

**Evolution of temperature tolerance in the introduced seaweed
*Gracilaria vermiculophylla***

An essay submitted in partial fulfillment of
the requirements for graduation from the

Honors College at the College of Charleston

with a Bachelor of Science in
Marine Biology

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MAY 2016

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Introduction

Biological invasions have increased in frequency in recent decades due to globalization and the alteration of ecosystems (Hufbauer et al. 2012). Introduced species impact biodiversity, ecosystem integrity, agriculture, fisheries, and public health (Lee 2002). Invasions result in a decrease of biological distinction between geographic regions of the world by replacing native flora and fauna through biological homogenization (Lee 2002). Yet, the majority of studies have focused on the role of broad physiological response or plasticity in facilitating successful invasions (Lee 2002). Nevertheless, the success of introduced species may also depend on genetic adaptation in response to natural selection (Lee 2002, Smith et al. 2009). For example, adaptation of increased temperature tolerance may aid in facilitating an invasion. Rey et al. (2012) documented this in the adaptation of cold tolerance in the *Wasmannia auropunctata* invasion (Rey et al. 2012).

Potential evolutionary pathways of temperature tolerance include pre-adaptation, post-adaptation, and bridgehead scenarios (Rey et al. 2012, Lombaert et al. 2010). In pre-adaptation scenarios, pre-existing traits in some native populations allow a species to successfully expand to new regions. This scenario occurred during the introduction of the ragwort *Senecio inaequidens* (Bossdorf et al. 2008) and of the ant *Wasmannia auropunctata* (Rey et al. 2012). In a post-adaptation scenario, a species is introduced to a new region then undergoes evolutionary change in response to new environmental pressures (Rey et al. 2012), as demonstrated in the introduction of the copepod *Eurytemora affinis* from estuarine to freshwater environments in North America, Europe, and Asia (Lee 1999, Lee and Petersen 2002, Lee et al. 2013). In a bridgehead scenario, a species adapts in a new region and this region serves as a launching point for subsequent invasions, as in the harlequin ladybird (Lombaert et al. 2010). When no genetic adaptation has occurred, phenotypic plasticity may allow for a species' introduction to novel

habitats, as with the alligator weed *Alternanthera philoxeroides* (Yu-Peng et al. 2006). Using a combination of genetic analysis and phenotypic tests is necessary for tracing the development of traits in introduced species and separating the effects of genetic adaptation and phenotypic plasticity (Rey et al. 2012).

We used the introduced red seaweed *Gracilaria vermiculophylla* as a model organism with which to investigate potential evolutionary invasion pathways and to distinguish between effects due to genetic adaptation versus phenotypic plasticity. *G. vermiculophylla* is native to the northwest Pacific and has been introduced to every continental margin in the Northern Hemisphere (Saunders 2009, Guillemin et al. 2008, Weinberger et al. 2008, Byers et al. 2012, Krueger-Hadfield et al. in press). Krueger-Hadfield et al. (in final prep) found a strong genetic break in the native range based on restriction length fragment polymorphism in the mitochondrial *cox1* gene that demonstrated the northern part of Honshu and Hokkaido served as the source of this widespread invasion. There have been at least two primary invasions across the northern hemisphere with many secondary introductions within and between coastlines (Krueger-Hadfield et al., in final prep). In the introduced range, thalli are found free-floating in high salinity estuaries along both coastlines of North America and throughout European coastlines (Krueger-Hadfield et al. in press). In contrast, in the native range, *G. vermiculophylla* is found attached to hard substratum on rocky shores of high salinity estuaries (Krueger-Hadfield et al. in press, Krueger-Hadfield and Sotka, unpubl. data). The timing of the invasions as well as the geographic area of origin in the native range suggests that aquaculture practices involving the translocation of the Pacific oyster *Crassostrea gigas* may have facilitated the invasion of each continental margin in the Northern Hemisphere (Krueger-Hadfield et al. in final prep). The Pacific oyster was introduced to bays and estuaries along the west coast of North America and

the Atlantic coast of France from the 1900's through the 1970's, and was sporadically planted along the Atlantic and Gulf coasts (Carlton 1992, Andrews 1980, Burreson et al. 2000).

Gracilaria vermiculophylla has expanded across wide latitudinal margins during its invasion. Fitness tradeoffs in these varying environments may have contributed to diversified selection among populations invading different habitats (Lee 2002). *G. vermiculophylla* covers 80-100% of some of the benthic habitats to which it is introduced (Byers et al. 2012). It affects its recipient ecosystems by outcompeting native macroalgae (Hammann et al. 2013), providing habitat for invertebrates (Nyberg et al. 2009), and altering community structure, species interactions, and trophic pathways (Byers et al. 2012). The success of introduced populations of *G. vermiculophylla* is likely attributable to several factors. These include high tolerance for physiological stress (Yokoya et al. 1999, Raikar et al. 2001) and high resistance to herbivores (Hammann et al. 2013). Introduced populations were likely also facilitated by a flexibility in reproductive mode associated with an ecological shift from hard to soft-substratum habitats (Krueger-Hadfield et al in press). The introduction of *G. vermiculophylla* from hard to soft substratum habitats resulted in a shift from haploid-diploid populations to diploid-dominated or fixed introduced populations (Krueger-Hadfield et al. in press). Finally, an absence of macroalgal competitors in estuarine habitats, such as the southeastern United States, aided in the successful introduction of the species (Byers et al. 2012).

Hammann et al. (2016) demonstrated higher recovery in introduced populations after heat shock when compared to native populations, suggesting that thermal tolerance may play a significant role in the species' successful spread within novel environments. The evolutionary pathway of thermal tolerance and the role of genetic adaptation versus phenotypic plasticity of the trait remains unclear. Moreover, though there is evidence that genetic adaptation aids

biological invasions, the roles of evolutionary processes in successful marine invasions have rarely been studied. There are few studies which have compared native and introduced populations of introduced marine species (Lee 2002, Tepolt 2015).

We tested thermal tolerance of native and introduced *Gracilaria vermiculophylla* thalli. Heat and cold assays were conducted on samples collected from the field and subsequently reared in a common garden. The purpose of these assays was to analyze the evolutionary pathway of temperature adaptation during the *G. vermiculophylla* invasion and to differentiate between the effects of genetic adaptation and phenotypic plasticity.

Methods: Sampling

Diploid thalli were collected from 15 native populations in Japan and 25 introduced populations along North American and European coastlines (Table 1). Only diploid thalli were sampled in order to reduce any variations in fitness occurring as the result of ploidy stages (SA Krueger-Hadfield and CJ Murren, unpubl. data) and due to diploid dominance in the introduced range (Krueger-Hadfield et al. in press).

Methods: Field-collected Thalli Assays

Within one week after collection, thalli were shipped to the Grice Marine Laboratory in Charleston, SC and placed in seawater with a salinity between 27-30 ppm, pH between 6.8 and 7.8, and temperature of 18-23° C. After each population was rehydrated for 24 hours, eight 1 cm tips were isolated from 10-30 thalli selected from the population. All isolated tips exhibited little to no bleaching before temperature experiments were conducted. Following tip isolation, thalli were incubated for one day at 16° C with a 12 hour light/dark cycle.

The 8 tips isolated from each thallus were used to conduct temperature assays. All tips were placed in sealed 250 μ l PCR tubes containing 200 μ l seawater. One of the 8 tips was kept in a 16 ° C incubator as a control during the duration of the assays. Two T100 Thermal Cyclers (Bio-Rad) were used to expose four tips to the following temperatures for 15 minutes each: 33.9, 39.1, 43.9, and 49.2 ° C. The remaining three tips were used for cold assays. They were placed in a -20° C freezer for 1, 2.5, and 4 hours.

After running temperature assays for all thalli from a site, each tip was assigned a random number and placed into CytoOne® 12-well tissue culture plates. Each plate contained 12 2.21 cm wells. Wells were filled with 4 mL of seawater with a salinity between 27-30 ppm and pH between 6.8 and 7.8. Bleaching scores were assigned to each tip. A score of 1 indicated no bleaching, 2 indicated partial bleaching, and 3 indicated full bleaching (Fig. 1). The well plates were incubated at 16 ° C for 8 days. On days 2, 4, 6, and 8, water was changed and bleach scores were assigned to each tip.

Methods: Common Garden Thalli Assays

The thalli from each population were cultured in a common garden for 3-4 months. Water was changed weekly and pumps were used to keep the thalli in motion. After samples were reared in the common garden, heat assays were repeated excluding the highest temperature treatment (49.2° C) because all thalli bleached at this temperature, regardless of geographic origin. Cold assays were not performed on common garden-reared thalli (Fig. 2). Results were analyzed using a mixed-model ordinal logistic regression and posthoc analysis.

Results

There was variance in the health and number of tips available on thalli collected from different populations. To account for varying degrees of bleaching present in the thalli before temperature assays were conducted, thalli were characterized based on the change in bleach score from day 0 to day 8. A thallus was included in the “proportion bleached” category if its bleach score increased from day 0 to day 8, meaning that it changed from a 1 to a 2 or 3, or from a 2 to a 3.

Among thalli in the field-collected assays, native thalli experienced a higher proportion of bleaching in response to both hot and cold temperature treatments. Introduced thalli did not exhibit more bleaching than native thalli in response to any of the temperature treatments (Fig. 4, 5). In response to field-collected cold assays, native thalli experienced a higher proportion of bleaching than introduced thalli in response to 1.5 hours ($p=0.006$) and 4 hours ($p=0.002$) at -30° C. Native and introduced thalli both exhibited a very low proportion of bleaching after exposure for 1 hour to -30° C, with no significant difference between the two groups ($p=0.783$; Fig. 5).

In response to heat assays, field-collected native thalli experienced a significant increase in bleaching compared to introduced thalli at 33.9° C ($p=0.001$) and 43.9° C ($p=0.010$). All thalli bleached completely at 49.2° C, the highest temperature treatment (Fig. 5). After rearing in a common garden, there was not a significant difference between the proportion bleached at 33.9° C or 39.1° C. At 43.9° C, introduced thalli had a slightly higher proportion of bleaching when compared to native thalli (Fig. 6).

Discussion

Field-collected thalli sampled from introduced populations exhibited a lower proportion of bleaching after both heat and cold shock. Bleaching is an oxidative stress response in which thalli expel photosynthetic pigments. Therefore, the bleaching response likely indicates the degree of stress occurring as a response to the temperature treatments (Hammann et al. 2016). A higher proportion of bleaching will indicate more stress in response to a temperature treatment. Our results demonstrate that field-collected thalli from the introduced range exhibit less stress in response to hot and cold temperatures when compared to thalli from the native range.

Among common garden-reared thalli, environmental history was presumably erased because thalli were cultured within the same environment. There was no significant difference between native and introduced common garden thalli in the proportion bleached between each group.

From assays conducted on field-collected thalli, there was significantly more bleaching among native than introduced thalli. This pattern did not persist in the assays conducted on common garden thalli. However, a past study suggested that thermal tolerance in introduced populations was likely induced by genetic adaptation (Hammann et al. 2006). This discrepancy may be explained by the loss of a significant number of the native Japanese thalli during the common garden rearing. The resulting smaller sample size of native thalli, especially the decrease in thalli originating from the source region, may have obscured the patterns that were evident in the field-collected thalli. The small sample size of native common garden-reared thalli inhibited our ability to robustly test the effects of phenotypic plasticity versus genetic adaptation using the common garden assays.

Besides work on the copepod *Eurytemora affinis*, few studies have compared native and introduced populations during marine biological invasions in order to identify differences

between them which may have influenced invasion success (Lee 1999, Lee and Petersen 2002, Lee et al. 2013). Observed differences between native and introduced populations of marine species may be due to adaptation that occurred during the invasion, causing genetic differentiation among native and introduced populations. However, these differences may also be attributable to the species' phenotypic plasticity. Few studies have elucidated the relative influence of these factors on the differences observed in native versus introduced populations (Lee 2002, Tepolt 2015). Results from our field-collected assays provide preliminary evidence that adaptation of temperature tolerance occurred during the *G. vermiculophylla* invasion and suggest that this adaptation occurred post-introduction. A larger sample size of native, common garden-reared thalli would be needed to robustly test the relative influence of genetic adaptation versus phenotypic plasticity on survivorship patterns resulting from temperature treatments.

Conclusions

Both native and introduced thalli exhibited wide thermal tolerance that may be attributable to acclimation to the fluctuating temperature conditions of estuaries as well as to habitats with high anthropogenic impact in both the native and introduced range. Lower bleaching proportions among introduced field-collected thalli suggest that introduced populations have a wider thermal tolerance than native populations. A larger sample size would be needed to thoroughly test the relative contributions of phenotypic plasticity and genetic adaptation to this pattern.

There have been numerous introductions of marine species along the coasts of North America via the movement of commercial oysters (Carlton 2001). The expansion of aquaculture industries will likely contribute to further human-mediated invasions (Carlton 1992). Models that characterize the traits of invasive species as static instead of adaptable often fail to predict their full introduced range and how this range changes over spatial and temporal shifts (Lee 2002). Understanding the role of evolution in facilitating marine invasions could aid in mitigation and management efforts of these species by improving our ability to predict which species are likely to adapt successfully to introduced environments. Studying adaptation of aquatic and marine introduced species is especially important. Introduced species often exhibit high degrees of plasticity and adaptability (Lee 2008); therefore, these species may be more resilient to climate change than terrestrial species (Tepolt 2015). Climate change is imposing more selective pressures on species and the rate of invasions is increasing (Tepolt 2015, Carlton 2001). In light of these trends, analyzing the evolutionary factors influencing invasive species may aid in determining the sources and pathways of invasions, identifying high-risk source populations and vectors, and identifying sustainable policies and management strategies (Geller et al. 2010).

Acknowledgements

This project was supported by NSF BIO-OCE (no.1357386) to E.E. Sotka, S.A. Krueger-Hadfield, A.E. Strand, and C.J. Murren and a SURF grant to S.J. Shainker. Many thanks to the Grice Marine Lab, the field helpers listed in Table 1, to the following mentors and fellow students for advice, lab and field assistance, and moral support: Erik Sotka, Stacy Krueger-Hadfield, Courtney Murren, Allan Strand, Paige Bippus, Ben Flanagan, Liz Duermit, Lauren Lees, Aaron Baumgardner, R. Terada, H. Endo, M. Kamiya, M. Nakaoka, M. Valero and C. Destombe.

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