RESEARCH ARTICLE

Thermal Constraints for Range Expansion of the Invasive Green Mussel, *Perna viridis*, in the Southeastern United States

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ABSTRACT

Cold temperatures are thought to be among the most important determining factors of geographic distribution for tropical and sub-tropical marine invertebrates. The Asian green mussel, *Perna viridis*, has been introduced into coastal waters of Florida where its current distribution is hypothesized to be limited by low temperatures during winter. Lethal and sub-lethal effects (heat shock protein/Hsp70 expression) of cold water and air temperatures were analyzed in two size classes of *P. viridis* from Florida in an effort to determine the effects of current and forecasted temperatures on the potential for range expansion. Mussels were exposed to water temperatures of 14, 10, 7 and 3°C for up to 30 days, or to air temperatures of 14, 7, 0 and −10°C for periods of 2 hr. Mortality was significantly increased at all water and air temperatures ≤14°C. No differences in mortality rates were observed between small (15–45 mm) and large (75–105 mm) size classes except after exposure to 7°C air, in which small mussels had higher mortality. Significant increases in Hsp70 expression were observed after a 2-hour exposure to 10°C water, but Hsp70 expression was not significantly increased at any temperatures in which mortality was not also significant. The temperature threshold for survival in this population appears to be between 10 and 14°C, suggesting that under current conditions *P. viridis* may already be at the northern edge of its potential range in the United States. If water temperatures increase with global climate change, northerly flowing currents may permit range expansion as temperatures allow. *J. Exp. Zool. 313A, 2010.* © 2010 Wiley-Liss, Inc.

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Temperature is often thought to be the predominant factor determining the geographic distribution of marine invertebrates (Hutchins, '47; Seed, '76; Hicks and McMahon, 2002). For tropical and sub-tropical species, it is predominantly cold winter temperatures that act as a limiting factor. Cold temperatures have been shown to increase mortality in a number of marine invertebrates, including the mollusks *Crepidula fornicata* (Thieltges et al., 2004) and *Perna perna* (Hicks and McMahon, 2002). Although cold-induced mortality may determine the absolute limits of a species potential range, typically species have a broad range of temperatures acceptable for survival (Delgado and Camacho, 2007). But even environmental stressors at nonlethal, yet extreme temperatures, can induce a stress response that may reduce long-term survival (Seed, '76; Krebs and Feder, '97; Krebs and Feder, '98; Beukema et al., 2009), growth (Feder et al., '92), and/or reproductive success (Krebs and Loeschcke, '94). Therefore, both the lethal and sub-lethal effects of

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temperature are likely to play a pivotal role in defining the observable patterns of species distribution.

Here the Asian green mussel, *P. viridis*, was used as a model to study the effects of cold temperatures on geographic distribution and range expansion. *P. viridis* is native to the Indo-Pacific region (Sidall, '80; Rajagopal et al., 2006), but in recent years, green mussels have been introduced to coastal waters of North America, South America, and the Caribbean (Agard et al., '92; Rylander et al., '96; Benson et al., 2001; Ingrao et al., 2001). Green mussels were first discovered in the United States as a fouling organism on the intake pipes of a power plant in Tampa Bay, FL in August 1999 (Benson et al., 2001). Since that time, they have also spread along parts of the Gulf coast and have been discovered on the east coast of the US in northern Florida and parts of Georgia (Power et al., 2004). The green mussel's potential impact on local ecosystems and native species is not yet fully understood; however, high densities have been reported to foul power plant intake pipes, ship hulls, and structures such as bridge pilings and are therefore likely to have an economic impact (Benson et al., 2001). The US Army Corp of Engineers has estimated that zebra and quagga mussels, which foul similar structures, cause up to $1 billion per year in damage and control costs. It has also been suggested that *P. viridis* may displace native species such as the oyster *Crassostrea virginica* (Baker and Benson, 2002).

Green mussels have become successful invaders in many locales due in part to their tolerance for environmental extremes (Rajagopal et al., 2006). For example, green mussels in the Indo-Pacific region experience an average annual water temperature range between 12 and 32°C (Rajagopal et al., 2006), with an optimal range between 26 and 32°C (Power, 2004). Previous experiments have shown that green mussels have a 50% mortality rate after 2-week exposures to water temperatures of 10 and 35°C (Sivalingam, '77). Water quality monitoring data from the Guana Tolomato Matanzas National Estuarine Research Reserve (GTMNERR) in St. Augustine, FL reports an average winter water temperature of 13.9°C, suggesting that local populations of green mussels may experience water temperatures well below their optimal range during winter months. In addition, intertidal green mussels in the southeastern United States may also be vulnerable to periods of acute stress from cold air when low spring tides correspond with sub-freezing overnight temperatures. During these periods, air temperatures are often substantially lower than the ambient water temperature. Therefore, chronic exposure to cold air and water temperatures is expected to limit the ability of *P. viridis* to expand its geographic range further north along the Atlantic coast of the United States.

Most studies on temperature tolerance in marine mollusks have focused on the critical temperature at which adult survival is no longer possible. More subtle sub-lethal effects that have been shown include reduction in growth and reproductive output in *Mytilus galloprovincialis* and *P. canaliculus* (Petes et al., 2007). Analysis of heat-shock protein (Hsp) induction can be a good model to monitor sub-lethal stress upon an organism (Dalhoff, 2004). Heat shock proteins (Hsp) are a highly conserved class of molecular chaperone that are up-regulated during periods of stress to repair damaged and denatured proteins (Lindquist and Craig, '88; Hendrick and Hartl, '93; Parsell and Lindquist, '93; Feder and Hofman, '99; Gonzalez-Riopedre et al., 2007). Heat shock protein induction due to temperature stress has been documented in numerous bivalve species, including *Mytilus* spp. (Buckley et al., 2001), *Dreissena polymorpha* (Singer et al., 2005), and *C. gigas* (Hamdoun et al., 2003); however, long-term effects on longevity and fecundity have not been widely studied in these species. Induced expression of heat shock proteins has been shown to decrease the larvae to adult survival rate (Krebs and Feder, '97), reduce fecundity (Krebs and Loeschcke, '94), and lower growth rates (Feder et al., '92) in *Drosophila melanogaster*. Although induced stress responses may increase survival in the short term, the costs may have long-term consequences on the potential for population growth and range expansion (Krebs and Feder, '97).

As temperature is likely to be among the most important factors determining the potential for range expansion of tropical exotic species, this study was designed to examine the effects of lethal and sub-lethal cold stress on two different size/age classes of the Asian green mussel, *P. viridis*. Based on volumetric difference, it is expected that larger mussels may exhibit a slower/reduced response to the effects of cold stress during aerial exposure due to a greater thermal inertia. Furthermore, subtidal mussels that have survived a previous winter may also be more likely to survive subsequent cold stress associated with low winter water temperatures. If there is differential survival with respect to size in cold conditions, the ultimate geographical distribution will be determined by the point at which young of the year can no longer survive the winter. In addition, heat shock protein expression was used as an indicator of physiological stress to examine sub-lethal effects of exposure to cold water and air temperatures.

MATERIALS AND METHODS

Sample Collection

Mussels for both water and air temperature trials were collected from three main sites in northeast Florida: Sister's Creek (30°23′31″N, 81°27′48″W), Matanzas Inlet (29°42′07″N, 81°13′43″W), and Whitney Lab (29°40′07″N, 81°12′49″W). Sites were selected due to a high density of *P. viridis* present on the underside of one or more floating docks. Additional mussels for the small size class were collected from PVC pipes attached to channel markers associated with a spat collection project in the Atlantic Intracoastal Waterway in the same region as the adult collections. All samples were collected from entirely subtidal habitats to minimize possible effects of varied thermal histories. Mussels

from different sites were acclimated together and were haphazardly assigned to experimental treatments.

P. viridis specimens of two size classes, 15–45 mm shell length ($n = 90$) and 75–105 mm shell length ($n = 90$), were collected in December 2007. The smaller size class was selected to represent "young of the year," or mussels that had not yet experienced a winter season. The larger size class represented mussels that had survived at least one winter season. Mussels were immediately transported to the lab in sea water. Macroscopic epibiotic organisms were removed and mussels were placed in an aquarium for acclimation. All experimental mussels were acclimated in 37.85 L aquaria in 35 ppt sea water at 14°C (simulating average winter water temperatures for St. Augustine) for 14 days. All aquaria were equipped with an undergravel filter and powerhead to generate current. Mussels were fed a diet of mixed phytoplankton (Kent Marine, Filter Feeding Invertebrate Diet) at a concentration of 3 μg/L, similar to the average chlorophyll concentration (3.1 μg/L) recorded by Guana Tolomato Matanzas National Estuarine Research Reserve for the months December 2005 through February 2006. Environmental chambers were maintained on a 12-hour light/dark cycle and a 50% water change was performed every other day.

Owing to significant mortality observed in the 14°C water and air trials (see *Results*), additional mussels were collected in March 2009. These mussels were used as a reference group and were maintained at 21°C for the course of 30 days to mimic nonstressful conditions. Reference mussels for the air temperature trials were exposed to 21°C air temperatures for 2 hr and were then returned to 21°C water for 30 days. Analysis of survivorship of these reference groups was used to determine whether the observed mortality in the 14°C groups was due to temperature or laboratory conditions. Mussels in these 21°C reference groups were not divided into size classes. Owing to differences in collection times, data from the 21°C trials were used for reference only and were not included in statistical analyses of other temperature trials.

Mortality

Chill Resistance (Cold Water Temperatures). To assess the lethal effects of cold water temperatures, a controlled laboratory experiment was conducted, comparing the mortality rate of mussels chronically exposed to sub-optimal temperatures. After acclimation, mussels were segregated into experimental groups. Groups of 30 mussels per treatment per size class were haphazardly assigned to one of three temperatures (14, 10, and 3°C). Experimental temperatures were selected to investigate the ability of *P. viridis* to expand its geographic range farther to the north. The average winter water temperature in St. Augustine, FL is 14°C. Charleston, SC has an average winter water temperature of 10°C and is located approximately 320 km north of the existing east coast population of *P. viridis*. The 3°C trial was selected as an extreme and corresponds roughly to the winter water temperatures near Boston, MA. Mussels for each experimental group were placed into a single 37.85 L aquarium and were placed into an environmental chamber maintained at constant temperature and a 12-h photoperiod. Temperatures were monitored using iButton temperature loggers and were maintained within ± 1°C of the reported experimental temperatures. All temperature trials were run simultaneously. Mussels in each trial were examined for viability every 24 hr. Viability was assessed by response to mechanical stimuli. When immersed, mussels typically display gaping valves. Viable mussels will close their valves when touched. Mussels that were unresponsive to stimulation with a dissecting probe for 10 sec or more were considered dead and were removed. Mussels were exposed to test temperatures for 30 days or until 100% mortality, whichever occurred first. Comparisons between size classes and temperature trials were made using a Kaplin–Meier survival analysis with Bonferroni's correction.

Freeze Resistance (Cold Air Temperatures). Mortality was assessed in relation to an acute exposure to one of four cold air temperatures. After acclimation, mussels were divided into experimental groups and sensitivity to a 2-hour exposure to cold air temperatures (14, 7, 0, and −10°C) was assayed. Experimental procedures were selected to represent ecologically relevant exposures of both temperature and time for the northeast Florida region. The average low air temperature in St. Augustine is 7.8°C in January. The St. Augustine area experiences a freeze (0°C) several times a year and the historical record low is −12.5°C. Therefore, field conditions in northeast Florida are such that green mussels are likely to quickly transition from the average 14°C water temperatures to the aforementioned colder air temperatures. During spring tides, mussels in the intertidal zone regularly experience emersion times of up to 4 hr (personal observation). Therefore, groups of 30 mussels from each size class were exposed to each temperature for 120 min, representing an average exposure time. Mussels were placed in a refrigerator set to the appropriate test temperature (± 1°C) and were suspended above the floor of the unit with nylon mesh to avoid direct contact with the refrigeration unit. Temperature was monitored using iButton data loggers. Mussels were removed at the end of the exposure period and returned to a recovery tank containing aerated sea water at 14°C. Viability was assessed as previously described. Dead animals were removed and live individuals were returned to an acclimation tank (14°C) and were examined daily for viability. Statistical analyses were conducted as described for chill resistance.

Sub-Lethal Stress Response

Cold Shock. To examine the sub-lethal stress response, up-regulation of heat shock proteins was analyzed in response to the two aforementioned cold stress factors. Mussel specimens were collected in December 2008 and March 2009 (21°C control samples) and acclimated as previously described. Only large mussels were used in these experiments to maximize the tissue

collected. Mussels ($n = 10$ per treatment) were exposed to water temperatures (14, 10, and 3°C) as described in the chill resistance experiment and air temperatures (14, 7, 0, and −10°C) as described in the freeze resistance experiment. Mussels were exposed to experimental treatments for 120 min, with the exception of the −10°C air temperature trial which was only exposed for 20 min because longer times were lethal (see *Results*). After cold shock exposure, mussels were removed and placed in a 14°C recovery tank. As cold temperatures are likely to slow protein synthesis, cold-shocked mussels were allowed to recover for 20 min before being dissected (Liu et al., '94).

Heat Shock Protein Analysis. Heat shock protein (Hsp70) expression was determined by Western Blot, as previously described by Hofmann and Somero ('95) with some adjustments. Several tissue types (gill, muscle, foot, and mantle) were assayed for Hsp70 expression. Mantle tissue generated the greatest induction of Hsp70 (unpublished data) and was therefore selected for analysis. After cold shock, mantle tissue was dissected from each individual and immediately frozen and homogenized in liquid nitrogen. Frozen tissue was then placed in 100 µL Tris-Buffered Saline (TBS) with protease inhibitor and centrifuged at 13,000 rpm for 5 min. Total protein content was determined by Bradford Assay (Bradford, '76) and 30 µg of total protein per sample was loaded onto an 8% polyacrylamide gel. Tissue pooled from mussels heat shocked in the laboratory at 39°C for 1 hr was used as a positive (stressed) control on each gel and tissue from mussels maintained at 21°C was used as a negative (unstressed) control. Proteins were separated by SDS polyacrylamide gel electrophoresis for 150 min at 110 V and then transferred to a nitrocellulose membrane (Whatman, Piscataway, NJ) using a semi-dry transfer apparatus at 18 V for 30 min. Membranes were incubated for 90 min in a 1:1,000 dilution of mouse anti-Hsp70 monoclonal antibody (Affinity Bioreagents, Golden, CO) that recognizes both the cognate and inducible forms of Hsp70. Membranes were washed in TBS with 0.05% Tween (TBST) and subsequently incubated for 30 min in a 1:5,000 dilution of alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Sigma, St. Louis, MO). Blots were washed again in TBST and then developed with alkaline phosphatase developing solution (Fisher, Pittsburgh, PA). Relative intensity of bands was determined using Kodak Molecular Imaging Software and mean band intensities for each treatment were compared using one-way ANOVA.

RESULTS

Mortality

Chill Resistance (Cold Water Temperatures). Chronic exposure to sub-optimal cold water temperatures increased the rate of mortality in *P. viridis* (Fig. 1). The median number of days to death did not vary significantly between size classes at 14°C ($\chi^2 = 16.8$, df = 29, $P = 0.965$) or 10°C ($\chi^2 = 11.9$, df = 29, $P = 0.997$). All mussels exposed to 3°C in both size class trials died within the first 24-hour period and therefore could not be analyzed due to a complete lack of variance among samples. Because no differences were detected between size classes, data were combined for further analysis of survival.

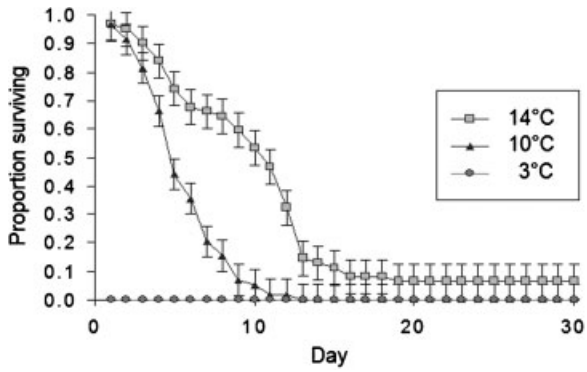

Figure 1. Proportion of *P. viridis* individuals surviving during a 30-day exposure to 14, 10, or 3°C water temperature. Error bars represent standard error of the mean survival.

Decreases in temperature caused a significant increase in the rate of mortality of *P. viridis* samples pooled over size classes. Mussels exposed to 14°C survived longer than those exposed to 10°C ($\chi^2 = 462.1$, df = 29, $P < 0.0001$) and 3°C ($\chi^2 = 297.5$, df = 29, $P < 0.0001$), whereas mussels exposed to 10°C also had a greater survival rate than those exposed to 3°C ($\chi^2 = 254.3$, df = 29, $P < 0.0001$). All mussels exposed to 3°C died within the first 24-hour period, wherteas those exposed to 10°C died in an average of 5.7 days, with 100% mortality after 13 days. The 14°C temperature trial served as an "unstressed" negative control in the original experimental design because green mussels in the northeast Florida region regularly experience this temperature during winter months. All other experimental manipulations were used to decrease temperatures below what is routinely experienced by mussels in this region. However, mussels exposed to 14°C in this study suffered significant mortality over the course of the experiment. Mussels chronically exposed to 14°C lived an average of 10.6 days, with 93.5% mortality after 30 days. Therefore, an additional reference trial was added with spring-collected mussels exposed to 21°C to recreate nonstressful conditions in an effort to determine whether the mortality observed in the 14°C treatment was due to exposure to cold temperatures or to the housing and husbandry conditions of the experiment. Mussels exposed to 21°C had an 88% survivorship over the course of the 30-day trial.

Freeze Resistance (Cold Air Temperatures). Two-hour exposures to cold, freezing, and sub-freezing air temperatures significantly decreased survivorship in *P. viridis* (Fig. 2). The median number of days to death decreased with a decrease in temperature for

both size classes. The 21°C reference mussels exhibited 84% survivorship 30 days after a 2-hour aerial exposure. As with the water temperature trials, no significant differences were observed between size classes at 14°C ($\chi^2 = 10.1$, df = 29, $P = 0.99$), 0°C ($\chi^2 = 0.6$, df = 29, $P = 1.0$), or −10°C ($\chi^2 = 4.2$, df = 29, $P = 1.0$). However, a significant difference between size classes was observed in the 7°C trial ($\chi^2 = 121.6$, df = 29, $P < 0.0001$) in which mussels in the smaller size class died an average of 3 days earlier than mussels in the larger size class. Therefore, further analysis of mortality rates associated with exposure to cold air temperatures was performed separately for both size classes at all temperatures.

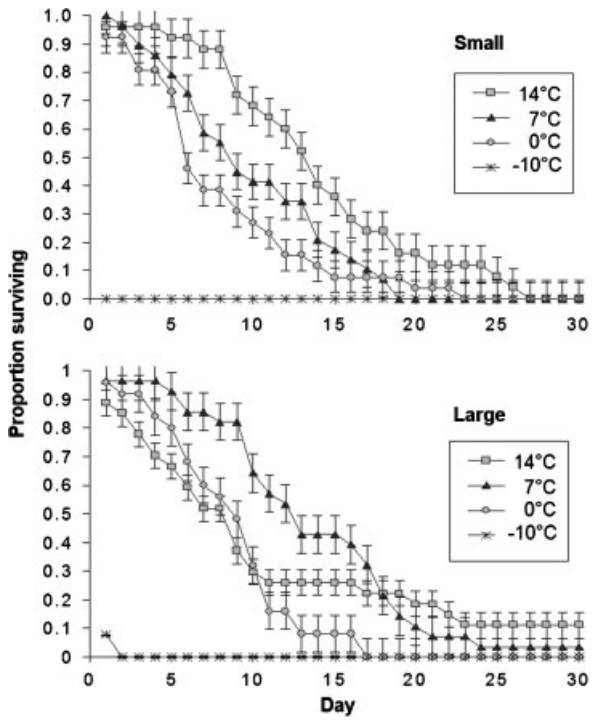

Figure 2. Proportion of *P. viridis* individuals surviving after a 2-hour exposure to 14, 7, 0, or −10°C air temperature. The large size class represents mussels 75–105 mm and the small size class represents 15–45 mm. Error bars represent standard error of the mean survival.

Both small and large size classes revealed a significant effect of temperature on survivorship. Analysis of the large size class showed that green mussels exposed to 14°C had significantly greater longevity than mussels exposed to −10°C ($\chi^2 = 112.1$, df = 29, $P < 0.001$). Exposure of large mussels to −10°C caused 100% mortality within 48 hours of exposure and was also significantly more lethal than 2-hour exposures to 7°C ($\chi^2 = 171.3$, df = 29, $P < 0.001$) and 0°C ($\chi^2 = 131.1$, df = 29, $P < 0.001$). Lower temperatures caused a decrease in survivorship in the large size between the 7°C and 0°C trials ($\chi^2 = 244.4$, df = 29, $P < 0.001$). However, no difference in survivorship was observed for large mussels exposed to 14°C and 7°C ($\chi^2 = 15.4$, df = 29, $P = 0.98$) or 0°C ($\chi^2 = 10.7$, df = 29, $P = 0.99$).

In the small size class, mussels exposed to 14°C had significantly greater longevity than mussels exposed to 7°C ($\chi^2 = 135.5$, df = 29, $P < 0.0001$), 0°C ($\chi^2 = 199.8$, df = 29, $P < 0.0001$) and −10°C ($\chi^2 = 134.9$, df = 29, $P < 0.0001$). All mussels exposed to −10°C died within 24 hr and this temperature was significantly more lethal than all other trials ($P < 0.001$ for all comparisons). Significant differences in survivorship were detected in all pair-wise comparisons except between the 7 and 0°C exposures ($\chi^2 = 13.9$, df = 29, $P = 0.99$).

Sub-Lethal Stress Response

The Hsp70 antibody used for western blotting recognized a single band in the 70 kDa range (Fig. 3). Mean relative band intensity differed significantly among temperature treatments ($F = 6.28$, $P < 0.0001$). All mussels tested in both the cold water and air trials produced significantly less Hsp70 than those heat shocked at 39°C. In the water temperature trials, the level of Hsp70 expression for cold-shocked individuals followed a bell-shaped pattern with a peak in expression observed at 10°C. This peak at 10°C represented a significant increase in Hsp70 expression relative to the 21°C control (Fig. 4). A slight but insignificant increase in expression relative to the 21°C control was also observed during exposure to 14°C water temperature. The air temperature trials resulted in a minimal increase in Hsp70

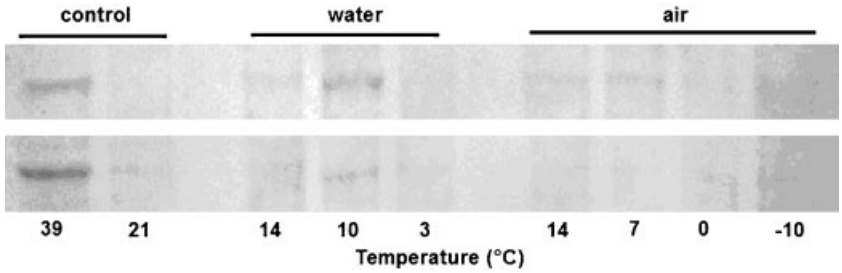

Figure 3. Western blots of Hsp70 expression. Lanes contain an equal amount (30 μg) of total protein.

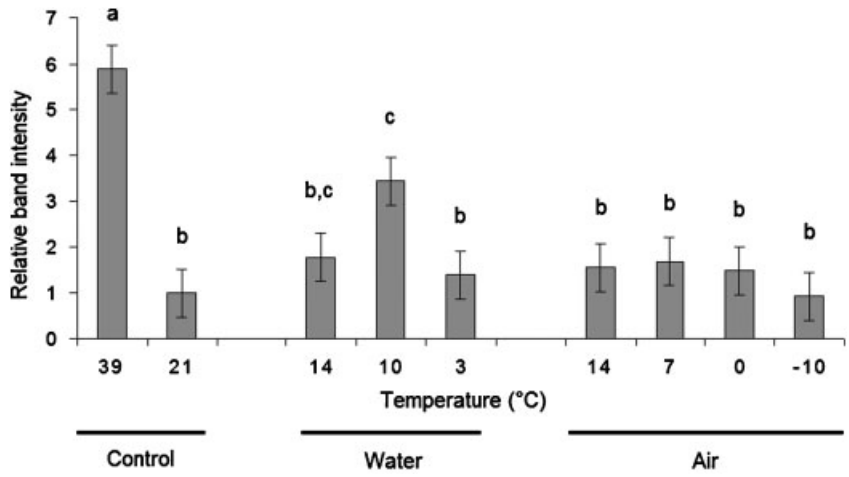

Figure 4. Intensity of Hsp70 production during heat shock, and cold shock via exposure to cold water and air temperatures relative to mussels maintained at 21°C. Error bars represent standard error of the mean relative intensity.

expression in 14, 7, and 0°C; however, none showed a significant increase in expression over the 21°C control.

DISCUSSION

Exposure to cold water temperatures decreases survivorship in *P. viridis*. Green mussels currently exhibit a patchy distribution in the coastal waters of northeast Florida (Baker et al., 2007). Over the past 25 years, this area has recorded an average January water temperature of 13.9°C (NOAA National Oceanographic Data Center). In this study, significant mortality (94% over 30 days) was observed at 14°C. In contrast, mussels exposed to 21°C had an 88% survival rate over the same time period, suggesting that more rapid mortality observed in the colder trials was predominantly due to the effect of temperature rather than laboratory housing conditions. Our data suggest that even in their current distribution, *P. viridis* is likely to experience winter die-offs that should limit population size under current conditions, and we have also observed some substantial overwinter mortality events in the field (personal observations).

In this study, 100% mortality was observed after 13 days exposure to 10°C water temperature. Sivalingam ('77), on the other hand, reported only 50% mortality after a 2-week exposure to 10°C water for cultured green mussels in Malaysia. Therefore, mortality rate in this study was nearly twice that of Sivalingam's ('77) study. In contrast to the sub-tropical Atlantic Ocean, the equatorial waters of the South China Sea near Malaysia generally range between 25 and 29°C in the cooler months (Mohsin and Ambak, '96). Therefore, the difference in temperature tolerances observed in the two studies has several potential explanations, including variations in acclimation, genetic adaptation, and experimental design. Regardless, the results of both studies suggest that significant mortality occurs at temperatures of 10°C, which corresponds with the average January water temperature in the coastal Savannah, GA and Charleston, SC region (NOAA National Oceanographic Data Center). Under the current conditions where coastal water temperatures are likely to remain at or below 10°C for a period of several weeks, green mussels introduced into northern Georgia and southern South Carolina are not likely to survive the winter, preventing the establishment of mature breeding populations of *P. viridis* in coastal waters of this region.

Despite numerous observations of individual green mussels and empty shells as far north as Charleston, SC (Power et al., 2004; Baker et al., 2007), no established coastal populations have been reported north of the Florida/Georgia border. However, established populations of green mussels have been observed 6–23 miles offshore attached to mooring buoys located at several of Georgia's artificial reefs (Georgia DNR, personal communication). Green mussel populations may be able to survive off the coast due to the warmer currents associated with the Gulf Stream. The Grays Reef Buoy (NOAA Station 41008) reports an average January water temperature of 13.6°C, over 3° warmer than coastal waters at the same latitude, but similar to the coastal water temperatures that support established populations in northeast Florida. The significant mortality observed in this study after chronic exposure to water temperatures of 14°C, coupled with the fact that no established populations have been reported in areas where average winter water temperatures are less than 13°C, suggests that the threshold for survival of *P. viridis* populations in the southeastern United States is likely between 10 and 14°C.

The effect of cold air temperatures on the survival of sub-tropical mussels has not been widely studied to our knowledge. As in the water temperature experiment, reference mussels exposed to 21°C had a high rate of survival (84% survival

30 days after aerial exposure), suggesting that the decreased survivorship observed in the air temperature trials was predominantly due to the effect of temperature rather than laboratory conditions. No significant differences in mortality were observed between mussels exposed to 14°C water temperatures and 14°C air temperatures. Additionally, green mussels exposed to 7°C air temperatures for 2 hr exhibited longevity similar to that of mussels kept submerged at 14°C. As 7°C air temperatures occur in St. Augustine simultaneously with 14°C water temperatures, it appears that exposure to typical low air temperatures does not further increase the rate of mortality in *P. viridis*. Freeze events, although infrequent, typically occur in northeast Florida an average of four times a year (GTMNERR monitoring data). Exposure to freezing air temperatures (0°C), reduced the mean survival time, suggesting that even one such cold shock event could cause a significant mortality event within the intertidal population. As aerial exposures are tide dependent, in some cases intertidal individuals could experience multiple exposures over the course of a few days, which could potentially increase the observed mortality rates. Owing to the fact that all mussels used in these experiments were from subtidal habitats, and therefore may be expected to be more sensitive to aerial exposure than the intertidal mussels that would routinely experience such conditions in the field and therefore these results may exaggerate the effect of aerial exposure. If intertidal mussels are less sensitive, colder air temperatures and/or longer exposure times may be required to induce significant mortality, which could expand the potential range of distribution. Additional experiments would be required to determine differences in tolerance between subtidal and intertidal sub-populations.

Previous studies on other bivalves have documented differences in temperatures sensitivity during periods of immersion and aerial exposure. During emersion, numerous species exhibit a gaping behavior when exposed to high temperatures. This gaping behavior permits evaporative cooling that keeps body temperatures several degrees below ambient air temperatures, increasing tolerance of extreme temperatures (Byrne et al., '88; Helmuth, '98). Conversely, *P. viridis* individuals generally exhibit a gaping behavior while submerged; however, when they are removed from the water they often quickly close their shells. It is possible that this behavior increases cold tolerance during periods of emersion by trapping water inside the shell. This would serve a dual purpose to prevent desiccation and to minimize the effects of rapid temperature change during short-term exposure to cold air temperatures.

Despite the fact that exposure to cold air temperatures resulted in significant mortality, the ability of *P. viridis* to tolerate chronic exposure to cold water temperatures is expected to be a more reliable predictor of its overall geographic distribution. Aerial exposure events require a specific set of circumstances (low spring tides corresponding with cold overnight temperatures) and these conditions are not likely to occur simultaneously very often in an area that typically experiences only four 0° days in an average winter. Additionally, although freeze events may occur occasionally all along the east coast, average January low air temperatures do not reach 0°C until the mid-Atlantic region near Virginia Beach, VA (NOAA; National Oceanographic Data Center). On the other hand, January water temperatures reach 10°C, which caused 100% mortality within 13 days in this study, much further south (near Savannah, GA). Therefore, green mussels would most likely experience population eradication via cold water temperatures before air temperatures could become a factor. Occasional freeze events in the southeastern United States are likely to generate significant mortality in intertidal sub-populations. However, exposure to cold air temperatures would not affect the subtidal portion of the population, making it more likely to determine vertical distribution as opposed to overall geographic distribution.

To our knowledge, the effect of age/size has not previously been analyzed in reference to cold tolerance for the green mussel, although significant size effects have been documented in the related *P. perna*, in which large individuals are reportedly more tolerant of high temperatures than small individuals (Hicks and McMahon, 2002). The small size class (15–45 mm) used in this study represents the "young of the year." Survival of "young of the year" is thought to be an important factor determining distribution patterns because larvae may be able to disperse and settle beyond their current range boundaries, but if they cannot survive the winter, permanent range expansion will not be able to occur. The effect of cold water exposure did not vary between sizes, indicating that small individuals were not more susceptible to cold water temperatures than larger individuals. This may be expected as green mussels typically exhibit gaping valves while submerged. It is likely that water flow during siphoning equilibrates the temperature throughout the mussel quickly enough that the effect of size becomes negligible. On the other hand, a significant effect of size was observed for the air temperature trials, where smaller mussels were more vulnerable to cold exposure at 7°C but no other temperatures. Based on volumetric differences and the principles of thermal inertia, if size were a factor, it would be expected that larger individuals would be more resistant to freezing, as observed here. This study suggests that size/age may influence survival at intermediate temperatures. However, the thermal inertia of larger individuals appears to be a relatively weak protective factor against environmental stress. Further replication of this study would be required to verify the effect of size on mortality at 7°C air temperature and rule out the possible confounding effect of pseudoreplication in this study.

Previous studies on cold tolerance in *P. viridis* have focused primarily on mortality (Sivalingam, '77; Nair and Appukuttan, 2003); however, sub-lethal effects of cold temperatures may also be an important factor in a species' ability to establish a viable population. This study used an analysis of heat shock protein production as a model to analyze sub-lethal effects of exposure to cold water and air temperatures. Previous studies have

documented seasonal increases in heat shock protein production in response to increased water temperatures during summer months in *Mytilus* species (Roberts et al., '97; Buckley et al., 2001). In this study, 2-hour exposures to 14 and 10°C water temperatures, and exposures to 14°C air temperature resulted in overall increases in Hsp70 production when compared with a 21°C control. Although only the 10°C water temperature resulted in a significant increase in Hsp70 expression, this study documents that green mussels do induce a stress response as a result of short-term exposure to cold temperatures. However, in this study, this sub-lethal effect was always accompanied by significant mortality. Therefore, although sub-lethal effects of cold stress may exist under some conditions, mortality events should supersede sub-lethal effects as the determining factor of range expansion in the southeastern region. Further studies are required to determine the duration of Hsp70 expression and its consequences on longevity, growth, and reproduction.

A thorough understanding of the thermal tolerance of invasive species may be crucial information for port cities that must determine which species to focus on with prevention programs. It may also be useful in the development of management strategies that rely on inducing sub-optimal environmental conditions. Although chlorine is often used in the management of aquatic invaders, inducing sub-optimal environmental conditions (heat) has also been successful in the localized eradication of species such as the introduced seaweed *Undaria pinnatifida* (Wotton et al., 2004). An understanding of thermal tolerance may also be important in light of the potential climatic changes associated with global warming. Experts suggest that air temperatures may become more erratic and extreme and that global sea surface temperatures could increase by 1.4–5.8°C over the next century (US National Climatic Data Center). Currently, green mussel populations are confined to a portion of the Gulf coast of Florida and the southeastern Atlantic coast as far north as southern Georgia. More extreme air temperatures would be likely to cause periodic mortality events within the intertidal portion of green mussel populations and may cause them to become predominantly subtidal along the northern edge of their range. Conservative estimates of a 1.4°C water temperature increase over the next 100 years would cause the coastal water temperatures near Savannah, GA to reach 12°C and could allow the current distribution of *P. viridis* to spread farther north into the coastal waters of Georgia. A more liberal estimate of a 5.8°C increase would raise the average January water temperatures near Cape Hatteras, NC to 13.7°C, making conditions suitable for green mussel survival throughout Georgia and the Carolinas. With the predominantly northward flowing currents it appears likely that *P. viridis* populations may continue to expand northward as water temperatures allow. Future studies will be needed to determine the availability of suitable substrate for *P. viridis* colonization in Georgia and the Carolinas, as well as their potential effects upon native species such as the Eastern Oyster, *C. virginica*.

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