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Potential for Range Expansion of the Invasive Green Mussel, Perna Viridis, in the Southeastern United States

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Potential for range expansion of the invasive green mussel, *Perna viridis,* in the southeastern United States

by

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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 two hours. Mortality was significantly increased at all water and air temperatures ≤ 14°C. No consistent differences in mortality rates were observed between small (15-45mm) and large (75-105mm) size classes after exposure to either cold water or air. Significant increases in Hsp70 expression were observed after a two 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 10-14°C which suggests that under current conditions, *P. viridis* may already be at the northern edge of its potential range in the United States. However, if water temperatures increase in association with global climate change, northerly flowing currents may permit range expansion as temperatures allow.

CHAPTER 1

POTENTIAL THERMAL CONSTRAINTS FOR RANGE EXPANSION OF THE INVASIVE GREEN MUSSEL, *PERNA VIRIDIS*, IN THE SOUTHEASTERN UNITED STATES

INTRODUCTION

Temperature is often thought to be the predominant factor determining the ultimate geographic distribution of marine invertebrates (Hutchins 1947; Seed 1976; 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). While 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). Environmental stressors at non-lethal, yet extreme temperatures, can induce a stress response that may reduce long-term survival (Seed 1976; Krebs and Feder 1998; Krebs and Feder 1997), growth (Feder et al. 1992), and/or reproductive success (Krebs and Loeschcke 1994). Therefore, both the lethal and sub-lethal effects of temperature are likely to play a pivotal role in defining the observable patterns of species distribution.

Here the Asian green mussel, *Perna viridis*, was used as a model to study the effects of cold temperatures on geographic distribution and range expansion. *Perna viridis* is native to the Indo-Pacific region (Sidall 1980, 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. 1992; Rylander et al. 1996; 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). *Perna viridis* has since spread along the Gulf coast and has also been discovered on the east coast of the US in St. Augustine, FL (2002) and Savannah, GA (2003) (Power et al. 2004).

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 26-32°C (Power 2004). Previous experiments have shown that green mussels have a 50% mortality rate after two week exposures to water temperatures of 10°C and 35°C (Sivalingam 1977). Water temperatures below 24°C have been shown to delay metamorphosis and larval development of *P. viridis* (Nair and Appukuttan 2003). 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. Chronic exposure to cold 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.

In addition to the chronic stress generated by exposure to sub-optimal winter water temperatures, 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. During spring tides, green mussels in the St. Augustine area are commonly exposed for periods of up to four hours (personal observation), presenting the potential for significant physiological stress. St Augustine, FL is near the northern edge of the current distribution of *P. viridis,* and therefore, northeast Florida presents a good location to study the potential for range expansion in this sub-tropical species.

Analysis of heat-shock protein (Hsp) induction can be a good model to monitor sub-lethal stress upon an organism (Dalhoff 2004). 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 have been shown to reduce growth and reproductive output in *Mytilus galloprovincialis* and *Perna canaliculus* (Petes et al 2007). 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 1988; Hendrick and Hartl 1993; Parsell and Lindquist 1993; Feder and Hoffman 1999; Gonzales-Riopedra et al 2007). Heat shock protein induction has been documented in numerous bivalve species including *Mytilus spp* (Buckley et al. 2001), *Dreissena polymorpha* (Singer et al. 2005), and *Crassostrea gigas* (Hamdoun et al. 2003). Induced expression of heat shock proteins has been shown to decrease the larvae to adult survival rate (Krebs and Feder 1997), reduce fecundity (Krebs and Loeschcke 1994), and lower growth rates (Feder et al. 1992) in *Drosophila melanogaster.* While 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 1997).

As temperature is likely to be among the most important factors determining the potential for range expansion of tropical exotic species, the present study was designed to examine the effects of lethal and sub-lethal cold stress on the Asian green mussel, *Perna viridis.* Mortality experiments were conducted on two size classes to investigate the effect of size/age on thermal tolerance. Based on volumetric difference it is expected that larger mussels may take longer to feel the effects of cold stress during aerial exposure. Mussels with a shell size greater than 70mm are likely to have survived a previous winter, and therefore may also be more likely to survive subsequent cold stress. 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.

METHODS

SAMPLE COLLECTION

For all experiments, samples 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 near St. Augustine. All samples were collected from entirely sub-tidal habitats to minimize possible effects of varied thermal histories. Mussels from different sites were acclimated together and were haphazardly assigned to experimental treatments.

Perna viridis specimens of two size classes, 15-45mm shell length (n=90) and 75-105mm 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 liter aquaria in 35ppt 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). Environmental chambers were maintained on a 12 hour light/dark cycle and a 50% water change was performed every other day.

Due to significant mortality observed in the 14°C water and air trials (see Results), additional mussels were collected in March 2009 and reference groups (21°C) were added to

mimic non-stressful conditions to determine if 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. Reference groups were acclimated and maintained at 21°C for the duration of the experiment. Data from the 21°C trials were used for reference only and were not included in statistical analyses.

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. Following 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°C, 10°C 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 320km 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 liter aquarium and were placed into an environmental chamber maintained at constant temperature and a 12 hour 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 24h hours. 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 ten seconds 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 two hour exposure to cold air temperatures (14°C, 7°C, 0°C, and -10°C) was assayed. Experimental procedures were selected to represent ecologically relevant exposures of both temperature and time. 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. During spring tides, mussels in the intertidal zone regularly experience emersion times of up to four hours (personal observation). Therefore, groups of 30 mussels from each size class were exposed to each temperature for 120min, 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°C, 10°C and 3°C) as described in the chill resistance experiment and air temperatures (14°C, 7°C, 0°C, and -10°C) as described in the freeze resistance experiment. Mussels were exposed to experimental treatments for 120 minutes, with the exception of the -10°C air temperature trial which was only exposed for 20 minutes because longer times were lethal (see Results). Following cold shock exposure, mussels were removed and placed in a 14°C recovery tank. Since cold temperatures are likely to slow protein synthesis, cold shocked mussels were allowed to recover for 20 minutes before being dissected (Liu et al 1994).

Heat Shock Protein Analysis

Heat shock protein (Hsp70) expression was determined by Western Blot, as previously described by Hoffman and Somero (1995) 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. Following cold shock, mantle tissue was dissected from each individual and immediately frozen and homogenized in liquid nitrogen. Frozen tissue was then placed in 100µL Tris-Buffered Saline (TBS) with protease inhibitor and centrifuged at 13,000rpm for 5 minutes. Total protein content was determined by Bradford Assay (Bradford 1976) 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 one hour was used as a positive control on each gel and tissue from mussels maintained at 21°C was used as a negative control. Proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) for 150min at 110V and then transferred to a nitrocellulose membrane (Whatman, Piscataway, NJ) using a semi-dry transfer apparatus at 18V for 30min. Membranes were

incubated for 90 minutes in a 1:1000 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 minutes in a 1:5000 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 *Perna viridis* (Fig. 1). The median number of days to death did not vary between size classes at 14°C (X^2=16.8 , df=29, p=0.965) or 10°C (X^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.

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 (X^2=462.1 , df=29, p<0.0001) and 3°C (X^2=297.5 , df=29, p<0.0001) while mussels exposed to 10°C also had a greater survival rate than those exposed to 3°C (X^2=254.3 , df=29, p<0.0001). All mussels exposed to 3°C died within the first 24 hour period, while 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 a negative control in the original experimental design, but 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 non-stressful 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 in the 21°C trial lived an average of 29 days.

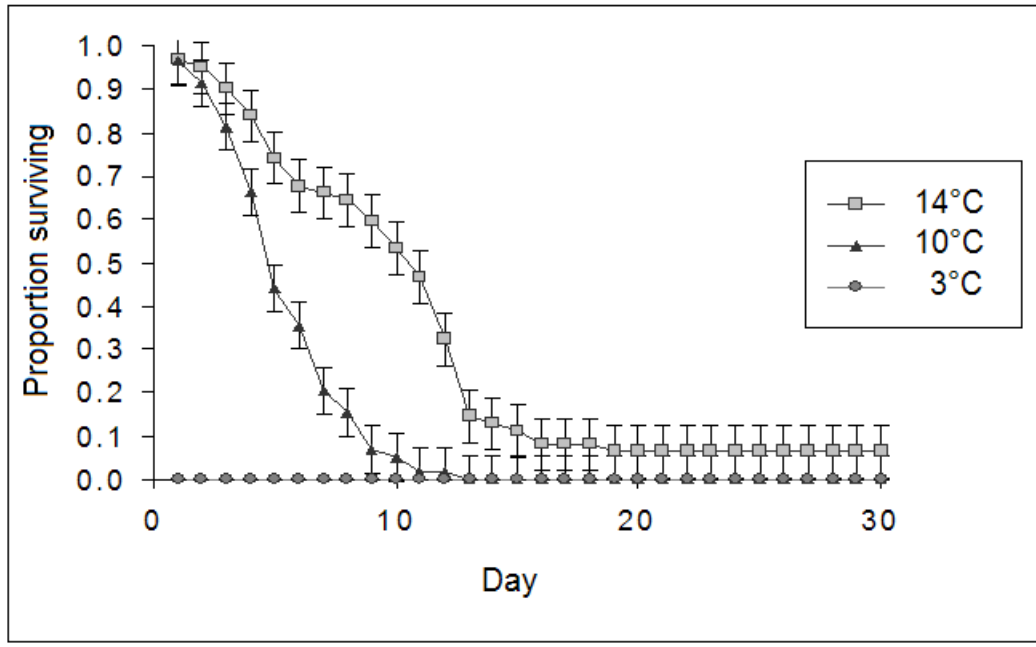

Fig. 1. Proportion of *P. viridis* individuals surviving during a thirty day exposure to 14°C, 10°C, or 3°C water temperature. Error bars represent standard error of the mean survival.

Freeze Resistance - (Cold Air Temperatures)

Two hour exposures to cold, freezing, and sub-freezing air temperatures significantly decreased survivorship in *Perna viridis* (Fig. 2). The median number of days to death decreased with a decrease in temperature for both size classes. As with the water temperature trials, no significant differences were observed between size classes at 14°C (X^2=10.1 , df=29, p=0.99),

0°C (X^2=0.6 , df=29, p=1.0), or -10°C (X^2=4.2 , df=29, p=1.0). However, a significant difference between size classes was observed in the 7°C trial (X^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.

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 (X^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 two hour exposures to 7°C (X^2=171.3 , df=29, p<0.001) and 0°C (X^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 (X^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 (X^2=15.4 , df=29, p=0.98) or 0°C (X^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 (X^2=135.5, df=29, p<0.0001), 0°C (X^2=199.8 , df=29, p<0.0001) and -10°C (X^2=134.9 , df=29, p<0.0001). All mussels exposed to -10°C died within 24 hours 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°C and 0°C exposures (X^2=13.9 , df=29, p=0.99).

When size class data were pooled for the air temperature trials, both 14°C and 7°C air temperatures resulted in mean survivorship of just over 12 days (12.3 and 12.2 days respectively). Both small and large size classes had an average survival of just over 8 days (8.4 days pooled average) when exposed to 0°C, with 100% mortality after 23 and 17 days respectively. The 21°C reference mussels exhibited an average survival of 28.1 days.

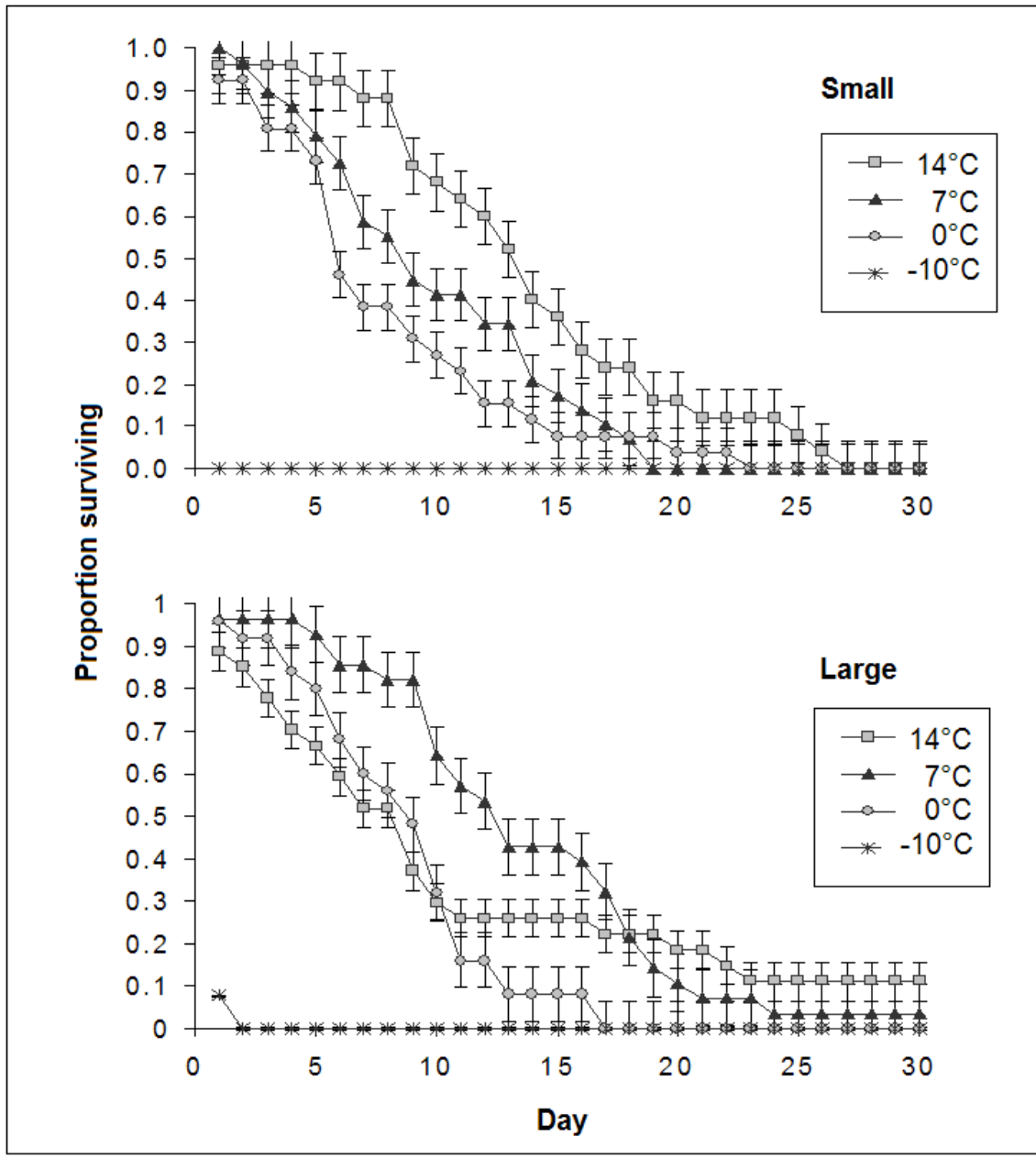

Fig. 2. Proportion of *P. viridis* individuals surviving following a two hour exposure to 14°C, 7°C, 0°C or -10°C air temperature. The large size class represents mussels 75-105mm and the small size class represents 15-45mm. Error bars represent standard error of the mean survival.

SUB-LETHAL STRESS RESPONSE

The Hsp70 antibody used for western blotting recognized a single band in the 70kDa 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 expression in 14°C, 7°C and 0°C, however none showed a significant increase in expression over the 21°C control.

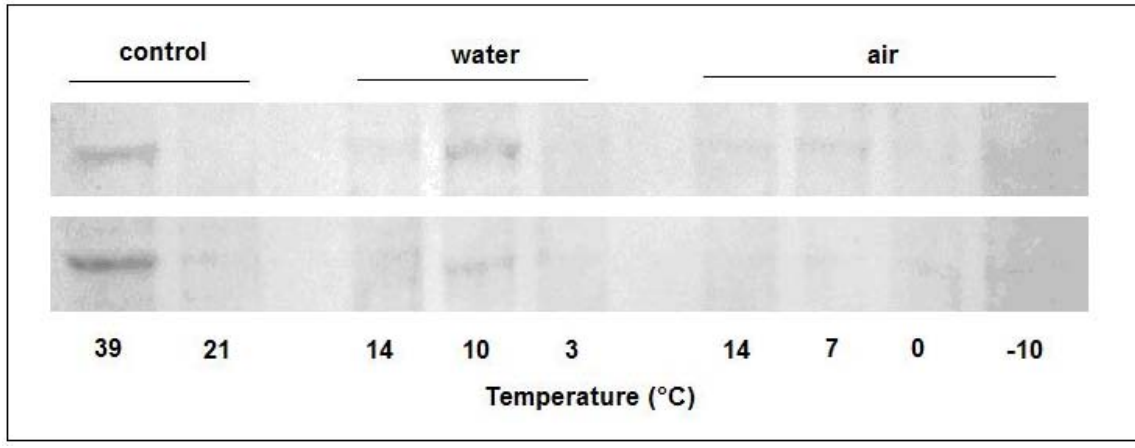

Fig. 3. Western blots of Hsp70 expression. Lanes contain an equal amount (30µg) of total protein.

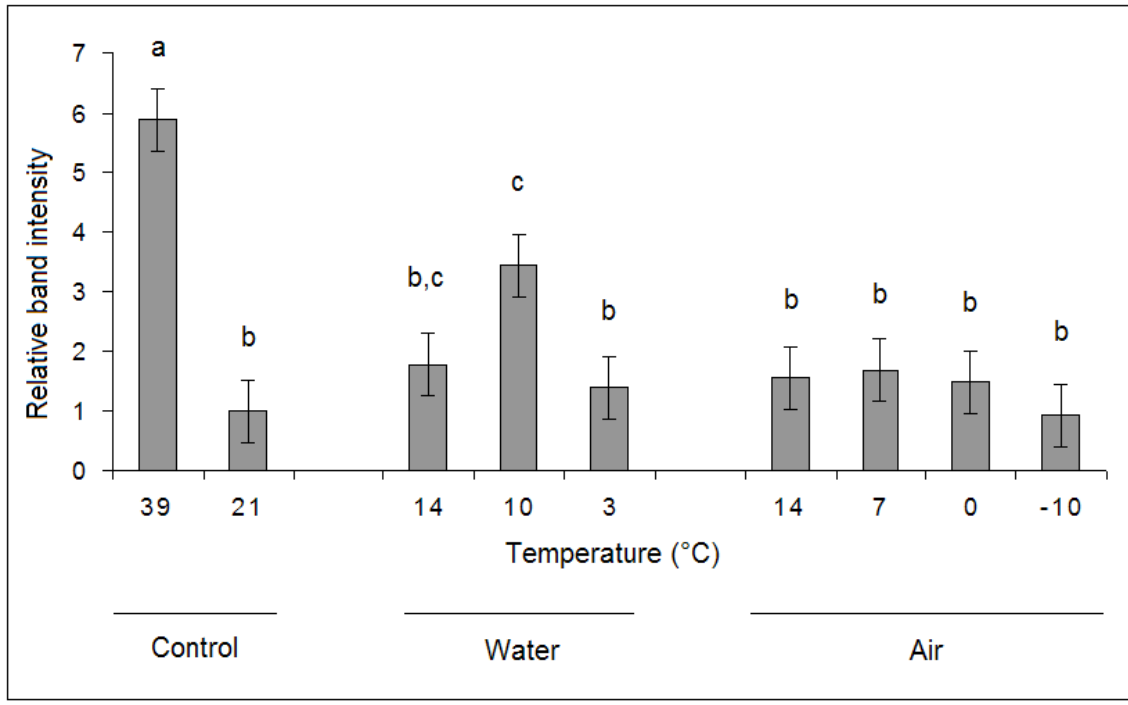

Fig. 4. Intensity of Hsp70 production during heat shock, and cold shock via exposure to cold water and air temperatures relative to mussels maintained at 21°C. Error bars represent standard error of the mean relative intensity and letters depict statistically significant results.

DISCUSSION

Exposure to cold water temperatures decreases survivorship in *Perna viridis*. Green mussels currently exhibit a patchy distribution in the coastal waters of northeast Florida (Baker et al. 2007), an area that has had an average January water temperature of 13.9°C over the past 25 years (NOAA National Oceanographic Data Center). In the present study, significant mortality was observed at 14°C with only a 6% of animals surviving for the full 30 days of the experiment in addition to the 14 day acclimation period. In contrast, mussels exposed to 21°C had an 88% survival rate, suggesting that more rapid mortality observed in the colder trials was 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 partial winter die-offs that should limit

population size under current conditions in this region, and we have observed some substantial overwinter mortality events in the field. Green mussels experienced 56% mortality after five days exposure to 10°C water. Sivalingam (1977), on the other hand, reported 50% mortality after a two week exposure to 10°C water for cultured green mussels in Malaysia. Therefore, mortality rate in the present study was nearly twice that of Sivalingam's (1977) study. 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. The average January water temperatures in the coastal Savannah, GA and Charleston, SC region are approximately 10-10.5°C (NOAA National Oceanographic Data Center). Despite numerous observations of individual green mussels and empty shells as far north as Charleston, SC (Baker et al. 2007; Power et al 2004), no established coastal populations have been reported north of the Florida/Georgia border. In the present study, 100% mortality was observed after 13 days exposure to 10°C water temperature. 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.

On the other hand, 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). These 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 three degrees warmer than coastal waters at the same latitude. Offshore temperatures in Georgia are similar to the coastal water temperatures that support established populations in northeast Florida. The significant mortality observed in the present study following 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 10-14°C. Consequently it is unlikely that *P. viridis* will become invasive further north than southeast Georgia under current climate conditions.

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. In the present study, green mussels exposed to 7°C air temperatures for two hours lived an average of 10-13 days following exposure, similar to the longevity of mussels kept submerged at 14°C. Exposure to typical low air temperatures does not seem to further increase the rate of mortality. Freeze events, while infrequent, typically occur in northeast Florida several times a year. Exposure to freezing air temperatures (0°C), reduced the mean survival time for *P. viridis* to 8 days, suggesting that even one such cold shock event could cause a significant mortality event within the intertidal population. As exposures are tide-dependent, in some cases intertidal individuals could experience multiple exposures to freezing air temperatures over the course of a few days, which could potentially increase the observed mortality rates. In comparison, the sub-tropical species *Perna perna* does not experience significant mortality until exposure to -2.5°C, with 100% survival observed after six hour exposures to 0°C air temperatures (Hicks and McMahon 2002). Therefore, green mussels appear to be less tolerant of cold air temperatures than the related *P. perna.*

Despite the fact that exposure to cold air temperatures resulted in significant mortality, cold shock via aerial exposure is not expected to be as important as chronic exposure to cold water temperatures in determining the geographic distribution of *P. viridis.* 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 four zero degree days in an average winter. Additionally, while 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, where average January water temperatures are a mere 5.8°C (NOAA National Oceanographic Data Center). In the present study, chronic exposure to 10°C water temperatures was lethal after only 13 days and therefore green mussels would most likely experience complete population eradication as a result of exposure to cold water temperatures before air temperatures could become a primary factor. Furthermore, only a portion of the population falls within the intertidal region. Therefore, exposure to cold air temperatures would not affect the sub-tidal portion of the population. Cold air temperatures may, however, influence the vertical distribution of *P. viridis*, causing populations to become predominantly subtidal in areas where tidal emersion during winter months results in increased mortality.

To our knowledge, the effect of age/size has not previously been analyzed in reference to cold tolerance for the green mussel. The small size class (15-45mm) used in the present 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 since water flow during siphoning should equilibrate the temperature throughout the mussel rather quickly regardless of size. 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, if size were a factor, it would be expected that larger individuals would be more

resistant to freezing, as observed here. However, if this were a real factor it should be observed at other temperatures, not just at 7°C. This suggests that this difference may be an artifact, potentially of the closed system design, rather than a real effect. Mussels for each trial were housed together in a single aquarium and therefore, death of a single individual could trigger mortality in other individuals. Further replication of this study would be required to verify the effect of size on mortality at 7°C air temperature. Effects of size have been documented in *P. viridis* when exposed to varying concentrations of chlorine, with larger/older mussels showing greater resistance (Rajagopal et al 2003) and large *Perna perna* individuals are reportedly more tolerant of high temperatures than small individuals (Hicks and McMahon 2002). Therefore, it appears that size can sometimes be an important factor in determining tolerance to physical conditions for some mussels. However, no consistent effect of size was observed in the present study and, therefore, it is unlikely that differential response of *P. viridis* of different sizes to cold temperatures will play any role in limiting their geographic range.

Previous studies on cold tolerance in *P. viridis* have focused primarily on mortality (Sivalingam 1977; 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. The present 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. In all cold shock trials, the observed expression of Hsp70 was significantly less than that observed in heat shocked individuals. This would be expected as cold shock is not as damaging to protein structure as heat shock. Additionally, the lower expression of Hsp70 at cold temperatures may be due to the reduction in metabolic functions and protein synthesis associated with cold temperatures in ectothermic animals. Two hour exposures to 14°C and 10°C water temperatures resulted in a two-fold and three-fold increase in Hsp70 production, respectively, relative to a 21°C control. While the observed trend may indicate some stress associated with exposure to 14°C, significant increases in Hsp70 expression in *P. viridis* only occurred during exposure to 10°C water temperatures. Such an

increase in Hsp70 expression indicates that individuals may have experienced an increase in protein damage as a result of exposure 10°C. The present study documents that green mussels do induce a stress response as a result of short-term exposure to cold water temperatures. In an ecological context, green mussels in northeast Florida would experience chronic exposure to winter water temperatures, and therefore further studies are required to determine the duration of Hsp70 expression. If heat shock protein expression remains elevated for the duration of exposure, the stress response could have deleterious consequences on longevity, growth and reproduction. As previously noted, water temperatures that remain at 10°C for longer than two weeks will not be able to support *P. viridis* populations. Significant sub-lethal stress does not appear to occur in the absence of significant mortality and therefore, mortality associated with chronic cold water exposure is likely to be a more important determining factor in defining the geographic distribution of *P. viridis* in the southeastern United States.

Exposure to cold air temperatures did not elicit as dramatic an increase in Hsp70 expression as exposure to cold water temperatures. Exposure to 14°C air temperatures induced nearly a two-fold increase in Hsp70 expression, similar to the effect observed after exposure to 14°C water temperature. However, exposure to 7°C air temperatures only generated a 50% increase relative to the control. *Perna 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 allows them to trap 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. The insulation generated by this behavior may prevent or delay the need for physiological defenses such as heat shock protein production. The production of such proteins is metabolically costly and, while the expression of Hsp70 helps to maintain survival, it may also divert resources from other functions such as growth and reproduction (Krebs and Feder 1997). Further studies are required to investigate the

impact of cold-induced heat shock protein production on longevity and reproductive activity/success in *P. viridis.*

A thorough understanding of the thermal tolerance of an invasive species is important not only for current prevention and management strategies, but also in light of the potential climatic changes associated with global warming. Experts suggest that the global sea surface temperatures could increase by 1.4°C – 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. 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. Therefore, it appears likely that *P.* viridis populations may continue to expand northward as water temperatures allow.

Under current conditions, *P. viridis* may already exist at the northern edge of its potential range along the east coast of the United States. However, if water temperatures increase in association with global climate change, northerly flowing currents would allow this potential range to expand in a northward direction throughout the southeastern United States. Future studies will be needed to determine the availability of suitable substrate for *P. viridis* colonization in Georgia and the Carolinas. Should green mussels become established in these areas, little is known about their potential effects upon native species. Future studies will be needed to address the interactions between *P. viridis* and economically important native species such as the Eastern Oyster, *Crassostrea virginica.*

CHAPTER 2

REPRODUCTIVE CYCLE OF THE ASIAN GREEN MUSSEL, PERNA VIRIDIS, IN ST AUGUSTINE, FL

INTRODUCTION

Increases in world trade, petroleum exploration, aquaculture industries and recreational activities have caused a breakdown in the natural boundaries that help maintain ecological stability and structure (Carlton 2001, Ray and McCormick-Ray 2004, Kraft et al 2002). Invasive species, following habitat destruction, are considered the second greatest cause of loss of biodiversity and extinction in native populations (Enserink 1999, Carlton 2001). Since the 18th century, the rate of known marine introductions has increased exponentially, a trend that shows no signs of slowing (Carlton 2001). There is speculation that green mussel population in St. Augustine was introduced via fouling on the hull of a Tampa-based barge that sat off the coast of St. Augustine for months during a beach re-nourishment project in 2002 (Maia McGuire, FL Sea Grant, personal communication). With the recent onslaught of tropical systems eroding the Florida coast and the increased focus on beach re-nourishment, this creates concern about the potential for future invasive species introductions.

As invasive species become established, they can have severely deleterious effects upon the native ecosystem by causing habitat loss, shifts in nutrient cycling, and the decline of native species (Carlton 2001, Bax et al. 2003). Invasive species have the potential to interact and compete with local economically important species (Simberloff et al 1997). The control and management of invasive species can also be financially burdensome. For instance, marine bio-fouling communities can drastically increase the maintenance costs for power plants and shipping companies when they use intake pipe or ship hulls as a substrate (Rajagopal et al 1995). For these reasons, the impacts of invasive species are both biologically and economically important. A thorough understanding of how these populations reproduce and disperse upon introduction to a new environment is an integral step in developing a plan for the management, control and prevention of invasive species.

Perna viridis, native to the Indo-Pacific region, is a sessile bivalve filter-feeder that attaches by means of byssal threads to hard substrates. They are generally found within a few meters of the surface (Yap et al 1979). When mature (20-30mm in length), they spawn, releasing eggs and sperm freely into the water column. After fertilization larvae remain free-swimming for 15-20 days before settling on a substrate (Yap et al 1979). In their native range, green mussels spawn twice a year, once in spring and once in the fall, with the precise timing dependent upon locality, water temperature, and salinity (Yap et al 1979).

In the absence of other limiting factors, the potential for population growth and geographic range expansion should be dependent upon two main factors, the frequency of spawning and the ability of larvae to disperse from the parental range. A number of factors, including temperature (Orton 1920) and resource availability (Newell et al. 1982), have been shown to influence the timing of reproduction in marine invertebrates. The goal of this study was to determine the peak spawning season(s) of the established population in St. Augustine, FL and to compare the observed reproductive cycle with local water quality monitoring data.

METHODS

SAMPLE COLLECTION

To analyze the frequency of spawning, mature mussels were collected on a monthly basis from Anastasia State Park (St. Augustine, FL) from January to December 2006. Ten individuals between 30-50mm were collected from the jetty on the south side of St. Augustine inlet. All mussels were collected from intertidal locations accessible during low spring tides. Individuals were returned to the University of North Florida where they were sacrificed.

ANALYSIS OF REPRODUCTIVE CONDITION

Gonad tissue was dissected and preserved in Baker's formalin. During dissection, the sex of each individual was determined by visual identification. The gonad tissue of female *P. viridis* individuals is bright orange in color, while males are a creamy white color. Histological preparations were made of the gonad tissue following the protocol of Walter (1982). Tissue was dehydrated and then imbedded in paraffin wax. Two slides were prepared for each individual using two 7µm sections from within the paraffin block per slide. Slides were stained with hematoxylin and counterstained with eosin and then were analyzed using a compound microscope. Two measures of reproductive maturity were calculated for each sample.

The gamete volume fraction (GVF) was determined for each month by using a dot matrix. Measurement of gamete volume fraction was used to determine the percentage of developing or ripe gametes in each sample. GVF ranges from a value of 0 (reproductively inactive) to 1 (maximum reproductive development). Five estimates were made and averaged for each individual. In addition, a gonad index was determined for each monthly sample. The gonad index was calculated for each individual using the methods of Walter (1982) along the following scale: Stage 1 (inactive), Stage 2 (active development), Stage 3 (ripe), and Stage 4 (spawning).

The proportion of mature gametes typically increases just prior to spawning and then sharply declines post-spawn. Observations of these trends were used to determine the peak spawning period(s) for the established population in St. Augustine. These measures of reproductive development were then plotted against water temperature and chlorophyll concentration data from Guana Tolomato Matanzas National Estuarine Research Reserve (GTMNERR) to investigate the influence of environmental factors on spawning.

RESULTS

A total of 97 slides were analyzed, of which 62 were male and 35 were female. The sex ratio for *P. viridis* individuals analyzed in the present study was significantly different from 1:1 (χ^2=7.515, p=0.0061), with more males than females.

Two measures of reproductive maturity were performed on each sample. The gamete volume fraction (GVF), which measures the percentage of mature and immature gametes in relation to other tissue types, showed very little variation over the 12 month study period (Fig. 1). A low value (.72) was recorded in January and a high value (.91) was observed in December. These data suggest that in Northern Florida green mussels retain a high level of reproductive maturity year-round.

A second measure of reproductive development was performed by assigning each sample to one of four developmental stages. The gonad index shows a similar trend except during the summer months (Fig. 1). The gonad index shows that reproductive maturity drops to around 50% from June through August. Two peaks in development can be identified from this measure, one in April/May and a second in September. The gonad index appears to be a more accurate assessment of actual reproductive development and this measure was used for comparison with environmental factors.

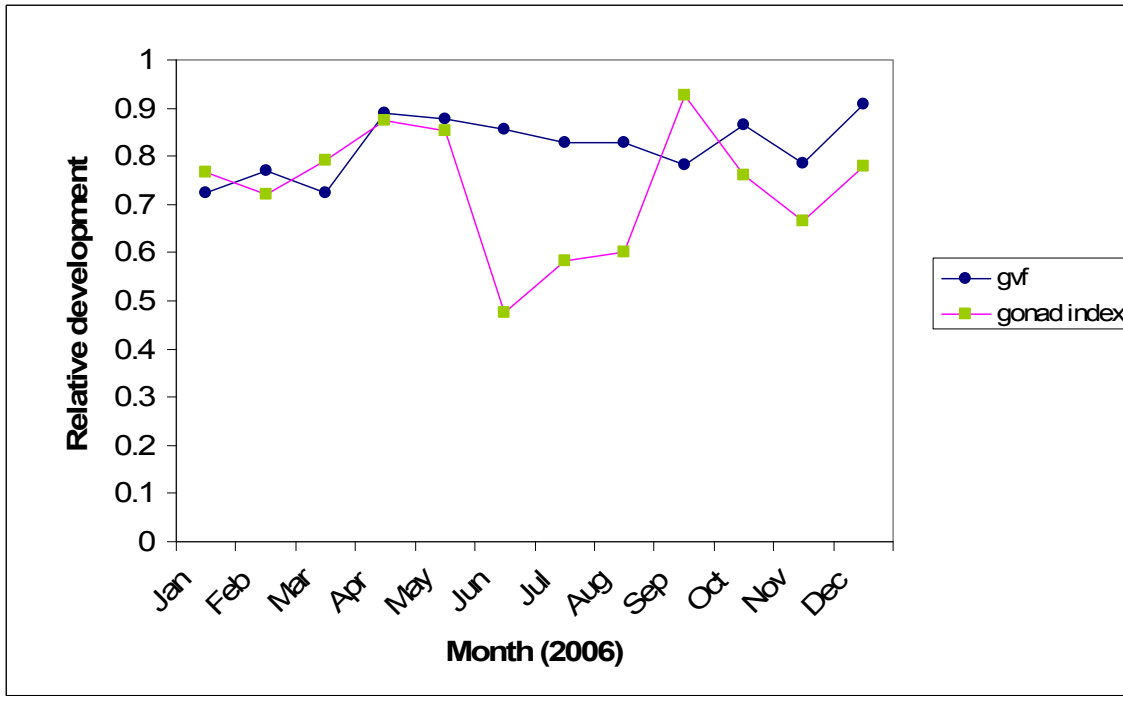

Fig. 1. Two measures of reproductive development in *Perna virids* from St. Augustine, FL. Gamete volume fraction measures the proportion of mantle tissue composed of developing and ripe gametes. The gonad index is a categorical analysis of the state of reproductive development.

The average gonad index for each set of monthly samples was plotted against 2006 monitoring data from Guana Tolomato Matanzas National Estuarine Research Reserve to assess the effects of temperature on reproductive development (Fig. 2). Temperature data shows a bell-shaped curve with a peak temperature (29.2°C) in July. Data from January and February 2006 was missing from this data set, so the low temperature (17.4°C) was observed in December. The relative reproductive development seems to follow a similar pattern to the increasing water temperatures during the early spring months and again in the late fall. However, temperatures continue to increase during the mid-summer while reproductive activity declines.

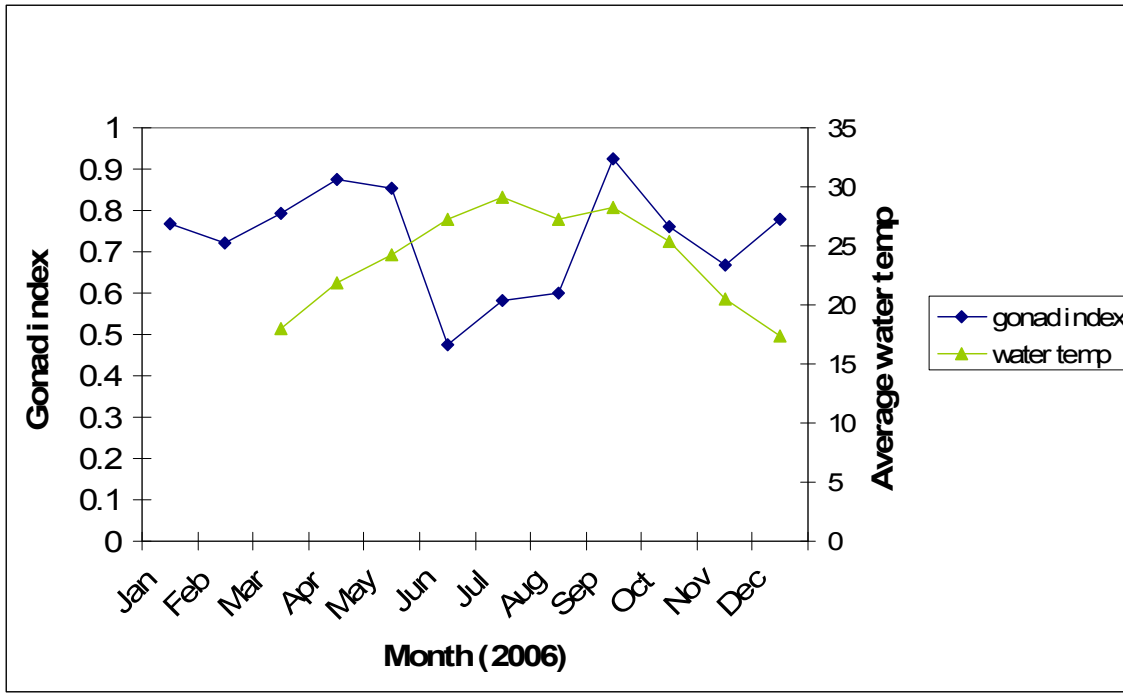

Fig. 2. Comparison of green mussel gonad index and average monthly water temperatures in St. Augustine, FL

The gonad index for each month was also compared to chlorophyll a levels (Fig. 3). Chlorophyll levels steadily increased to a maximum of 12.5 µg/L in August 2006 and then began to decline again in October. As with temperature, increases in reproductive development appear to coincide with increases in chlorophyll concentration in the early spring. The second spawning peak occurs simultaneously with the peak chlorophyll concentration.

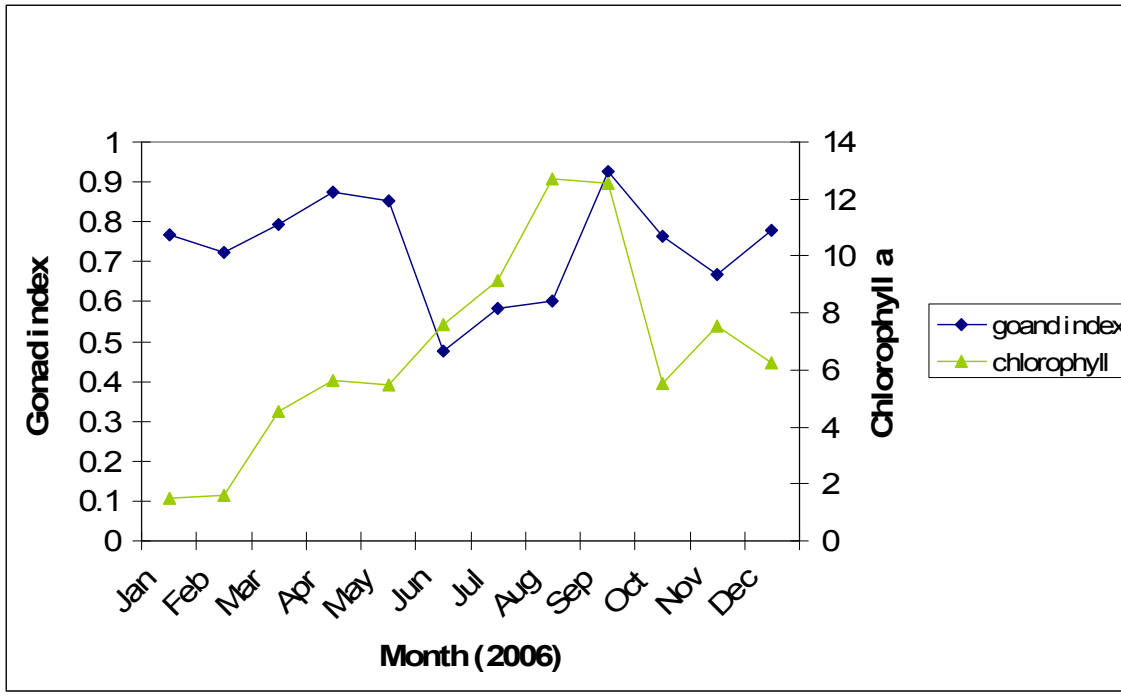

Fig. 3. Comparison of gonad index and monthly average chlorophyll a levels in St. Augustine, FL.

DISCUSSION

The data from the gonad index estimation of reproductive development was selected for analysis with environmental conditions because this measure appears to be a more accurate representation of the actual developmental state. The design of the gamete volume fraction can overestimate the level of development due to the differences in size between male and female gametes. To use this method, a dot matrix is overlaid on top of a projection of the histological preparation. The measure analyzes the percentage of mature and immature gametes that fall beneath these dots. Due to the size differential between male and female sex cells, this method consistently records a high level of development in male specimens. The sex ratio in this study

was not constant, and therefore months with more males reported higher development levels. By categorizing the specimens into relative development levels, this problem was resolved and a more accurate gonad index was used to compare to environmental factors such as temperature and chlorophyll concentration.

The gonad index recorded for this 2006 data set was bimodal with two mild peaks in spawning, once in the spring (April/May) and once in the fall (September). This is consistent with data from mussels in their native range. Sivalingam (1977) reported two peaks in spawning for cultured mussels in Malaysia, the first in March/April and the second in October/November. In India, green mussels also show two peaks in May/June and October/November (Rajagopal et al. 1998). The introduced population in Tampa, FL has also been shown to have two peaks in reproductive activity, the first in April and a later peak from September to November (Barber et al. 2005).

Both the gamete volume fraction and the gonad index show that *P. viridis* maintains a high level of reproductive development for a majority of the year. None of the individuals analyzed in the present study could be classified as inactive. Several live mussels collected in December 2007 in association with a separate mortality study were observed to spawn upon transport to our laboratory (personal observation). Hence, we can conclude that the environmental conditions in St. Augustine are favorable for reproductive development and sub-optimal temperatures do not appear to limit the production of mature eggs and sperm in this region.

When compared to the average monthly water temperature, the gonad index does not initially appear to follow the same trend. However, it is possible that temperature does play a role in triggering reproductive activity. Peak reproductive activity appears to occur at temperatures above 20°C. It is possible that there is a threshold temperature that triggers development and spawning in the spring. The low reproductive activity observed during the mid-summer months is then likely to be a result of synchronized spawning. Following the peak spawning in

April/May, spawned mussels must redevelop mature reproductive cells. It is probable that this process would take several months. Given suitable conditions, redeveloped mussels would then spawn a second time before temperatures dropped to sub-optimal levels.

Chlorophyll a is a good estimator of the food resources available to mussels. An adequate food supply would be necessary for the development of gonad tissue. Hence, increasing levels of chlorophyll seen in the early spring, coupled with increasing temperatures, could act as a stimulus for reproductive development. The peak chlorophyll concentration that appears to correspond to the second peak in spawning may, however, be more important to settlement patterns than for spawning. Spat settlement typically occurs roughly a month after spawning. Therefore, high levels of chlorophyll would be present following the first spawning event, but much lower levels would be available for the spat of the second spawning cycle. We would expect that the survival rate for the first spawning event would be greater than for the second event if this was true. Previous reports have shown a correlation between chlorophyll levels and settlement patterns, indicating that this food source is important for the growth and survival of young mussels.

This study was conducted as a pilot study for a larval dispersal/settlement study. Given the small sample sizes used here, it is advisable that future studies of this nature be conducted with more individuals and over multiple years. These data would show year-to-year variation in reproductive development and would provide a more accurate representation of typical reproductive behavior in Northeast Florida. Additional studies should also be conducted to analyze the patterns of larval settlement and their potential correlation to environmental factors and distance from the source population. All of these data would provide valuable information in regards to the potential for range expansion of *Perna viridis* in Northeast Florida and the Southeastern United States.

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PUBLICATIONS and PRESENTATIONS

Whipple, A.V., W.G. Abrahamson, M.A. Khamiss, P.L. Heinrich, A.G. Urian & E.M. Northridge. 2009. Host-race formation: promoted by phenology, constrained by heritability. Journal of Evolutionary Biology. 22(4):793-804.

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