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RESEARCH ARTICLE

Michael S. Berger · Craig M. Young

Physiological response of the cold-seep mussel *Bathymodiolus childressi* to acutely elevated temperature

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Abstract It is predicted that deep-sea animals adapted to thermally stable conditions should be highly sensitive to temperature change and should not have inducible heat-shock responses. This premise was tested with the cold-seep mussel *Bathymodiolus childressi* Gustafson, 1998 from 750 m depth in the Gulf of Mexico at a site known as Brine Pool NR-1 (27°43.4157N, 91°16.756W). Mussels were collected during February 2003. Site temperature, measured in different months between 1995 and 2005, ranged between 6.5 and 7.2°C. Although Brine Pool NR-1 is stenothermal, hydrogen sulfide, oxygen, and salinity vary over temporal and spatial scales. In laboratory experiments, *B. childressi* survived increases up to approximately 20°C above ambient temperature for 6 h before suffering greater than 50% mortality. Although a high thermal tolerance was observed, *B. childressi* did not express an inducible 70 kDa heat-shock protein. However, high constitutive levels of hsp70 were present in *B. childressi* suggesting a necessity to remediate protein damage from stressors other than elevated temperature; these constitutive proteins probably confer an indirect thermal tolerance.

Introduction

A very large percentage of the Earth's biosphere is found in the deep oceans and polar seas, where waters are cold and thermally stable and seasonal changes seldom involve significant temperature excursions (Littlepage 1965; Gage and Tyler 1991). Ectothermic organisms living in cold and thermally stable habitats have evolved novel physiological adaptations such as reduced metabolic costs (Marsh et al. 2001), microtubule formation at low temperatures (Williams et al. 1985; Detrich et al. 2000), and the production of anti-freeze glycoproteins (DeVries 1988). Many of these cold-adapted organisms have also lost traits that are typical of non-cold-adapted species. For example, Antarctic fish over evolutionary time have lost respiratory protein expression (Ruud 1954; Sidell et al. 1997). A few species adapted to thermally stable habitats, such as an Antarctic fish (Hofmann et al. 2000; Buckley et al. 2004), a fresh water *Hydra* sp. (Bosch et al. 1988), and an Antarctic ciliate (La Terza et al. 2001), no longer have the ability to upregulate heat-shock proteins when exposed to sub-lethal temperatures. Loss of the heat-shock response is atypical and limited, to the best of our present knowledge, to a few species found in thermally stable environments.

Apart from the few exceptions noted above, heat-shock proteins (molecular chaperones) are ubiquitous across phyla and virtually always upregulated when exposed to elevated temperatures (Feder and Hofmann 1999) or other cellular stressors that result in protein denaturation (Lindquist 1986; Morimoto et al. 1994; Feder and Hofmann 1999). Heat-shock proteins rescue and prevent the aggregation of denatured proteins, thereby helping to conserve the existing pool of proteins from irreversible damage (Parsell and Lindquist 1993; Buchner 1996; Fink 1999). The magnitude of the heat-shock response reflects the level of stress an organism is exposed to (Hofmann and Somero 1995; Tomanek and Sanford 2003; Dahlhoff 2004), and organisms without a heat-shock response are sensitive to relatively small increases in temperature (Somero and DeVries 1967; Bosch et al. 1988).

When irreversible protein damage does occur, ubiquitin, a low molecular weight protein, binds to damaged protein, marking it for degradation by cytoplasmic proteases (Hershko and Ciechanover 1992; Hochstrasser 1995). The

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M. S. Berger · C. M. Young
Oregon Institute of Marine Biology, University of Oregon,
Charleston, OR 97420, USA

Present address: M. S. Berger (✉)
Whiteley Center, Friday Harbor Laboratories,
620 University Road, Friday Harbor,
WA 98250, USA
E-mail: mikeberger@bms.bc.ca
Tel.: +1-503-8304460
Fax: +1-206-5431273

concentration of ubiquitin conjugates has been used to quantify protein damage resulting from thermal stress (Lee et al. 1988; Hofmann and Somero 1995; Spees et al. 2002a).

In contrast to many other thermally stable deep-sea environments, multiple physiological stressors that vary temporally and spatially are present in the cold-seep habitat of *Bathymodiolus childressi* on the upper Louisiana slope of the Gulf of Mexico (Nix et al. 1995; Bergquist et al. 2005). Stressors include elevated hydrogen sulfide, hydrocarbons, low oxygen levels, and high salinity (Anderson et al. 1983; Kennicutt et al. 1985; MacDonald et al. 1990; Nix et al. 1995; Smith et al. 2000). Low oxygen levels, high salinity, and toxic chemicals all have been demonstrated to induce the upregulation of heat-shock proteins (Airaksinen et al. 1998; Vijayan et al. 1998; Feder and Hofmann 1999; Spees et al. 2002b). In species that have lost the heat-shock response, exposure to stressors that promote protein denaturation did not result in the upregulation of heat-shock proteins (Hofmann et al. 2000; Buckley et al. 2004).

The present study tested the prediction that animals adapted to a cold, thermally stable environment may have lost the inducible heat-shock response and therefore be more stenothermal in their tolerances. The cold seep mussel *B. childressi* lives in a thermally stable deep-sea environment and is an excellent species to address this prediction. Specifically we address the following questions. What is the upper lethal temperature of *B. childressi*? Does a species adapted to a thermally stable seep habitat lack a functional heat-shock response? Does extensive irreversible protein damage occur as a result of exposure to elevated temperature?

Materials and methods

Collection

Bathymodiolus childressi Gustafson, 1998 were collected in the middle zone (Smith et al. 2000) at Brine Pool NR-1 site in the Gulf of Mexico (27°43.4157N, 91°16.756W) using a *Johnson Sea-Link* submersible (Harbor Branch Oceanographic Institution) during February 2003. Water temperature at the collection site was 7.25°C at the time of collection. Clusters of *B. childressi* were returned to the surface in an insulated box to reduce thermal stress. Mussels were allowed to recover for at least 3 days at 8°C on board the RV *Seward Johnson*, prior to any experimentation.

Submersible dives were made to the Brine Pool NR-1 site during many different months between 1995 and 2005. We measured water temperature on the bottom each time we visited to determine the temporal stability of temperature at this site.

Thermal incubation

To measure thermal tolerance and physiological response of *B. childressi*, ten individuals (90 to 126 mm shell length) were submerged in seawater at 8, 12, 16, and 20°C for 6 h during experiment 1 and at 8, 20, 25, and 30°C in experiment 2. *Mytilus* species, the sister taxa to *B. childressi* (Distel et al. 2000), exposed to elevated temperature for 2 h results in a quantifiable heat-shock and ubiquitin response (Hofmann and Somero 1996; Roberts et al. 1997; Buckley et al. 2001). An air pump was used to mix and oxygenate the water continuously during the exposure. Mortality was assessed every 30 min for the first 3 h, then hourly thereafter. Mussels were considered dead when the valves did not close following repeated stimuli to the mantle. Following thermal incubation, all surviving *B. childressi* were allowed to recover at 8°C for 2 h. After recovery, foot tissue from six *B. childressi* in each temperature treatment was removed, frozen with liquid nitrogen, and stored at −80°C for immunochemical analysis.

Endogenous hsp70 levels

Analysis of hsp70 levels followed the methods and reagents of Hofmann and Somero (1995) with the following modifications. Samples were homogenized in buffer. An aliquot of the supernatant was removed to determine sample protein concentration (Coomassie Blue assay, BioRad). Ten micrograms of total protein per sample were separated electrophoretically on a 7.5% SDS-polyacrylamide gel (Laemmli 1970). Following electrophoresis, proteins were transferred to a presoaked nitrocellulose membrane (0.45 μm), blocked, incubated for 1.5 h with a primary antibody diluted 1:2,500 (anti-hsp70, rat monoclonal, MA3-001, Affinity Bioreagents), incubated for 30 min with a secondary antibody diluted 1:2,000 (rabbit anti-rat, AI-4000, Vector Laboratories), and then incubated for 1 h with protein-A horseradish peroxidase conjugate diluted 1:5,000 (BioRad, 170-6522). To visualize the proteins, an enhanced chemiluminescence detection method (ECL reagents, Amersham) was used, followed by exposure of the membrane to X-ray (Kodax X-OMAT) film. Optical density of each visualized band was quantified using Gel-Pro Analyzer software (version 4.0, Media Cybernetics). Since multiple gels were compared within an experiment, all bands on each gel were normalized to a positive control prepared from heat-shocked mussel (*Mytilus californianus*) so relative comparisons could be made.

Ubiquitin conjugate analysis

Ubiquitin conjugate analysis followed the methods of Hofmann and Somero (1995) with the following exceptions. Samples were diluted to a concentration of 5 μg ml^{-1} in a saline solution, loaded in triplicate 100 μl volumes onto a presoaked nitrocellulose membrane (0.45 μm) in a dot blot vacuum apparatus (BioRad), and gravity fed through the membrane. The membrane was blocked, incubated for 1.5 h with a polyclonal rabbit anti-ubiquitin conjugate primary antibody diluted 1:2,500 (provided by Dr. Gretchen

Hofmann), and then incubated for 1 h with protein-A horseradish peroxidase conjugate diluted 1:5,000 (Vector Laboratories, PI-1000). Visual detection and analysis were identical to methods previously outlined for hsp70 Western Blotting.

Data analysis

All analyses were conducted using Systat (version 9.0, SPSS Inc.) and Statistica (version 6.0, Statsoft). The assumptions of normality and homoscedasticity, tested with a Kolmogorov–Smirnov test with a Lilliefors option and a Cochran's C test, respectively, were not violated. Separate one-way ANOVAs were used to test the effect of incubation temperature on heat-shock protein and ubiquitin conjugate levels.

Results

Temperature at the brine pool site ranged from 6.48 to 7.55°C with a mean of 6.96°C. Variances were low and nearly all values were within 0.5°C of the overall mean (Fig. 1).

Exposure to temperatures between 8°C and 20°C for 6 h in experiment 1 resulted in no mortality of *B. childressi* (Fig. 2). However, when exposed to higher temperatures for 6 h in experiment 2, mortality was 30% at 25°C and 90% at 30°C (Fig. 2). The estimated temperature of 50% mortality (LT_{50}) was 27°C.

Only one resolvable band in the 77 kDa region was observed for *B. childressi* in both experiments (Fig. 3). Two bands, one in the 77 kDa region and the other in the 72 kDa region, were resolvable in the positive control prepared from heat-shocked *M. californianus* tissue (Fig. 3).

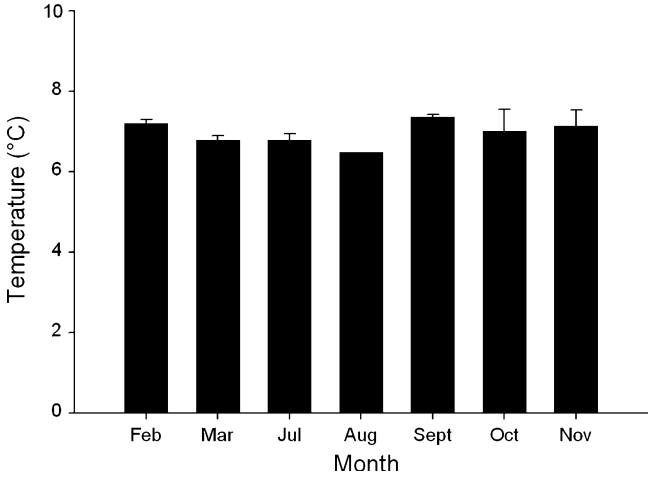

Fig. 1 Average (+1 SD) monthly temperature measurements taken from submersibles at the Brine Pool NR-1 during dives. Only one data point was taken per submersible dive. Months were in various years between 1995 and 2005

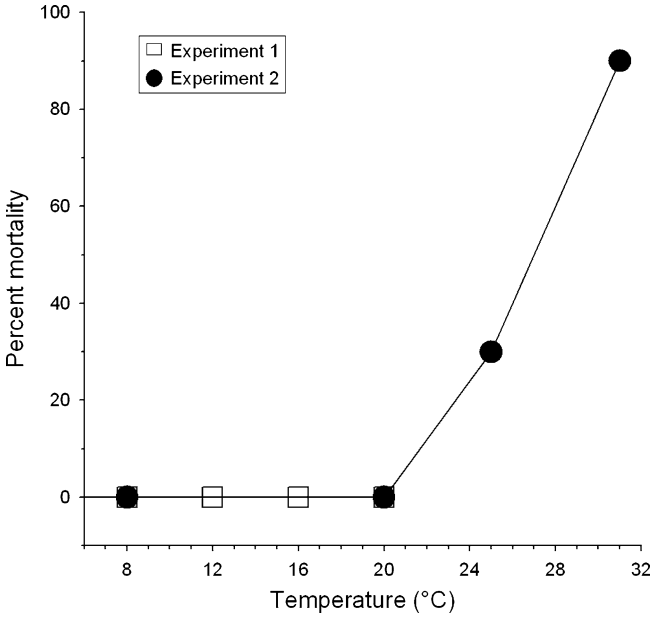

Fig. 2 *Bathymodiolus childressi*. Percent mortality after 6 h of thermal incubation during experiments 1 and 2. Each data point represents the percent mortality of ten individuals

Endogenous levels of hsp70 in the control treatment of 8°C were not significantly different (ANOVA, $F_{3,20}=1.43$, $P=0.26$) from hsp70 levels in higher incubation temperatures of 12, 16, and 20°C in experiment 1 (Fig. 4a). In experiment 2, endogenous hsp70 levels in the 8°C control treatment were also elevated and not significantly different (ANOVA, $F_{2,12}=1.33$, $P=0.30$) from exposure to higher temperatures of 20 and 25°C (Fig. 4b).

Irreversible protein damage did not occur in *B. childressi* exposed to elevated temperatures for 6 h. Significantly elevated levels of ubiquitin conjugate were not observed when comparing the 8°C control treatment to elevated temperatures of 12, 16, and 20°C (ANOVA, $F_{3,20}=1.57$, $P=0.23$; Fig. 5).

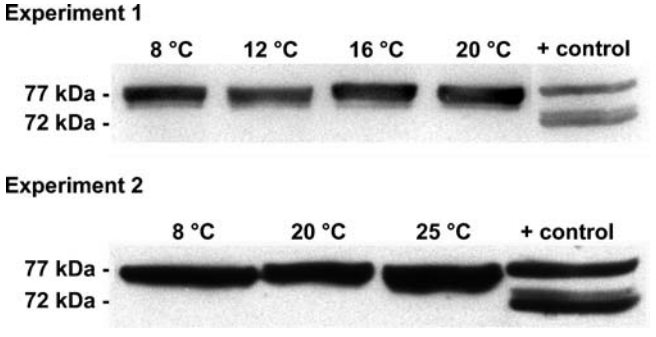

Fig. 3 *Bathymodiolus childressi*. Western Blot of hsp70 expression during experiments 1 and 2. *Captions* above each *lane* correspond to incubation temperature of *B. childressi* foot tissue and *Mytilus californianus* positive control (+ control)

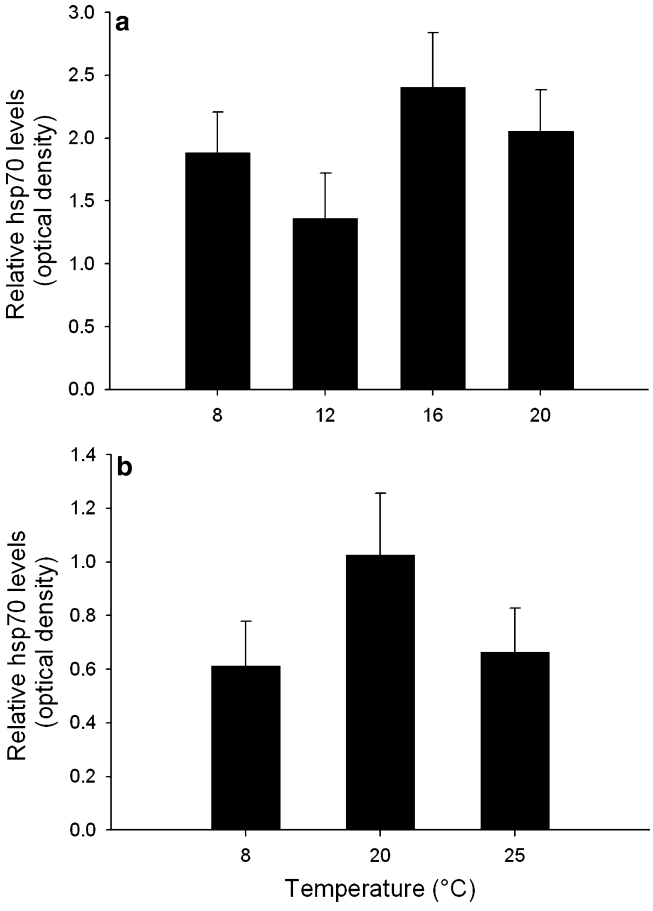

Fig. 4 *Bathymodiolus childressi.* Levels of endogenous hsp70 from **a** experiment 1 and **b** experiment 2 following a 2 h recovery from thermal incubation. Each *bar* represents the mean (+1 SE) of six individuals

Discussion

The aim of this study was to determine whether a species, such as *B. childressi*, evolving in a thermally stable habitat loses its ability to respond physiologically and to survive elevated temperatures. The few examples in the literature suggest that species adapted to thermally stable environments display temperature sensitivity and a loss of the heat-shock response (Bosch et al. 1988; Hofmann et al. 2000; La Terza et al. 2001). However, our results indicate that *B. childressi* from a thermally stable environment tolerates temperatures almost 20°C above ambient for 6 h before suffering mortality greater than 50%. There was no evidence for an inducible heat-shock response, but a high thermal tolerance in concert with no irreversible protein damage suggests that *B. childressi* has retained a heat-shock response or an equivalent mechanism to reduce thermally denatured protein damage.

In some organisms that experience regular physiological stress, such as desert ants, levels of endogenous hsp70 were high prior to encountering high temperatures (Gehring and Wehner 1995). Additionally, an Antarctic fish that does not have an inducible heat-shock response has been shown to constitutively express heat-shock protein gene products at a constant rate (Buckley et al. 2004). Our data are consistent with the hypothesis that *B. childressi* constantly expresses high constitutive levels of heat-shock proteins that remediate irreversible protein damage from stressors other than temperature. The presence of high background levels of hsp70 resulting from exposure to high temperatures has been suggested to increase the upper thermal limit of an organism (Clegg et al. 1998; Hamdoun et al. 2003; Sorte and Hofmann 2004). Therefore, it is plausible that repeated exposure to any of the potential stressors at seep habitats could result in an adaptation to produce high, yet non-detrimental levels of hsp70, which would indirectly confer an increased temperature tolerance in *B. childressi.*

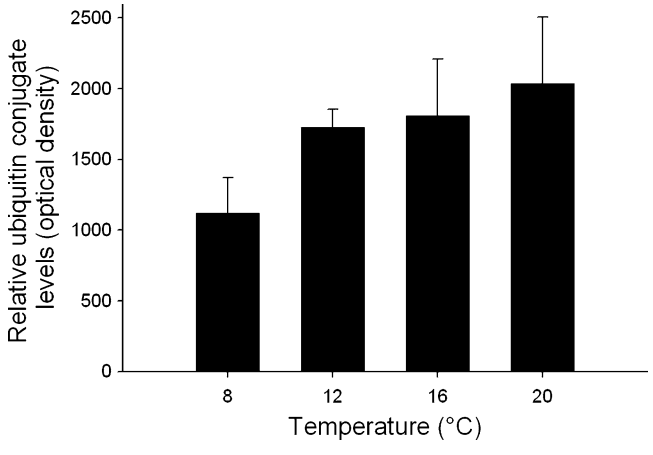

Fig. 5 *Bathymodiolus childressi.* Levels of ubiquitin conjugate following a 2 h recovery period from thermal incubation during experiment 1. Each *bar* represents the mean (+1 SE) of six individuals

It is entirely possible that *B. childressi* does not have an inducible heat-shock response. Results from other mytilid mussel species indicate that two hsp70 isoforms are expressed: a constitutive (i.e., background) isoform and inducible isoform (Hofmann and Somero 1995; Roberts et al. 1997; Helmuth and Hofmann 2001; Halpin et al. 2002). Two resolvable bands representing constitutive and induced hsp70 levels were observed in our positive control prepared from *M. californianus* tissue. A band in the 72 kDa region that would represent an inducible hsp70 isoform analogous to *M. californianus* was not observed in *B. childressi.* Based on this fact alone, we can conclude that *B. childressi* does not have an inducible heat-shock response.

An antibody has been used to quantify the upregulation of hsp70 in other mussels of the family Mytilidae (Hofmann and Somero 1995; Roberts et al. 1997; Chapple et al. 1997; Snyder et al. 2001), and it reacts with both constitutive and induced hsp70 isoforms (Helmuth and Hofmann 2001). Since a heat-shock response is highly conserved across taxa (Lindquist 1986), and *B. childressi* has only recently diverged from its sister taxa in the genus *Mytilus* (Distel et al. 2000), non-specificity of the antibody used to detect induced isoforms in

this study is an unlikely explanation for the lack of an inducible response.

This study has addressed the physiological response of an unusual species from a highly variable deep-sea environment. Future work should examine the physiological response of *B. childressi* to other ecologically relevant stressors such as hypoxia, hydrogen sulfide, high salinity, or hydrocarbons. Further examination of the mechanisms regulating the heat-shock response are necessary to conclusively determine if an upregulation of hsp70 is induced when *B. childressi* is exposed to multiple physiological stresses.

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