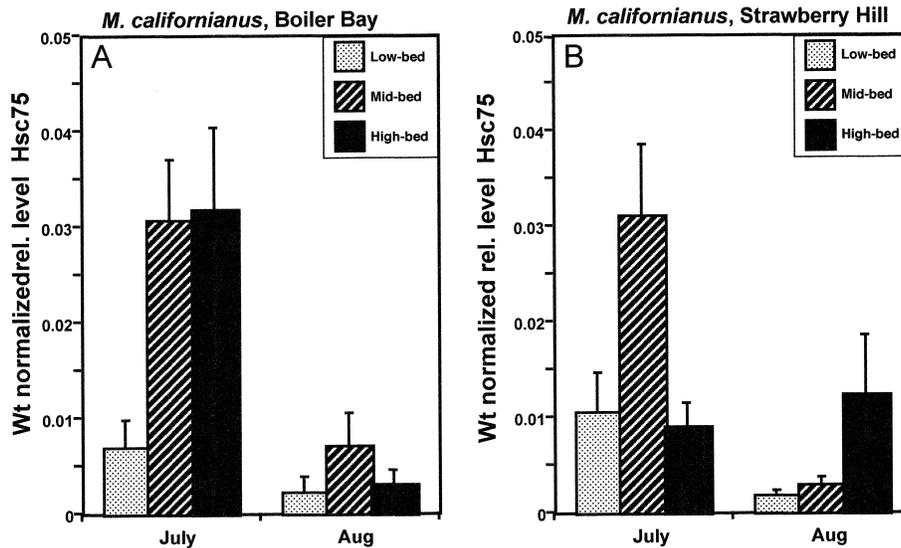

FIG. 3. Weight-normalized relative level of Hsc75 in *M. californianus* by collection month at (A) Boiler Bay, OR and (B) Strawberry Hill, OR. Bars indicate standard error.
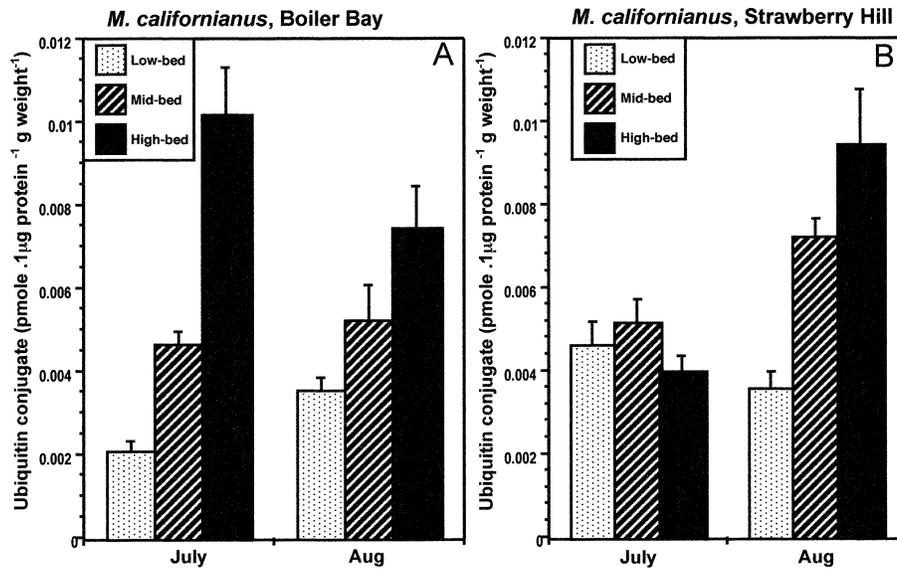
M. californianus, Boiler Bay

M. californianus, Strawberry Hill

FIG. 4.　Weight-normalized concentrations of ubiquitin conjugate in pmoles/.1 μg of protein for the July collection at Boiler Bay and Strawberry Hill, OR. Bars indicate standard error.

in the winter. Hsp70 summer levels at Boiler Bay were also higher than at Strawberry Hill in both summer and winter (Tukey-Kramer, $P < 0.05$). Looking at the season × zone interaction, *L. digitalis* collected from the high zone in summer/fall, had higher Hsp70 levels than those collected from the mid zone in summer/fall, and both mid and high zones in winter/spring (Tukey-Kramer, $P < 0.05$).

DISCUSSION

Microhabitat variation

The rocky shore contains a variety of microhabitats, such as tidal pools *vs.* emergent habitats, tops *vs.* undersides of rocks, and use of biotic habitat-forming species such as mussel beds and algal canopies. An example of differential physiological response to such small-scale variation in conditions was seen in the New Zealand limpet, *C. ornata*. Of the two Hsp70 isoforms distinguishable on a western blot, only one, the HMW isoform, varied significantly between crevice and open flat microhabitat areas. Levels were higher in the open, flat area population than in the crevice population (Fig. 1, *t*-test, $P = 0.0036$), consistent with the expectation that these areas are more thermally stressful. The lack of difference in the LMW isoform, indicates that the two isoforms may differ in expression patterns (*e.g.,* inducibly *vs.* constitutively expressed), and thus potentially in function. These data, though promising some interesting insights into microscale physiology of *C. ornata*, are preliminary. Little is known about either the ecology or the heat shock response in this limpet and more work on both would be necessary to establish the functional significance of our observations. Variation in expression and function of Hsp70 isoforms can severely hamper ecological interpretation of results.

Mussels in the genus *Mytilus* are better studied, both from an ecological and a physiological standpoint (Hofmann and Somero, 1995, 1996*a*, *b*; Simpfendorfer *et al.*,1995; Dahlhoff and Menge, 1996; Navarrete and Menge, 1996; Arifin and Bendell-Young, 1997; Roberts *et al.*, 1997; Helmuth, 1998, 1999; Helmuth and Hofmann, 2001). Over the course of a year, Helmuth and Hofmann (2001) examined variation in *M. californianus* between two emergent intertidal habitats expected to differ in thermal stress: a north facing vertical rock face, and a horizontal rock face. These emergent habitats were compared to a continuously submerged tidepool population. They combined measurement of Hsp70 pools with accurate thermal models of *M. californianus* body temperature. For the 72 kDa isoform present in mussels, inferred to be an inducible isoform from prior work (hereafter Hsp72, Hofmann and Somero, 1995; Roberts *et al.*, 1997; Helmuth and Hofmann, 2001), results revealed substantial variation among microhabitats, with the north facing population levels of Hsp72 consistently lower than the horizontal face. The tidepool population did not have lower levels of Hsp72, but neither was it the thermal refuge that was predicted. The 75 kDa isoform, inferred to be the constitutive isoform (hereafter Hsc75), varied among microhabitats, but not in a way matched with either *a priori* predictions of thermally stressful habitats, or with thermal modeling of mussel body temperatures (Helmuth and Hofmann, 2001). This may be due to the multiple functions of constitutive isoforms within the cell (Hochachka and Somero, 2002, Hofmann, 2002). Alternatively, difference in response may reflect the complexity of incorporating thermal history into measurement of the HSR, *i.e.,* responses to longer term ''chronic'' *vs.* shorter term ''acute'' thermal stresses (Helmuth and Hofmann, 2001).
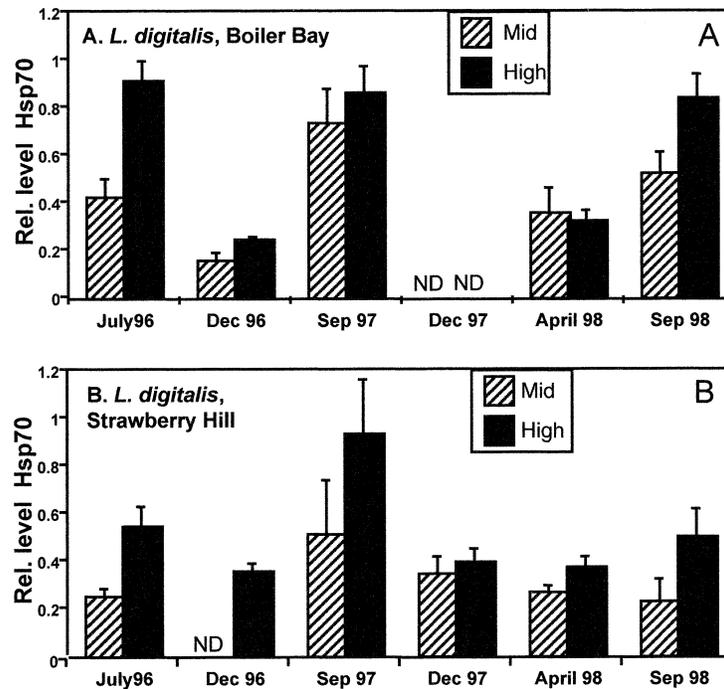
FIG. 5.   Relative level of Hsp70 in *L. digitalis* for 6 collections from July 1996–Sept. 1998 in the high and mid zones at (A) Boiler Bay, OR and (B) Strawberry Hill, OR. Bars indicate standard error.

Within zone variation

Within a biotically defined rocky intertidal zone, thermal and other physical stress is predicted to increase with tidal height. Our sampling scheme for *M. californianus* incorporated both this increasing stress gradient and variation within a site and tidal height, *i.e.,* between transect variation. Our results indicate the potential nested character of physiological variation. Weight corrected relative levels of both Hsp72 and Hsc75 varied between transects, even though efforts were made to have comparable sampling areas (*e.g.,* exposure and aspect). However, despite variation at the small scale, variation also occurred at larger scales, among site, tide-level, and collection for the inducible isoform Hsp72 (Table 1, Fig. 2, ANOVA, site × zone × collection $P = 0.0432$), and between collections for the constitutive isoform, Hsc75 (Table 2, Fig. 3, ANOVA, $P = 0.0013$). Highest levels of both isoforms were observed during the July collections. For Hsp72, relative levels increased with increasing tidal height at Boiler Bay in July, but August collections at Boiler Bay and all collections at Strawberry Hill did not vary in relation to tide-level (Tukey-Kramer, $P > 0.05$). Thus, within zone variability, as demonstrated by between transect variation, may be greater at certain times and sites than the physical forcing on physiology associated with increasing tidal height. Previous work at one of the sites, Strawberry Hill, demonstrated that Hsp72 (identified as a 66–68 kDa isoform at that time) and Hsc75 (identified as a 69–73 kDa isoform) was higher at higher tide-levels in summer (Roberts *et al.,* 1997). Thus, variability at small scales does not pre-clude detection of these responses at the larger scale when the physiological signal is strong and when a sampling scheme is tailored to assess these levels of variation.

Ubiquitin conjugate levels provide another measure of the cellular response to protein damage. While Hsps function to refold proteins, ubiquitin conjugates target irreversibly damaged protein for destruction by the proteosome (Rechsteiner, 1987; Ciechanover, 1998; Buckley *et al.,* 2001), and thus is another useful measure of organismal response to protein damage. In the *M. californianus* data from Boiler Bay and Strawberry Hill, ubiquitin conjugate levels showed a similar pattern as Hsp72 (Figs. 2, 4), and the two measures were correlated (regression $r^2 = 0.5983$, $P < 0.0001$ for July and $r^2 = 0.5570$, $P < 0.0001$ for August). The correspondence of Hsp72 levels to ubiquitin conjugate levels in *M. californianus* indicate that inducible isoforms of Hsps are useful indicators of organismal stress under field conditions. The looser relationship between ubiquitin conjugates with Hsc75 (Figs. 3, 4, regression, $r^2 = 0.3069$, $P < 0.0001$ for July and $R^2 = 0.3055$, $P < 0.0001$ for August) may reflect the more varied chaperoning function of constitutive isoforms in the cell.

Between zone variation

The most dramatic habitat comparison may be between intertidal and subtidal populations. Sharp *et al.* (1994) sampled the "snakelocks" anemone, *Anemonia viridis,* from both intertidal and subtidal populations. Collected anemones were exposed to a gradient of

temperatures (one temperature/individual) before analysis via western blotting. Results indicate that intertidal individuals were expressing a heat inducible protein prior to collection, while the subtidal population only produced the protein after exposure to elevated temperatures. These data indicate that intertidal anemones had already been naturally 'heat shocked' and initiated the HSR in the field while subtidal individuals had not experienced thermal stress severe enough to induce Hsp70 synthesis until exposure in the laboratory.

Although not as dramatically different as subtidal *vs.* intertidal comparisons, different intertidal zones differ in more than just their submergence time. Tidal zones are defined biotically as well as by physical tidal height. So an organisms' thermal environment will change not only due to changes in exposure time, but also due to alterations of the biotic habitat, resulting in differences much more profound than moving, for example, between high and low ends of the mussel bed. *L. digitalis* showed significant variation in expression of Hsp70 in both time and space (Fig. 5). Limpets collected from high, summer habitats showed significantly higher total pools of Hsp70 than those in the mid zone or in winter-spring months, indicating these individuals may be under the greatest thermal stress.

Between site variation

Mesoscale variation occurs at the "between site" level where study populations are separated by 10–100s of kilometers. Helmuth (1999) established that the environmental parameters driving organismal body temperature: solar radiation, temperature, and wind, vary at this scale in a fashion great enough to create unique microclimates between sites separated by <100 km. Careful modeling of organismal body temperatures demonstrated that mussel body temperatures can also vary at this scale (Helmuth, 1998, 1999). Therefore, when looking at the population level, it could be predicted that Hsps and other stress proteins would vary at this scale, in addition to variation at smaller scale.

Temperature loggers at Boiler Bay and Strawberry Hill recorded higher temperatures at Strawberry Hill, creating the expectation that organismal body temperatures would be higher at Strawberry Hill and subsequently Hsp levels would be higher as well. In both *M. californianus* and *L. digitalis* the highest levels of Hsp70 were recorded at Boiler Bay, contrary to predictions (Figs. 2, 3, 5). Conversely, Dahlhoff *et al.* (2001) recorded both higher body temperatures and higher Hsp70 levels for *Nucella emarginata* at Strawberry Hill. Our data indicate that thermal stress and its physiological response varies at the site level, but these responses are organism-specific. Our predictions were based on aerial temperature data for Boiler Bay and Strawberry Hill, not on organismal body temperatures. Aerial temperature alone is not sufficient to predict body temperature, when organismal thermal properties differ greatly from those of the temperature logger

(Helmuth, 1998, 1999, 2002). Data such as wind speed and solar radiation were not available to more accurately model organismal body temperatures, and body temperatures were not measured directly. Currently, loggers specifically designed to record temperatures that closely match mussel body temperatures are being used in the field (Helmuth and Hofmann, 2001).

While levels of Hsps varied between sites, these effects interacted with other effects such as tide-level and season. Clearly, as is true for other types of ecological and physiological sampling, assessing variation at the level of site requires careful consideration of other variation sources. Additionally, there is a need to find methods to accurately measure body temperature at multiple sites whether through more comprehensive physical measurements at sites of interest (*i.e.,* setting up weather stations), using accurate thermal proxies (*e.g.,* the mussel loggers developed by Helmuth and Hofmann, 1998), or methods for implanting temperature loggers or probes within organisms. Collection of relevant environmental parameters such as body temperature are a crucial link in development of indicator molecules for ecological use.

General remarks

The data presented here and in previous work (Sharp *et al.*, 1994; Roberts *et al.*, 1997; Helmuth and Hofmann, 2001) demonstrate that organisms within a species vary in levels of Hsp70 isoforms under natural conditions and at a variety of scales. These data point to the potential utility of molecular tools in examining not only how the natural environment modulates physiological responses but also future work on how physiological responses translate to larger scale patterns of population dynamics and community structure. However, these data also point out some of the qualifications necessary when using physiological measures, most of which are common to any organismal or environmental measure.

Most obviously, and the point of the data and studies cited here, assessing at what levels variation occurs is a requisite to assessing the utility of physiological measures. All scales examined have shown significant variation, from the microhabitat to site. In this way they are no different than any other commonly measured within species variable, such as size distribution, density, growth, etc. (Sutherland, 1996). And just as with those measures, researchers looking to molecular tools need to tailor sampling schemes, especially in the early stages of investigation, to accurately assess this variation. Models for these sampling schemes come from both classical community ecology and genetic research. More peculiar to Hsps is the context of organismal physiology, plasticity of the response, and the role of the HSR in the natural environment.

The HSR is a complicated physiological mechanism involving a suite of cellular mechanisms (see Feder and Hofmann, 1999; Hochachka and Somero, 2002). Hsp total pool reflects one aspect of this response, the amount of Hsps in relation to other proteins within the

organism. While this metric is functionally significant, it can be difficult to interpret in isolation. One avenue of 'calibration' of the system is comparison with other measures of organismal condition. We would recommend that measurements of Hsps be placed in context of other measures of organismal condition, such as metabolic rate, growth, thermotolerance, or other physiological measures, at least in the preliminary phases of investigation if researchers are interested in using Hsps as a biomarker.

Additionally, the HSR is a highly plastic response dependent on thermal history (Lindquist, 1986). The synthesis of new Hsps is dependent not only on the amount of damaged proteins within a cell but also the amount of existing Hsps in the cell. So the timing and duration of prior heat stress will influence the production and amount of Hsps in an organism. How the timing and duration of thermal stress under natural conditions alters the HSR remains a complicated issue (Buckley *et al.*, 2001; Helmuth and Hofmann, 2001). Finally, researchers must be aware that molecules in the Hsp family are involved a variety of cell functions and can have genetic variation within and between populations, vary with developmental status, and with nutritional status.

Evidence to date demonstrates that an increasingly popular metric of the HSR, the total pool of Hsp70 isoforms, is a potentially powerful tool in linking the ecology of organisms in natural populations to their physiological responses. Potentially, the metric varies at the same scales as body temperature, from the microscale to the mesoscale. While part of what makes this a useful tool, that variation should be taken into account when designing a sampling scheme. Additional research into the plasticity and functioning of the HSR in the field will yield greater insight into how Hsps operate in nature and ultimately into a greater synthesis of physiological and ecological tools.

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