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Understanding The Invasion Of Florida's Intertidal Crassostrea Virginica Reefs By Non-native Marine Invertebrate Species

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UNDERSTANDING THE INVASION OF FLORIDA'S INTERTIDAL
CRASSOSTREA VIRGINICA REEFS BY NON-NATIVE MARINE
INVERTEBRATE SPECIES

by

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ABSTRACT

Predicting the locations of new biological invasions has become a high priority for biologists as well as trying to predict if newly introduced species will become damaging to native ecosystems. Reefs of the eastern oyster *Crassostrea virginica* in Mosquito Lagoon, Florida have been highly disturbed in recent years resulting in dead reefs (piles of dead, disarticulated shells) some of which have been restored. I conducted oyster reef surveys for non-native invertebrates to determine if disturbance on these oyster reefs might assist invasion by two species, *Mytella charruana* and *Perna viridis*, recently introduced to the southeastern coast of the United States. Next, I investigated if *M. charruana*'s temperature and aerial exposure tolerance limits may allow for it to establish permanently on intertidal oyster reefs. Temperature and aerial exposure tolerance experiments were conducted and oyster reef temperatures were collected. Oyster reef surveys could not predict if reef disturbance is assisting in the invasion process because only two non-native individuals (*P.viridis*) were found, one on a restored reef and one on a natural (reference) reef. Tolerance experiments showed that some *Mytella charruana* survived even after 7 days of 8° C temperatures if the mussels are exposed to air for 4 hours or less per day. *Mytella charruana* had near 0% survival after 4 hours of 44° C. However, only disturbed reefs reached this temperature in the field. It is likely that *M. charruana* could survive in the low intertidal zone on restored or reference reefs. This information is important for understanding the introduction of *M. charruana* in Mosquito Lagoon and also provides a data set of temperature tolerances for better understanding of whether the species might be able to invade other areas.

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INTRODUCTION

Worldwide shipping increases have led to the spread of many marine organisms, some of which have become invasive. An invasive species is a species which, after being introduced to a novel area by humans either directly or indirectly, has been demonstrated to have caused either economic or ecological harm or both (Lockwood et al. 2007). Invasive species have been shown to negatively affect ecosystems by altering structure, functioning and dynamics of an ecosystem (Mooney et al. 2005). Invasive species cost the United States nearly $120 billion each year through non-native species control costs, lost production due to their effects, as well as other associated costs of non-natives (Pimentel et al. 2005).

Biologists have devoted a lot of attention to being able to predict if a non-native species will become invasive. This is important because prediction will help allocate valuable prevention and eradication efforts toward species that require the most attention. However, prediction can be difficult. Initially, biologists tried to predict invasiveness by looking for a set of characteristics which were assumed to be typically found in invasive species (Ehrlich 1998, Lodge 1993, Ricciardi and Rasmussen 1998). A problem that has been cited with this method is that these types of lists tend to be too general and make it difficult to predict invasiveness in specific ecosystems (Marchetti et al. 2004). Another problem associated with predicting invasiveness and the spread of a non-native species is the frequent occurrence of a lag time. This occurs between the point at which a species is introduced and when it becomes invasive (Lockwood et al. 2007, Kowarik 1995). Lag times can create problems for native species because seemingly harmless non-native introductions may eventually lead to problems for native ecosystems. Lag times can

occur for a few reasons, including growth trajectories, threshold levels of detection, and Allee affects (Lockwood et al. 2007). Another factor that can affect lag times and cause non-native species to eventually become invasive is propagule pressure. Propagule pressure involves both the number of individuals released (propagule size) and the number of separate introductions events (propagule density) (Beirne 1975). The larger the propagule density and number, the greater the chance of establishment success of an invading species (Beirne 1975).

Another factor that can increase invasion success for non-native species is the alteration of natural disturbance regimes (Marchetti et al. 2004, D'Antonio 2000, Minchinton 2002). A popular definition of disturbance developed by White and Picket (1985) is "a discrete event in time that disrupts ecosystem, community or population structure and changes resources, substrate availability, or the physical environment." Alteration of natural disturbance regimes has been shown to promote invasions across several different habitat types. Terrestrial plants provide examples in habitats where fire regimes have been altered (D'Antonio 2000). Disturbance of aquatic habitats has also been shown to promote invasibility for species through processes like urbanization and creation of water diversions (Marchetti 2004). There is not a lot of evidence to suggest that non-native species out-compete native species after disturbance occurs (Lockwood 2007). Rather, domination by non-native species typically occurs due to disturbance that alters a habitat enough so that conditions are outside the realm for which the native species is adapted to survive (Lockwood 2007). The combination of many factors such as disturbance, propagule pressure, and lag times can make prediction of invasiveness difficult. Attempts to simplify this by finding general sets of characteristics held by all

invasive species have sometimes led to oversimplifications (Rejmanek and Richardson 1996, Kolar and Lodge 2001). I have chosen to look closely at an intertidal system and determine invasibility in this system.

The intertidal, or littoral, zone can be described as the area along the shoreline that lies between the mean high and low tide lines (Dame 1996). Sessile organisms that live in intertidal zones must be able to withstand a difficult set of stressors such as repeated exposure to air based on body position and tidal cycles, reduced feeding time, and often drastic changes in temperature (Widdows and Shick 1985). Distribution patterns of organisms often coincide with temperature gradients that occur within the intertidal (Somero 2002). Temperatures while submerged can be quite different from temperatures during aerial conditions, which may be affected by ground and air temperature, wind, cloud cover, solar radiation and relative humidity (Johnson 1975, Bell 1995, Helmuth 1998, 1999). Being able to tolerate intertidal conditions requires special adaptations for heat and cold stress, dessication stress, exposure to fresh water in the form of rainfall, and reduced feeding time (Levinton 2001, Tomanek and Helmuth 2002). Mussels and oysters, the focal organisms of this study, are ectotherms. While living in the intertidal, their bodies are subjected to temperature regimes that occur there since they are unable to regulate their own body temperature. There are several factors that control the temperature regime in the intertidal and, more importantly, the actual body temperature that the organisms experience (Helmuth 2002). Visible short wave solar radiation, infrared long wave radiation transmitted between the sky and earth, infrared radiation that travels between the ground and the air, conductive heat traveling to and from the ground, heat traveling between the air and an organism via convection, and

evaporative heat loss are all factors that affect the temperature of an organism at low tide (Porter and Gates 1969). Knowing the temperature tolerance levels of an organism while it is exposed to air may help us understand whether or not it can become established in a particular habitat. If one knows the tolerance limits of the organism as well as the actual temperatures that it would experience in a novel habitat, while barring other factors such as competition, it can be estimated whether it might be possible for the organism to live there successfully.

Two non-native species of concern, *Mytella charruana* and *Perna viridis,* have recently been introduced to the southeastern coasts of the United States, including my study locations in Mosquito Lagoon and near St. Augustine, Florida. *Mytella charruana*, a mussel native to Central and South America, invaded the southeastern Atlantic coast of the United States, most likely via ballast water (Carlton 1992, Gillis et al. 2009). It was first found in the U.S. in Jacksonville, Florida in 1987 (Lee 1987, Carlton 1992). *Mytella charruana* was subsequently found in Mosquito Lagoon, Florida in 2004 (Boudreaux and Walters 2006) and has been found in coastal areas from Mosquito Lagoon in Florida to South Carolina as of 2009 (Gillis et al. 2009). The green mussel, *P. viridis*, is a mussel of Indo-Pacific origin (Baker et al. 2007). It first invaded Florida's Gulf Coast in 1999, and later invaded the east coast of Florida in 2002 (Baker et al. 2007). *Perna viridis* was first found in Mosquito Lagoon in 2004. By 2007 it had been found from the IRL northward to Charleston, South Carolina (Baker et al. 2007). This species was most likely transported via ballast water or boat hull fouling (Baker et al. 2007).

Mytella charruana and *Perna viridis* are both species that can survive intertidally (Pereira et al. 2003, Rajagopal et al. 2006). They are also species that could have

detrimental effects on ecosystems in their invaded ranges. Baker et al. (2007) suggested

native oysters were likely suppressed by high densities of *P. viridis* in northern Tampa

Bay and compared the invasion of green mussels to the invasion of the zebra mussel,

Dreissena polymorpha, in freshwater areas of the northern United States. However, no

research group has experimentally investigated this possibility. Both *P. viridis* and *M.*

charruana have been found on oyster reefs in Mosquito Lagoon and in close proximity to

reefs in St. Augustine, Florida (Boudreaux and Walters 2006, Walters unpublished data).

My study locations included Mosquito Lagoon near New Smyrna Beach (Figure

1), Florida and the coastal waters of the Guana Tolomato Matanzas National Estuarine

Research Reserve (GTMNERR) in St. Augustine, Florida (Figure 2). These locations

provided a good model because of their high levels of biodiversity and the prevalence of

intertidal *C. virginica* reefs (Dybas 2002, Frazel 2009). *Crassostrea virginica* reefs in

both locations are also home to native mussel species (Boudreaux et al. 2006, Pers. obs.).

Reefs included in my study contained predominantly *Brachidontes exustus* in Mosquito

Lagoon and *Geukensia demissa* in GTMNERR waters. Reefs in Mosquito Lagoon have

undergone much degradation in recent years which has been shown to be caused by

repeated exposure to recreational boat wakes (Grizzle et al. 2002, Wall et al. 2005). This

has resulted in the presence of dead reefs as well as reefs not disturbed by boat wakes

which I will refer to as reference reefs. A third type of reef present in Mosquito Lagoon

are restored reefs which include reefs previously dead but that have been covered with

oyster shells that have been anchored in place via Vexar mesh and concrete weights.

This provides substrate for new *C. virginica* to settle. Dead reefs and reference reefs are

also found within the GTMNERR (Pers. obs.). Both *P. viridis* and *M. charruana* have

been found in these locations either on or near (< 1 km) native *C. virginica* reefs (Pers. obs.). Reefs of the oyster *C. virginica* provide a useful model habitat due to the three-dimensional structure created by oysters that is utilized by a wide variety of associated organisms (Boudreaux et al. 2006, Barber et al. 2010).
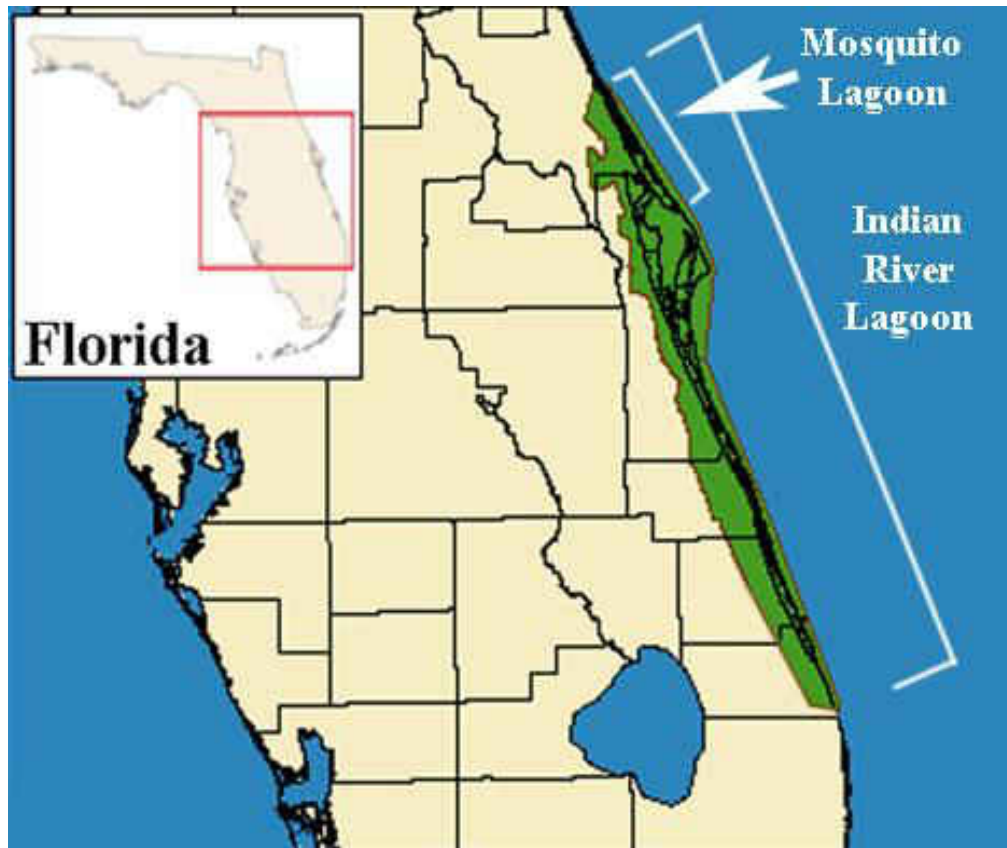
Figure 1: Map of the Indian River Lagoon (The Nature Conservancy 2010).
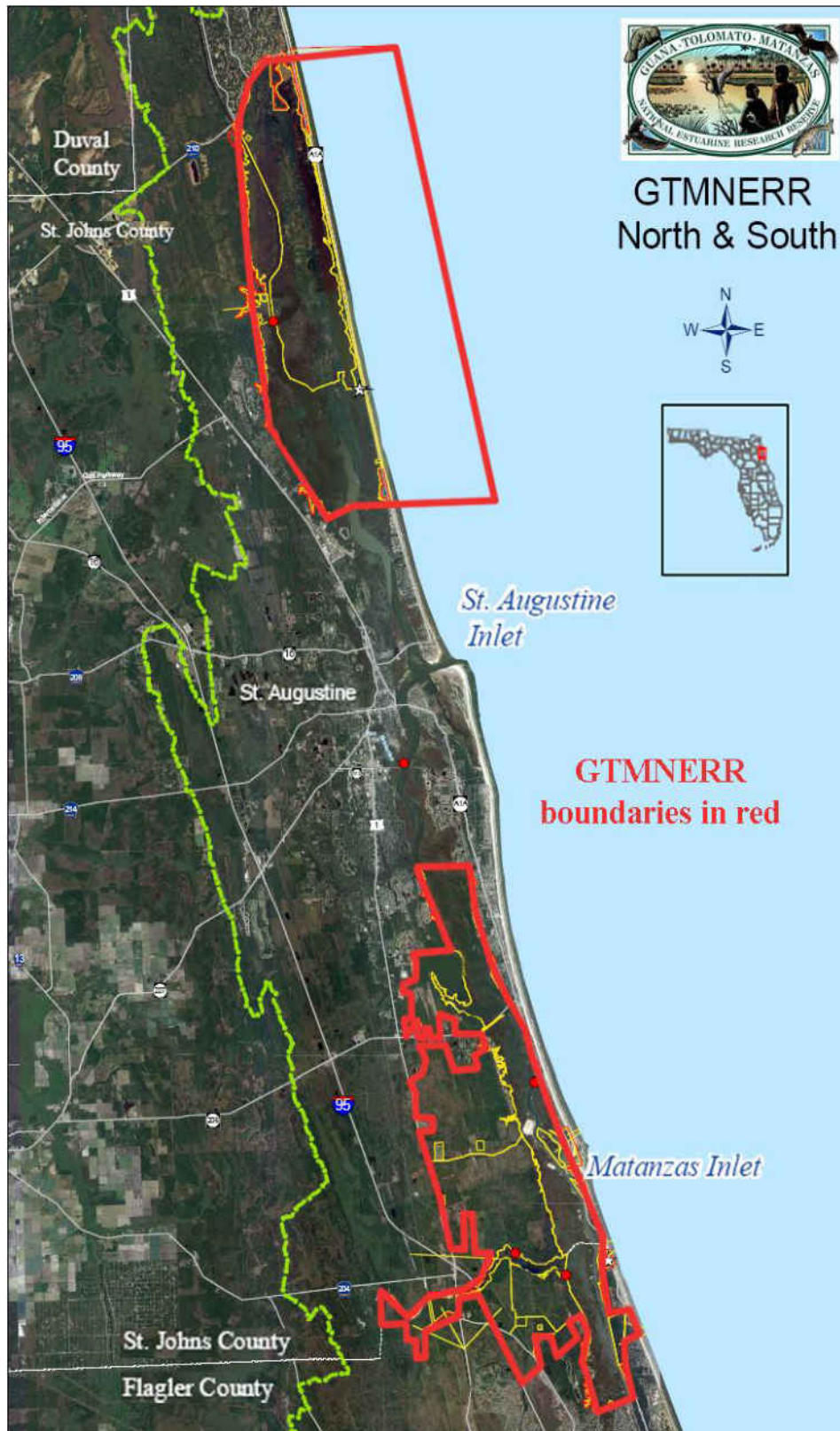
Figure 2: Map of GTMNERR, including northern and southern portions (Florida Department of Environmental Protection 2008).

There were two goals for this study. The first goal was to track the presence of non-natives on oyster reefs in both study locations to test the hypothesis that boat wake disturbance on oyster reefs increases invasibility leading to larger populations of invaders on dead reefs. The second goal was to investigate whether or not *M. charruana* has the potential, based on its aerial exposure and thermal tolerances, to establish on *C. virginica* reefs and compete with natives in Mosquito Lagoon. *Mytella charruana* was chosen for this question because it had higher survival in preliminary aerial exposure tolerance experiments than *P. viridis*. This question was addressed by collecting environmental temperatures from oyster reefs and comparing them to aerial exposure and temperature tolerances of *M. charruana*, which were found through laboratory experiments. Achieving these goals is important for helping to conserve native *C. virginica* as well as native mussel species.

<u>Research Questions and Hypotheses</u>
1. **Will there be any difference in the recruitment of non-natives on each of the 3 reef types: reference, restored, and dead reefs?**

   H$_O$: Each of the three types of oyster reefs will have similar abundances of non-native species.

   H$_A$: The intertidal zones of dead reefs will harbor more invaders than the other two reef types. This should be followed by restored reefs, and reference reefs with the fewest non-native mussels.

Reasoning: Alteration of disturbance regimes has been shown to increase invasiveness in a system (Hobbs and Huenneke 1992). Based on this prediction, reefs that have been disturbed by humans should have higher levels of invasion.

Purpose: Answering this question will help us predict where future invasions should occur. Results will also help determine the value of oyster reef restoration as a means of invasion prevention.

2. **Will the combined thermal and aerial exposure tolerances of *Mytella charruana* allow this species to permanently establish on intertidal oyster reefs in Mosquito Lagoon, Florida?**

H_O: *Mytella charruana* will be able to withstand air temperatures that occur on oyster reefs in Mosquito Lagoon, Florida for periods of time equal to or exceeding intertidal exposure periods.

H_A: *Mytella charruana* will not be able to withstand air temperatures that occur on oyster reefs during low tide aerial exposure in Mosquito Lagoon and its establishment will therefore be limited by environmental tolerance levels.

Reasoning: Although *M. charruana* has been found on oyster reefs in the past, populations were not sustained (e.g. die off event in Mosquito Lagoon during winter 2004/2005) (Boudreaux and Walters 2006). The lack of tolerance for the temperatures and exposure times that occur on oyster reefs seemed a likely explanation for lack of sustained invasion.

Purpose: Understanding the range of aerial temperatures and exposure times that *M. charruana* can withstand will help us predict whether it is likely that the

species can permanently establish on native oyster reefs. This data will also

provide a known range of survival to be compared with temperature data from

other intertidal locations in the southeastern United States.

METHODS

<u>Oyster reef surveys</u>

Oyster reefs were monitored every 6 months, in summer and winter, in Mosquito Lagoon

and in St. Augustine waters from July 2008 through March 2010. Three types of reefs

were surveyed in Mosquito Lagoon: dead reefs (reefs with seaward margins of dead,

disarticulated shells), restored reefs, and reference reefs. In Mosquito Lagoon, seven of

each type of reef were surveyed for the presence of *M. charruana* and *P. viridis*. Dead

reefs and reference reefs were surveyed in the GTMNERR, as no restored reefs existed

there at the time of the surveys. In the GTMNERR, six of each type of existing reef were

monitored. On each reef, 0.5 m x 0.5 m quadrats were used to standardize each search

area. Thirty points were chosen on each reef and the area inside of each quadrat was

surveyed. Diagrams showing the layout of each reef type are shown in Figure 3, Figure

4, and Figure 5. On restored reefs, similar size areas had already been placed in the form

of restoration mats. On these reefs, mats were assigned numbers *a priori*, and then 30

mats were chosen using a random number generator (Figure 5). On reference and dead

reefs, I haphazardly chose the 30 sampling points (Figure 3 and Figure 4). The numbers

of live oysters, native ribbed mussels and any live *P. viridis* or *M. charruana* in each

quadrat were recorded. Any non-native species found were quantified, removed and

preserved. The number live *C. virginica* was also counted in each quadrat.

One-way analysis of variance was used to test for significant differences in the

average number of individuals of each invader across the 3 reef types. Analysis of

variance tests were also used for numbers of live oysters as well as number of native

ribbed mussels found on each reef type. Tukey's *post hoc* test for Honestly Significant

Differences was used to determine differences among reefs.
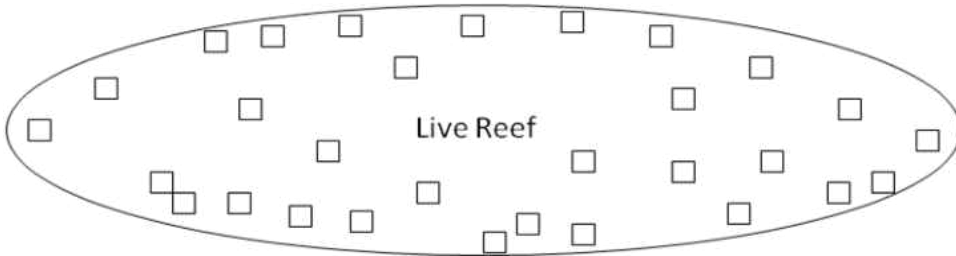
Figure 3: Diagram of a reference reef showing haphazard placement of 30 quadrats. Note that quadrats can be placed at any location on the reef surface.
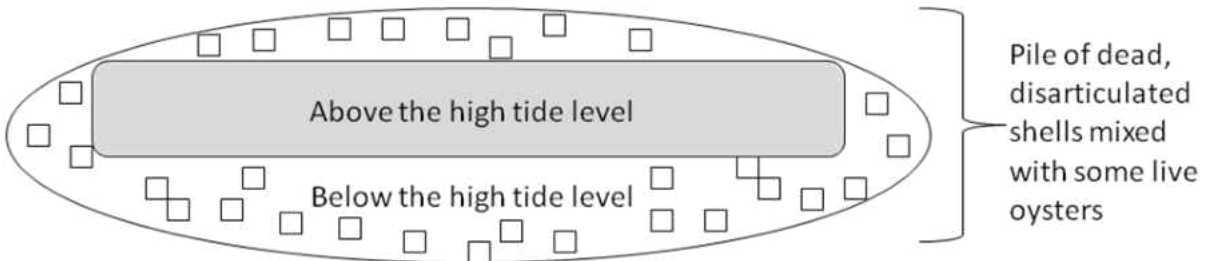
Figure 4: Diagram of dead reef showing haphazard placement of 30 quadrats. Areas below the high tide line that were sampled are shown as well as the area that was always above the high tide line (identifiable by sun-bleached shells) where quadrats were not placed.

1	2	3	4	5	6	7	8	9	10	11	12	13	14
28	27	26	25	24	23	22	21	20	19	18	17	16	15
29	30	31	32	33	34	35	36	37	38	39	40	41	42
56	55	54	53	52	51	50	49	48	47	46	45	44	43
57	58	59	60	61	62	63	64	65	66	67	68	69	70
84	83	82	81	80	79	78	77	76	75	74	73	72	71

Figure 5: Diagram of a restored oyster reef on which mats were used as counting units instead of quadrats. Diagram shows numerical ordering of mats from which 30 random sample points were taken.

To learn if the internal temperature of *M. charruana* when exposed to air differed from surrounding air, mussel loggers were constructed to mimic the thermal dynamics of *M. charruana* using methods developed by Helmuth and colleagues (Helmuth 2002, Schneider 2008). This was done by filling cleaned and dried *M. charruana* shells with silicone and inserting an iButton[®] temperature logger into the silicon (Helmuth 2002, Schneider 2008). The silicone was then allowed to dry for 24 hours. Only extremely large *M. charruana* were large enough to encase iButton[®] temperature loggers. Mussels greater than 40 mm in length were needed to encase an iButton (17.35 mm in diameter,5.89 mm thick). For this reason, loggers could not be matched to the mussel sizes that have been found in Mosquito Lagoon.

Mussel mimic accuracy test

After construction of the mussel mimics, a field experiment was completed on oyster reefs in Mosquito Lagoon to find out if there was a difference in temperature between the iButtons[®] placed in mussel shells (mimics) and iButtons[®] not encased in silicon and mussel shells (controls). This was to determine if mussel mimics were more useful for predicting mussel temperature than an iButton[®] alone. One mussel mimic logger and one exposed (without mussel mimic) iButton[®] temperature logger were attached to concrete donut weights using cable ties on each of 7 reefs in Mosquito Lagoon during February 2009. The loggers and mussel mimics were always in contact with the substrate. Each logger was placed at an equivalent elevation in the mid-intertidal zone so all loggers would be submerged and exposed at the same times. Although this did not capture the longest possible time that mussels might have been exposed on a reef, it did ensure that loggers were consistently exposed at low tide and

submerged at high tide. All loggers were left on oyster reefs for 8 days. Data were then collected and t-tests were performed to test for significant differences in average, maximum, and minimum temperatures collected by iButton® controls and those collected by mussel mimic loggers using R (Version 2.7.1) statistical software. This logger test data was then used to decide which logger type was best for collecting temperature data from oyster reefs so that it could be compared to the temperature tolerance data for *M. charruana* while being exposed to air.

<u>Mussel mimic logger test results</u>

One donut weight, with an iButton[®] and mussel mimic attached, was lost during the experiment, reducing the number of replicates to 6 reefs of each type. The test results showed that there was no difference in average temperature between the mussel mimic iButtons[®] and the iButtons[®] alone (p = 0.5508). Average temperature for iButtons[®] was 18.12° C (± 0.17 S.E.) while average temperature of mussel mimics was 18.01° C (± 0.01 S.E.). The averages of the daily maximum and minimum temperatures were also not significantly different (p values of 0.4855 and 0.06695, respectively). Mussel mimics had an average daily maximum temperature of 25.58° C (± 0.95 S.E.) and an average daily minimum temperature of 8.17° C (± 0.25 S.E.), while iButtons[®] had an average maximum of 24.92° C (± 0.74 S.E.) and an average minimum of 8.83° C (± 0.17 S.E.). This suggested that there would be no need to use the mussel mimic loggers because they did not produce significantly different results than iButtons[®] alone (Figure 6).
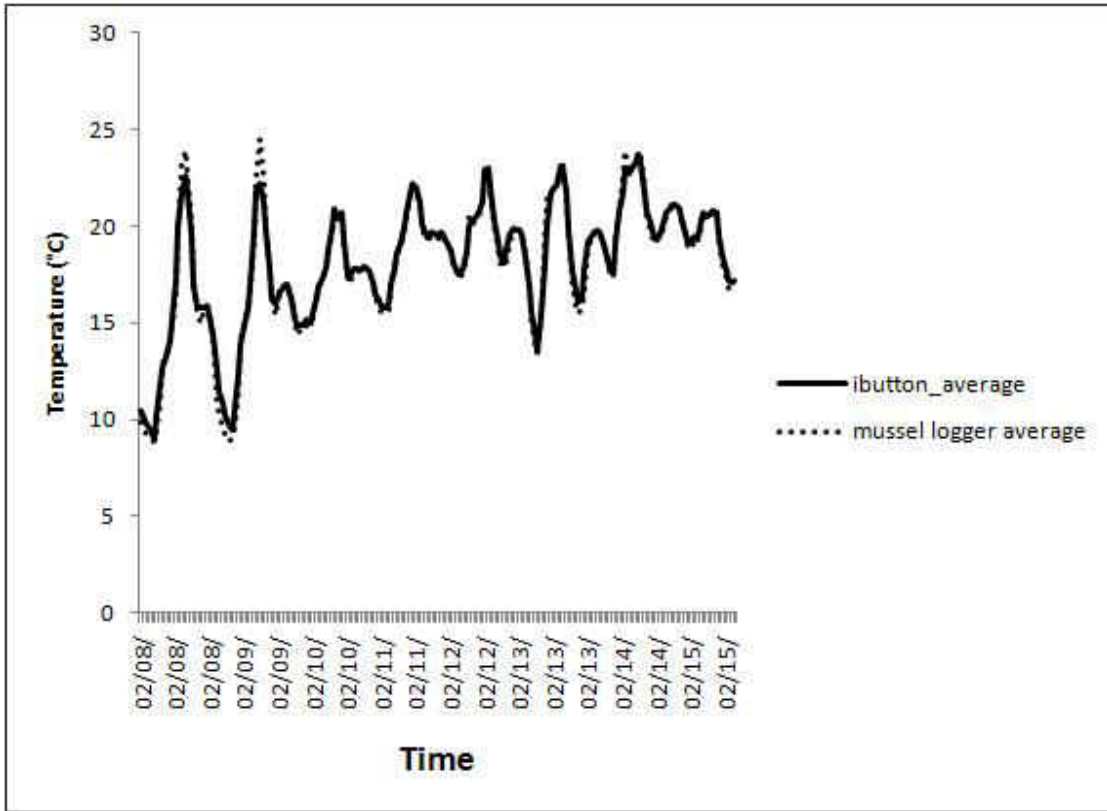
Figure 6: Average temperatures collected by iButton temperature loggers versus mussel mimic loggers. Data show average temperature on six different oyster reefs across 8 days in February 2009.

Long-term oyster reef temperature data collection

Low tide air temperatures from oyster reefs in Mosquito Lagoon, FL were

collected using iButton® temperature data loggers. Loggers were attached to concrete

donut weights using cable ties so that the loggers were allowed to contact the reef

surface. Each temperature logger was placed on a unique oyster reef. All loggers were

placed at a similar elevation in the intertidal zone and each logger was placed at an

elevation which allowed it to be exposed at low tide and submerged at high tide. Loggers

were therefore at the mid-intertidal zone ensuring that both submersion and aerial

exposure would occur. Each logger was set to record temperature readings at 30 minute

intervals. Loggers were placed on a total of 15 oyster reefs (5 dead reefs, 5 restored

reefs, and 5 reference reefs). In the event that loggers were lost or malfunctioned, these

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data were removed from analyses so that averages were calculated with fewer replicates. Temperature data was graphed and examined to separate periods of exposure from temperatures that were recorded while the loggers were submerged.

Temperature data collected from loggers while they were exposed at low tides were averaged across days from 7 – 24 July 2009, and again from 11 January through 16 February 2010. Analysis of Variance (ANOVA) tests were used for the warm and cold temperature data to test for significant differences in temperature between the three types of oyster reefs (dead, restored, and reference reefs). Tukey *post hoc* comparison tests were then used to find differences amongst the reef types. Separate ANOVAs were used for average temperature while submerged, average temperature while exposed, average maximum temperature and overall maximum temperature. Averages while exposed and submerged were calculated by finding the mean for each reef on each day and then averaging across all days. Averages were then calculated across all replicate reefs of each type to obtain one average value for each reef type. Average maximum values were calculated by taking the highest value for each day for each reef followed by an average across all the days. Values were then averaged across all replicate reefs to obtain one average value for each reef type. Overall maximum values were calculated by taking the hottest temperature that occurred on each reef during any of the collection days. These maximum values were averaged across each replicate reef to obtain one average value for each reef type. Cold average temperature while submerged and exposed, average minimum, and overall minimum temperatures were calculated for winter temperature data in the same manner. Data collected from the temperature loggers was used in conjunction with the results of the aerial exposure laboratory experiments to help predict

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if *M. charruana* will be able to survive in the temperatures that occur on oyster reefs in Mosquito Lagoon.

<u>Room temperature aerial exposure tolerance experiments</u>

Seven day repeated exposure trial methods

Before combined temperature and air exposure experiments were conducted, I needed to be sure that the mussels would be able to withstand the experimental design air exposure at mild (room) temperatures. Results of these tests would also be used to decide which mussel species to use in the thermal aerial exposure tolerance experiments. First, *M. charruana* was collected from the Lion's Club dock in Jacksonville, Florida (30°22.691 N, 81°37.238 W). After collection, mussels were transported to the University of Central Florida in seawater from the collection site. The mussels were placed in filtered, aerated aquaria filled with additional seawater from the site of collection. The mussels were acclimated to laboratory conditions for 10 days. These acclimation methods were carried out for both large (28-44 mm) and small (7-12 mm) size mussel trials.

After acclimation, the mussels were subjected to 5 different time treatments of aerial exposure (0, 4, 8, 12, and 18 hours) at laboratory room temperature (22° C). These treatments were used in two separate large and small mussel trials. To accomplish this, mussels were haphazardly placed in plastic, 2-liter aquaria with aeration. Five mussels were placed in each tank, and there were 5 replicate tanks for each treatment. The mussels were set on top of clay tiles for easy removal without stressing the mussels by breaking byssal threads. Each treatment was carried out once each day for 7 days. This

was accomplished by removing the tiles with mussels attached from all tanks except the 0-hour treatment (continuously submerged) and exposing them to air. At the end of each time treatment, the mussels for that treatment were placed back into the tanks. Survival was checked at the beginning of each day to allow the mussels to re-acclimate to water overnight and ensure accurate survival assessments. Any mussels that gaped and did not respond to stimulation were considered dead. The water was changed in each tank on the fourth day of each trial.

To understand the tolerance limits of *Perna viridis*, repeated exposure experiments were conducted with *Perna viridis* in a similar fashion as those for *M. charruana* except for a few key differences. These methods were again carried out in two separate experiments for both small (25 – 62 mm) and large mussels (82 – 121 mm). *Perna viridis* were collected from a dock in St. Augustine, Florida and transported to the University of Central Florida in the same manner as with the *M. charruana* experiments. All mussels were then placed into large (110 gallon) tanks with aerated seawater from the collection site and allowed to acclimate to aquarium conditions for 7 days. Due to the larger size of *P. viridis*, only one mussel was placed in each tank. The mussels were then exposed to the same time treatments that were used in the *M. charruana* repeated exposure experiment (0, 4, 8, 12, and 18 hours). However, for the *P. viridis* experiments, each treatment contained 20 replicate tanks instead of 5. Survival analysis was used in both *M. charruana* and *P. viridis* experiments to test for significant differences among treatments and identify which treatments were different (SPSS Version 17.0.2).

Continuous exposure at room temperature trials

For *Mytella charruana*, acclimation was carried out in the same method as in the repeated exposure experiments described above. Both large (18 – 35 mm) and small (5 –

12 mm) size mussels were tested in separate experiments. After acclimation, mussels were taken out of the acclimation tanks and exposed to their respective treatments. These treatments included 0 (continuous submersion in 2L tanks as in repeated exposure trial), 1, 2, 3, 4, 5, 6, and 7 days of continuous aerial exposure. All mussels were placed on petri dishes (5 mussels per dish) and each dish was assigned a number. At the end of each treatment time, 5 petri dishes were randomly chosen and submerged in tanks for 6 hours to allow the mussels to re-acclimate to the water. After the 6-hour period, survival was assessed as in Trial 1. *Perna viridis* continuous exposure trials were conducted similarly but again with some differences from *M. charruana* experiments. Large (80 – 110 mm) and small (11 – 58 mm) mussels were tested in separate trials. For *P. viridis*, only 1 mussel was placed in each petri dish after the acclimation period of 7 days. The same exposure treatments were used as with *M. charruana* except that each treatment contained 20 mussels, each in separate tanks. Thus, each day, 20 dishes were randomly removed at the end of each treatment and returned to the 2-liter plastic aquaria and later checked for survival. For the *M. charruana* experiments, one-way ANOVA (Analysis of Variance) was used to test for significant differences among treatments followed by a *post hoc* Tukey's Significant Differences test to find which treatments were significantly different from one another (SPSS, Version 17.0.2 2009). *Perna viridis* experiments resulted in binary data which was analysed using logistic regression (SPSS, Version 17.0.2 2009).

Thermal aerial exposure tolerance experiments on *M. charruana*

Mytella charruana were collected from Fire Station 38 Marine Unit in Jacksonville, Florida (30°23'24.02"N, 81°38'29.81"W) because this location provided a large enough

population to supply all of the needed individuals for the experiments. This site is located less than 2.5 km from the Lion's Club dock collection site. The mussels were acclimated using the same methods as the *M. charruana* experiments at room temperature except for one difference. For this experiment the mussels were acclimated for 12 days, which included a 3-day period of acclimation to aerial exposure. This was important because they were collected from the bottoms of floating docks, which remain permanently subtidal (Schneider 2008). Aerial exposure acclimation was accomplished by removing all mussels from the acclimation tanks during the last 3 days of the laboratory acclimation period for increasing time intervals. The mussels were exposed to air on the first day for 1 hour, then 2 hours on day 2, and 3 hours on the last day before the experiment began (Schneider 2008). *Mytella charruana* were then exposed to a combination of experimental treatments of air exposure time and temperature. This included both a warm temperature experiment designed to mimic summer temperatures and a cold temperature experiment for assessing winter temperature tolerances. For each of the two experiments, a randomized block, two-factor, design was used. The first factor was temperature. Four temperature treatments were included in each experiment (warm: 25, 29, 35, and 44°; cold: 25, 20, 15, and 8°C). The room temperature treatment (25° C) was intended to control for added heat or cold beyond what the mussels would experience simply by being in the laboratory. This made it possible to delineate between differences in survival that were simply from exposure to air (emersion alone) and differences that could be attributed to the combined temperature and aerial exposure treatments. The second factor was air exposure time. Treatments of 0, 4, 8, and 12 hours of aerial exposure were completed each day for 7 days. The 0-hour treatment consisted of mussels

that were continuously submerged in room temperature water. This treatment acted as a control for exposure to air. Because mussels were exposed to both temperature and time treatment combinations for 7 repeated days, a repeated factor of number of days was produced. However this factor could not be included via a repeated measures ANOVA because the data did not meet the sphericity assumption. Thus all data included in the ANOVA were taken from the results of the last day of each trial. The blocking factor was made up of 3 separate, week-long runs that occurred across time, with the first run representing one block, a second run representing the second block, and the third run representing the third block. Three separate runs, completed one after another, were used because only four incubators were available. Since each incubator would represent an experimental unit, the block design was used to eliminate pseudoreplication that may have occurred inside each incubator if only one week-long run had been used.

Mytella charruana were placed in plastic, 2 liter, aerated aquaria in their respective treatments (8 tanks per treatment, 5 mussels per tank). Air exposure at treatment temperatures were achieved by placing the mussels in incubators for both hot and cold temperature trials. A diagram showing the complete layout of these treatments is shown in Figure 7. Three runs using separate mussel collections, and lasting 7 days each, were carried out for both warm and cold temperatures. Runs represented time in the block design. The separate, week-long runs were used to eliminate pseudoreplication (Figure 7). Each run represented 1 replicate for each treatment. This design resulted in three replicates of each treatment with subsampling within replicates.

All time and temperature treatments began at the same time. All mussels were removed from their tanks and placed in the incubators containing their respective

temperature treatments (except for air exposure controls which remained submerged at all times). At the end of each time period, all mussels for that treatment were returned to the water in their respective tanks. Mussels were kept on petri dishes to lower stress caused by pulling and breaking byssal threads. This also standardized the temperature of the substrate. Survival was assessed after mussels had time to re-acclimate to the water and therefore occurred at the beginning of each day (starting after day 1 of exposure) before mussels were removed. Thus, any mortality observed was attributed to the exposures carried out during the previous 24 hours. Any mussels that gaped and did not respond to stimulation were considered dead. Water was changed in all tanks on the fourth day of each 7-day trial. One iButton® temperature logger was used to monitor the air temperature inside each of the 4 incubators. Water temperature inside aquaria was monitored as well with iButton® loggers placed inside 4 aquaria spaced throughout the tanks. One more temperature logger was placed in the open air in the laboratory to monitor the air temperature in the room.

Data for both warm and cold tolerance experiments was transformed using the arcsine square root transform in order to meet the normality and homogeneity of variance assumptions of ANOVA. Box plots, histograms, and residual plots were used to check for normality and homogeneity of variance. R statistical software (R version 2.7.1) was used to perform the randomized block, 2-factor ANOVA to test for significant differences among treatments as well as effect of block and interaction effects. If the test for differences among treatments was significant, then *a posteriori* Tukey's tests were used to identify where the differences existed among treatments within the temperature and time factors. For the warm tolerance experiment, the data was also analyzed as a

one-way ANOVA (treatments were analyzed as treatment combinations of time and temperature) with a *post hoc* Tukey test to test for differences between specific treatment combinations. This was used because interaction effects did not allow for the interpretation of main effects of the time and temperature treatments in the two-factor ANOVA design.

Week 1		Week 2		Week 3	
Room temperature (25°C)		**Room temperature (25°C)**		**Room temperature (25°C)**	
4 Hour X 8 tanks 8 Hour X 8 tanks 12 Hour X 8 tanks	Submerged 0 Hour X 8 tanks	4 Hour X 8 tanks 8 Hour X 8 tanks 12 Hour X 8 tanks	Submerged 0 Hour X 8 tanks	4 Hour X 8 tanks 8 Hour X 8 tanks 12 Hour X 8 tanks	Submerged 0 Hour X 8 tanks
Incubator 1 (29°C)		**Incubator 1 (29°C)**		**Incubator 1 (29°C)**	
4 Hour X 8 tanks 8 Hour X 8 tanks 12 Hour X 8 tanks	Submerged 0 Hour X 8 tanks	4 Hour X 8 tanks 8 Hour X 8 tanks 12 Hour X 8 tanks	Submerged 0 Hour X 8 tanks	4 Hour X 8 tanks 8 Hour X 8 tanks 12 Hour X 8 tanks	Submerged 0 Hour X 8 tanks
Incubator 2 (35°C)		**Incubator 2 (35°C)**		**Incubator 2 (35°C)**	
4 Hour X 8 tanks 8 Hour X 8 tanks 12 Hour X 8 tanks	Submerged 0 Hour X 8 tanks	4 Hour X 8 tanks 8 Hour X 8 tanks 12 Hour X 8 tanks	Submerged 0 Hour X 8 tanks	4 Hour X 8 tanks 8 Hour X 8 tanks 12 Hour X 8 tanks	Submerged 0 Hour X 8 tanks
Incubator 3 (44°C)		**Incubator 3 (44°C)**		**Incubator 3 (44°C)**	
4 Hour X 8 tanks 8 Hour X 8 tanks 12 Hour X 8 tanks	Submerged 0 Hour X 8 tanks	4 Hour X 8 tanks 8 Hour X 8 tanks 12 Hour X 8 tanks	Submerged 0 Hour X 8 tanks	4 Hour X 8 tanks 8 Hour X 8 tanks 12 Hour X 8 tanks	Submerged 0 Hour X 8 tanks

Figure 7: Layout of experimental design for warm air exposure and thermal tolerance experiments. Each box represents a separate incubator. 0-hour tanks are tanks from which the mussels were never removed from the water and therefore were not placed in an incubator (control). For this reason they are not shown inside of a box but rather beside the corresponding box. Cold experiments utilized the same design but with temperatures of 25, 20, 15, and 8°C. Note that each week represents one replicate of each treatment. Incubator temperature assignments were randomized, as well as the placement of mussels inside incubators.

RESULTS

Oyster reef survey results

Throughout all of the times surveyed, there were only two instances of a live, non-native invertebrate being found on an oyster reef. This occurred when one live *P. viridis* individual was found on a restored reef and another on a reference reef in Mosquito Lagoon during the January 2009 survey.

Surveys for the number of live *C. virginica* and live *B. exustus* showed that, for the Mosquito Lagoon reefs monitored, the factor of reef type was significant. The ANOVA for number of *C. virginica* resulted in a p value of <0.0001 for the factor of reef type, while the ANOVA for number of *B. exustus* had a p value of 0.001 (Table 1 and Table 2). Thus a post hoc Tukey's test was used in both cases. Quadrats placed on reference reefs resulted in an average of 37.45 ($\pm$ 3.08 S.E.) live oysters per 0.25 m^2. This was a significantly higher average than that found on dead reefs (Figure 8). These reefs averaged just 1.36 ($\pm$ 0.35 S.E.) live *C. virginica* per quadrat. Restored reefs also had a significantly higher number of *C. virginica* than dead reefs with an average of 38.34 ($\pm$ 3.08 S.E.) live *C. virginica* per 0.25 m^2. Restored reefs were not significantly different than reference reefs for number of live *C. virginica* on the reefs tested in Mosquito Lagoon (Figure 8). In Mosquito Lagoon, reference reefs held a significantly higher number of the native mussels than dead reefs. However, restored reefs were not significantly different from either of the other two reef types for the presence of live *B. exustus* (Figure 9). *Brachidontes exustus* were found on reference reefs at an average of 0.34 ($\pm$ 0.05 S.E.) per 0.25m^2. This was significantly higher than the 0.03 ($\pm$ 0.03 S.E.)

found on average on dead reefs. Restored reefs had an average of 0.18 (± 0.06 S.E.) *B. exustus* per quadrat (Figure 9).

Table 1: Analysis of variance table for the test of reef type on the dependent variable of number of live *C. virginica* for oyster reefs in Mosquito Lagoon.

Source	Df	SS	MS	F value	P value
Reef type	2	6231.27	3115.63	76.575	<0.0001
Within Groups	18	732.374	40.687		

Table 2: Analysis of variance table for the test of reef type on number of live *B. exustus* for oyster reefs in Mosquito Lagoon.

Source	Df	SS	MS	F value	P value
Reef type	2	0.337	0.168	10.377	0.001
Within Groups	18	0.292	0.016		
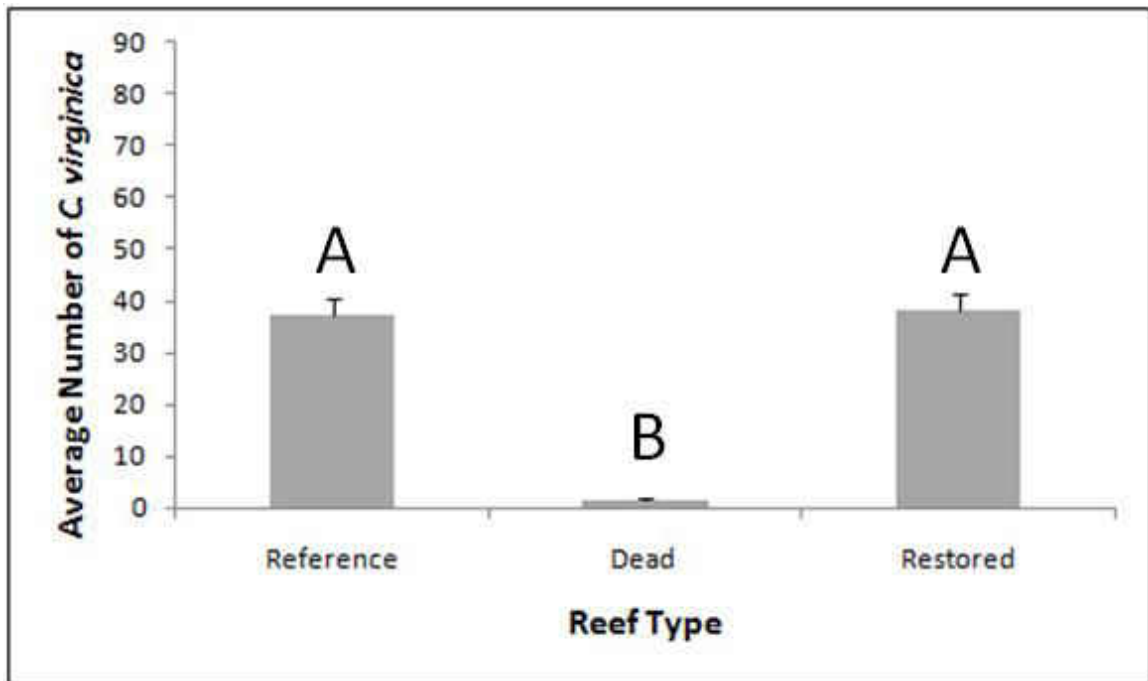
Figure 8: Average number of live *C. virginica* per 0.25m^2 on each of three reef types in Mosquito Lagoon. Uppercase letters denote significant difference between reef type treatments as determined by a *post hoc* Tukey test. Error bars represent standard error.
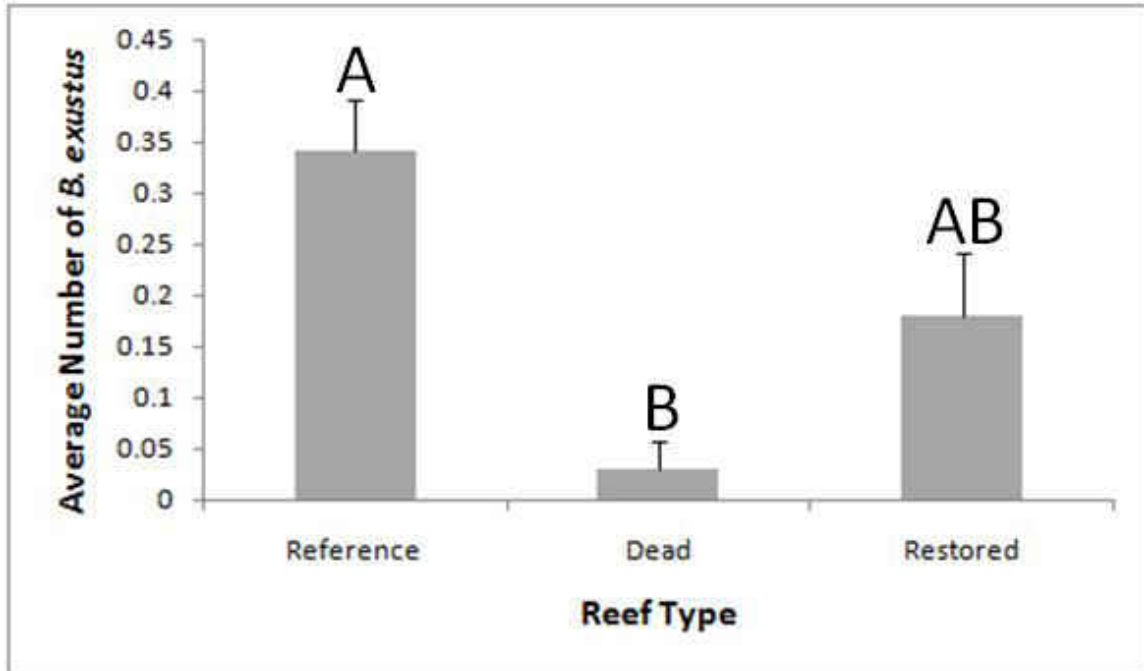
Figure 9: Average number of *B. exustus* found on each of three reef types in Mosquito Lagoon. Uppercase letters denote significant difference between reef type treatments. Error bars represent standard error.

Oyster reef monitoring in the GTMNERR yielded similar results. Reef type once again had a significant effect on the number of live *C. virginica* as well as the number of live, native mussels (*G. demissa*) (Table 3). The t-test for *C. virginica* found a p value of <0.0001 for the factor of reef type while the t-test for *G. demissa* had a p value of 0.002 (Table 3). Reference reefs had a higher number of live *C. virginica* per $0.25m^2$ with an average of 80.13 (± 5.79 S.E.) (Figure 10). Dead reefs had an average of 2.22 (± 0.89 S.E.) live *C. virginica* per quadrat. *Geukensia demissa* were more prevalent on reference reefs as well. This was shown by an average of 3.95 (± 0.97 S.E.) live *G. demissa* on reference reefs and 0.03 (± 0.02 S.E.) on dead reefs (Figure 11). Although *Mytella charruana* and *Perna viridis* were not readily found on oyster reefs in either of the two study locations, it is important to note that the lack of significant decline in native

individuals found on dead reefs would suggest that these reefs present ample open space

for the non-native mussels to settle.

Table 3: Results of t-tests for both number of live *C. virginica* and number of live *G. demissa* found on dead and reference reefs in the GTMNERR.

Test	t Statistic	Df	Sig. (2-Tailed)
C. virginica	13.288	10	<0.0001
G. demissa	4.061	10	0.002
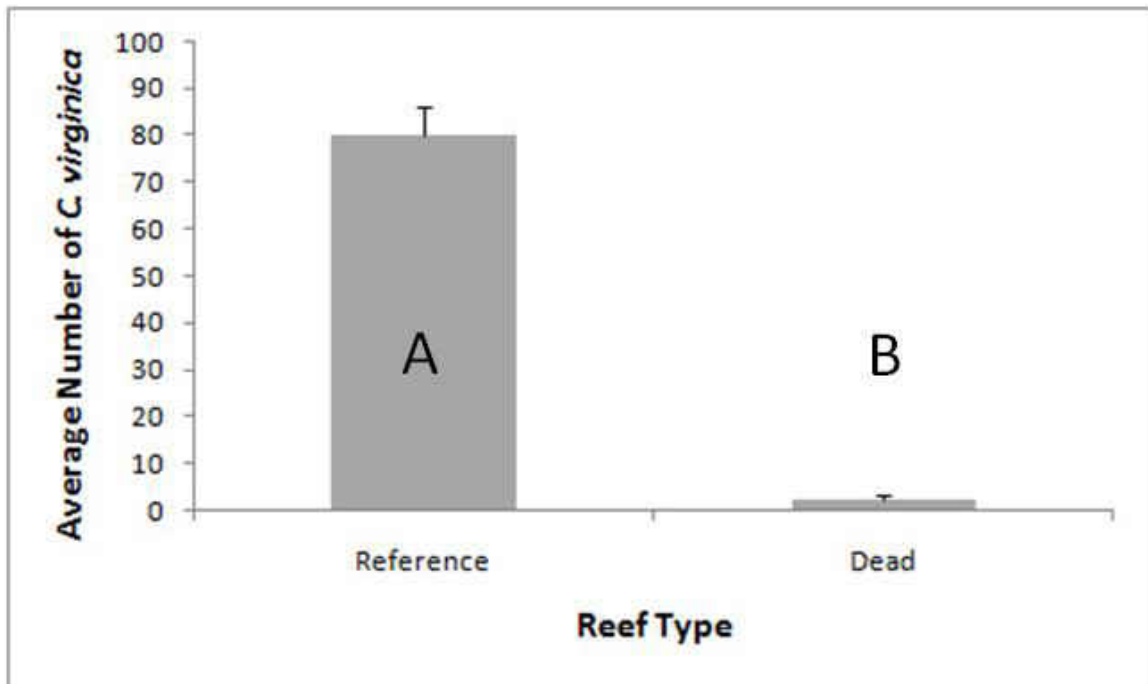
Figure 10: Average number of live *C. virginica* per 0.25m² found on two reef types within the GTMNERR. Uppercase letters denote significant difference between treatments. Error bars represent standard error.
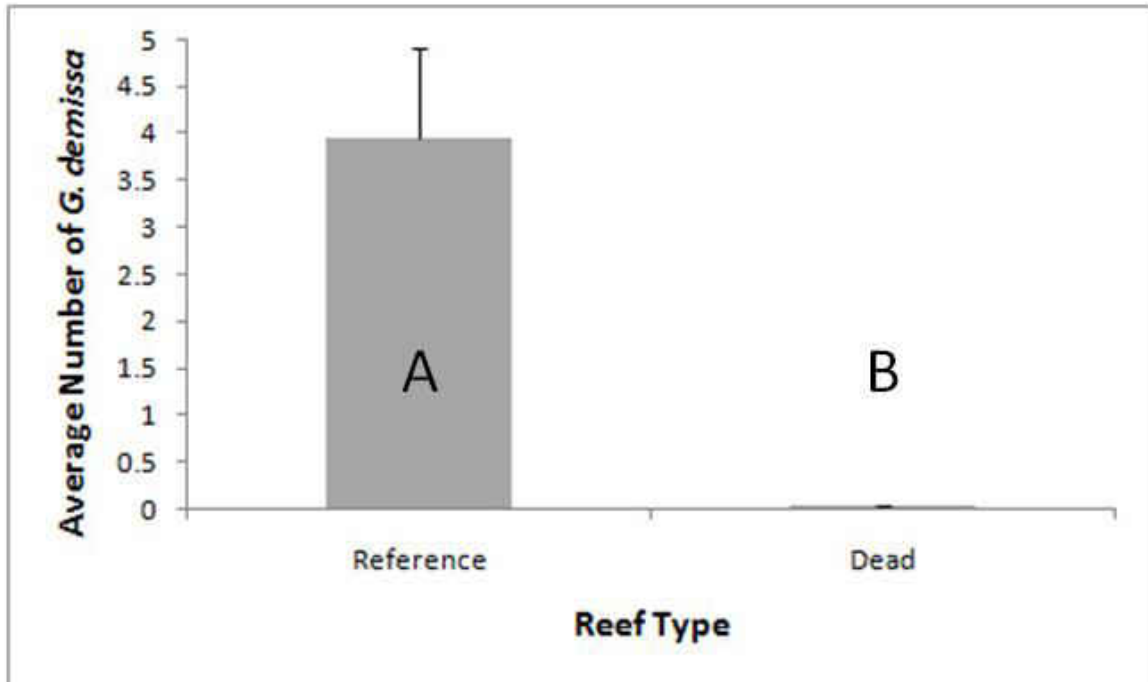
Figure 11: Average number of live *G. demissa* per 0.25m^2 found on two reef types within the GTMNERR. Uppercase letters denote significant difference between treatments. Error bars represent standard error.

Room temperature aerial exposure tolerance experiments

In the *M. charruana* repeated exposure experiment, large mussels did not show a significant difference in survival between the control treatment (mussels that were submerged continuously) and mussels that were exposed for 4, 8, 12 or 18 hours per day with survival in all treatments exceeding 90% after 7 days (Figure 12). Small *M. charruana* did not experience a significant change in survival when exposed to air for 12 hours per day or less, but did have statistically lower survival when exposed for 18 hours per day (Figure 13). The *M. charruana* extended exposure trial showed that 100% of large *Mytella* survived 24 continuous hours of exposure, with 8% of large *M. charruana* surviving 4 continuous days of aerial exposure (Figure 14). After 48 hours, survival dropped below 50%. Only 8% of small mussels survived after 24 hours of aerial

exposure, with 0% survival after two days (Figure 15). *Mytella charruana* was able to

survive long periods of air exposure at room temperatures and these preliminary

experiments showed that exposure experiments in the laboratory can be completed

successfully with this species.
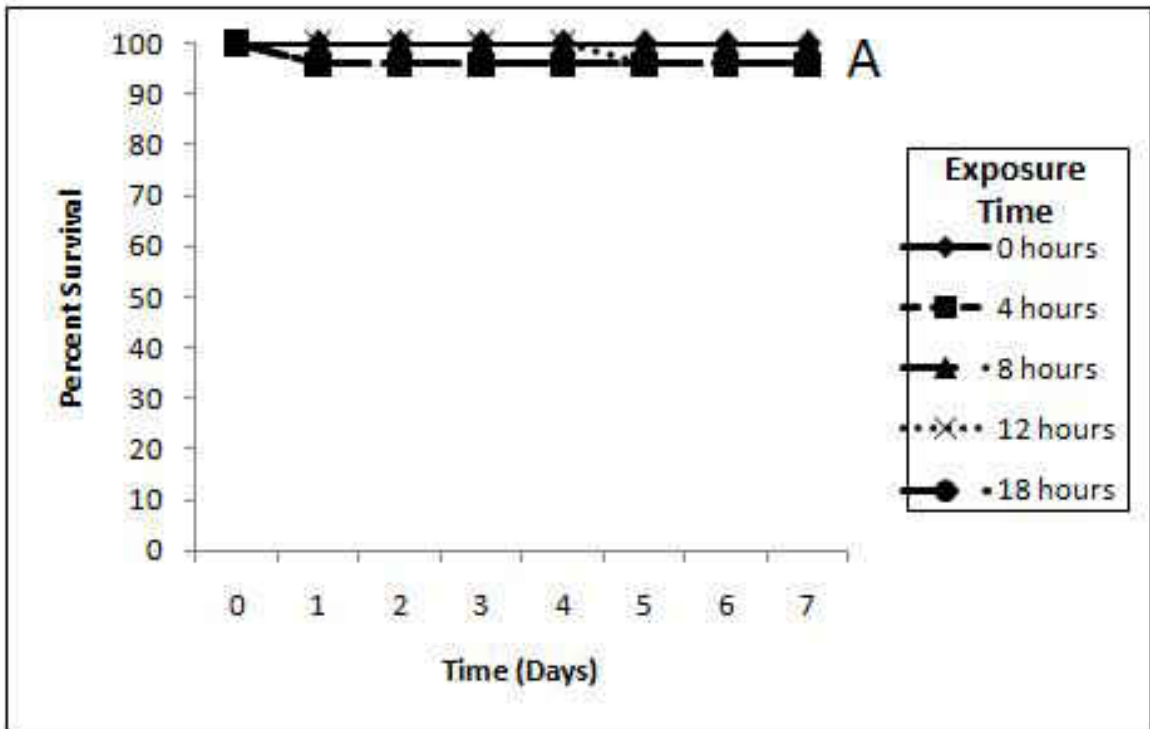
Figure 12: Large *Mytella charruana* repeated exposure experiment average survival over time results for large mussels at five different treatment times. Note that the 0 hours line is not visible due to overlapping of lines.
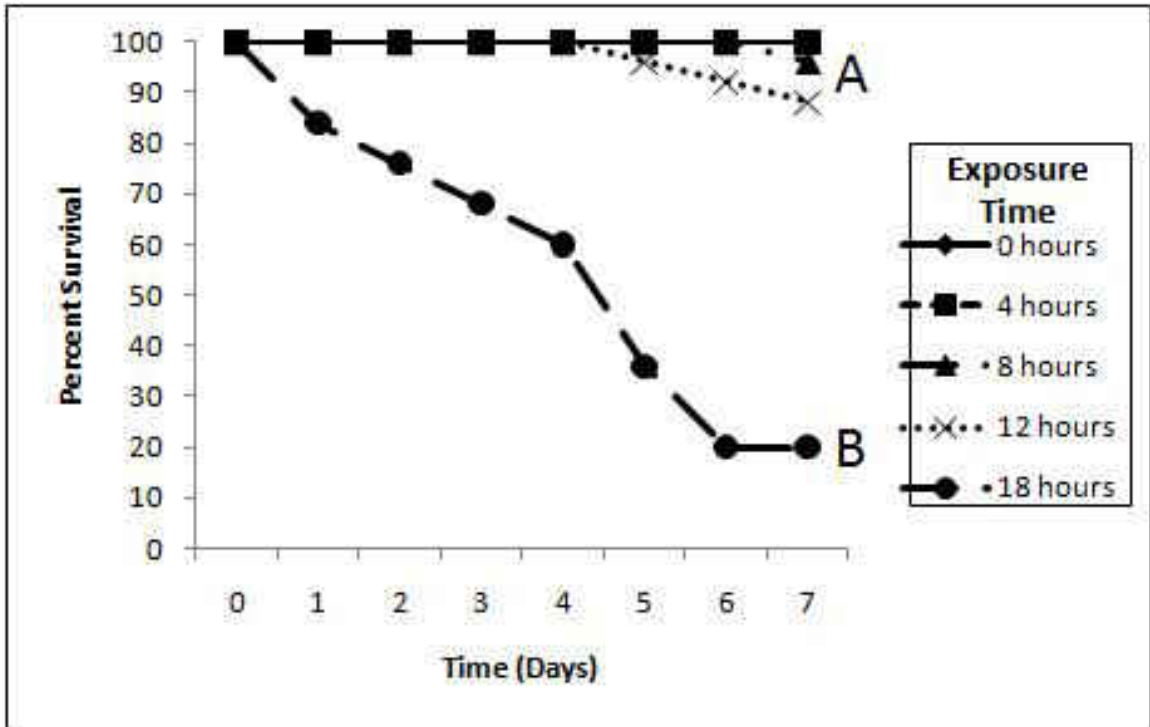
Figure 13: Small *Mytella charruana* repeated exposure experiment average survival over time results at five different treatment times. Note that the 0-hour line is not visible due to overlapping of lines.
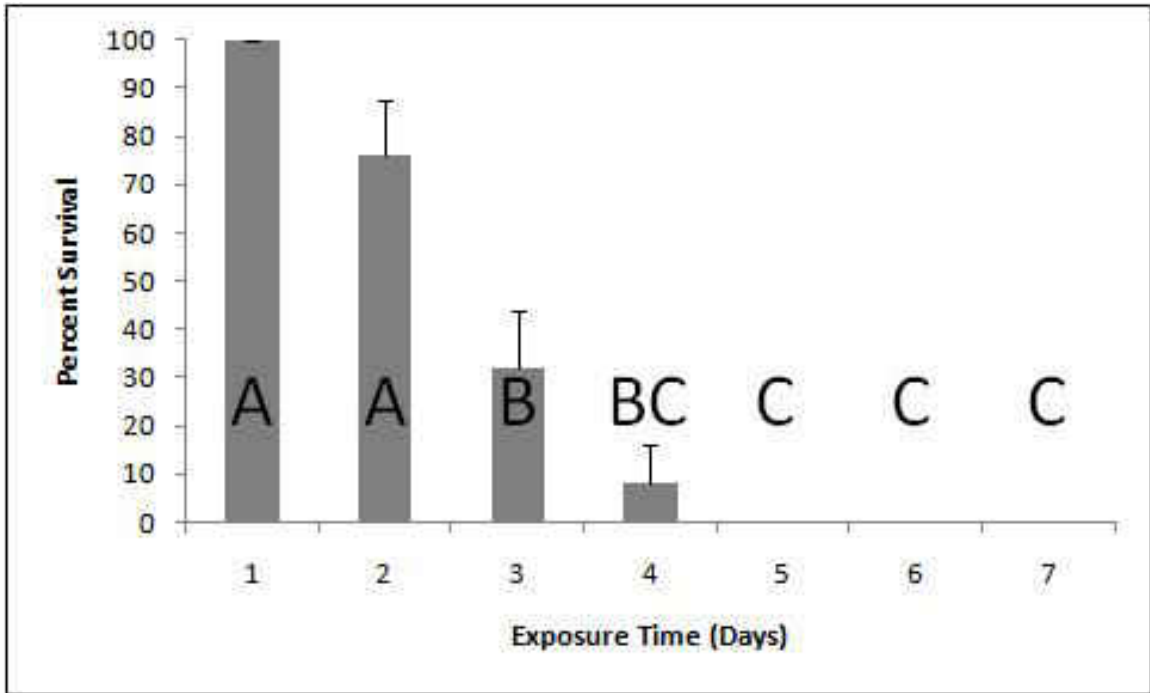
Figure 14: Extended exposure experiment results including mean (± S. E.) survival across time treatments for large *Mytella charruana*.
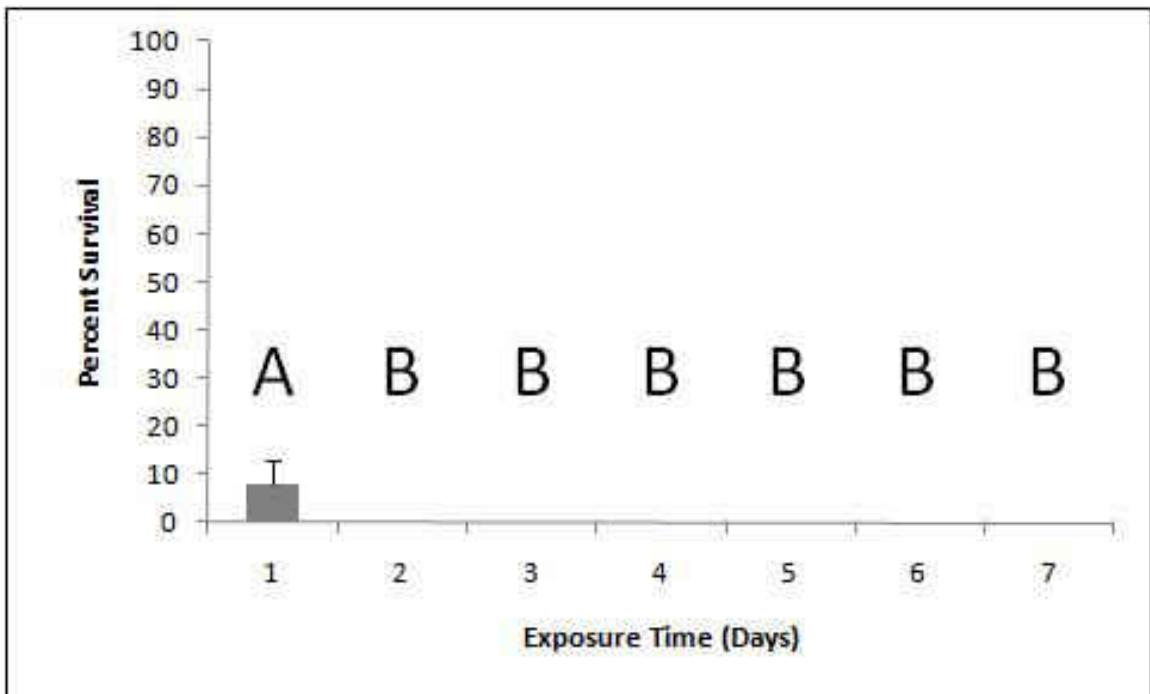
Figure 15: Extended exposure experiment results including mean (± S. E.) survival across time treatments for small *Mytella charruana*.

The *P. viridis* repeated exposure experiment for large mussels resulted in statistically similar survival between the control and the 12-hour and less exposure treatments. However, survival was significantly reduced in the 18-hour treatment with only 50% survival after 7 days (Figure 16). Small *Perna viridis* fared even worse in the repeated exposure experiment. By the end of the week, the 12-hour treatment only had 30% survival and the 18-hour treatment had 0% survival starting on day 5, both being significantly lower than the 80% survival in the control (Figure 17).

The extended exposure results for large *P. viridis* showed that green mussels can live exposed at room temperature in a laboratory continuously for up to 5 days, with 5% survival. Survival did not drop below 50% until mussels had been exposed for 3 days, at which time survival was 25% (Figure 18). Small *P. viridis* had 100% survival after 1 day of exposure. This matched the submerged controls which also had 100% survival. However, survival dropped to 10% in the 2-day treatment and other treatments of 3 days or more resulted in 0% survival (Figure 19).
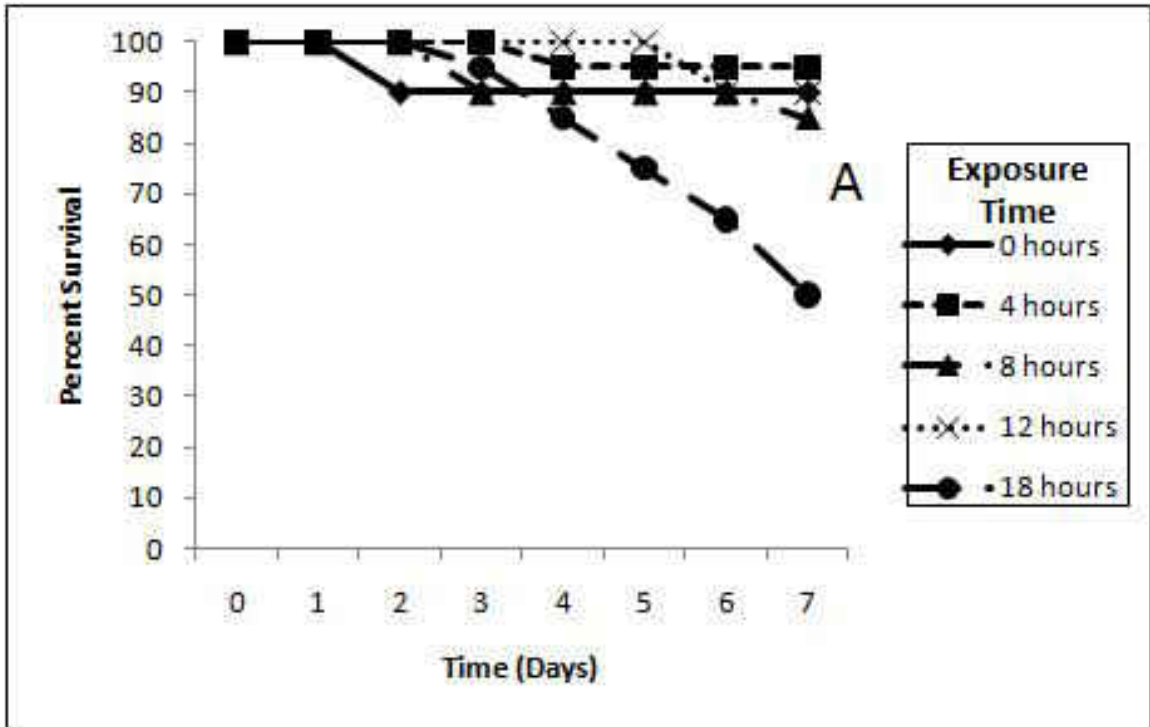
Figure 16: Large *Perna viridis* repeated exposure results with lines showing average survival over time for large mussels at five different treatment times.
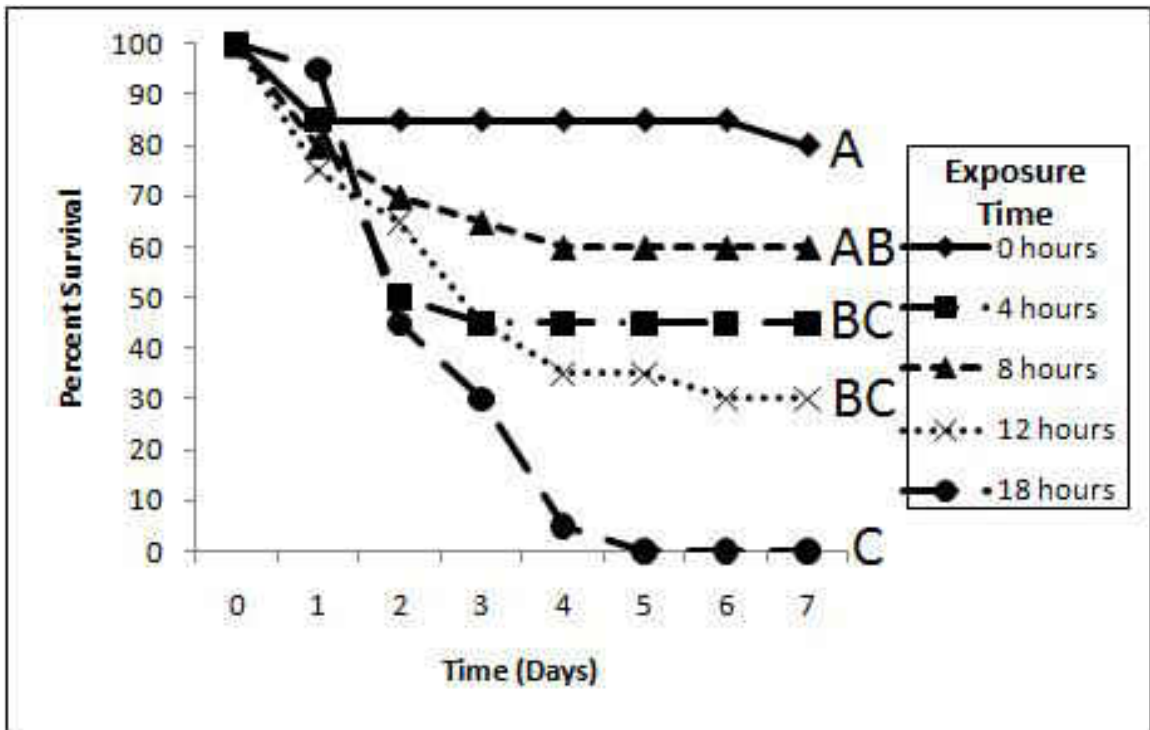
Figure 17: Small *Perna viridis* repeated exposure results with lines showing average survival over time at five different treatment times.
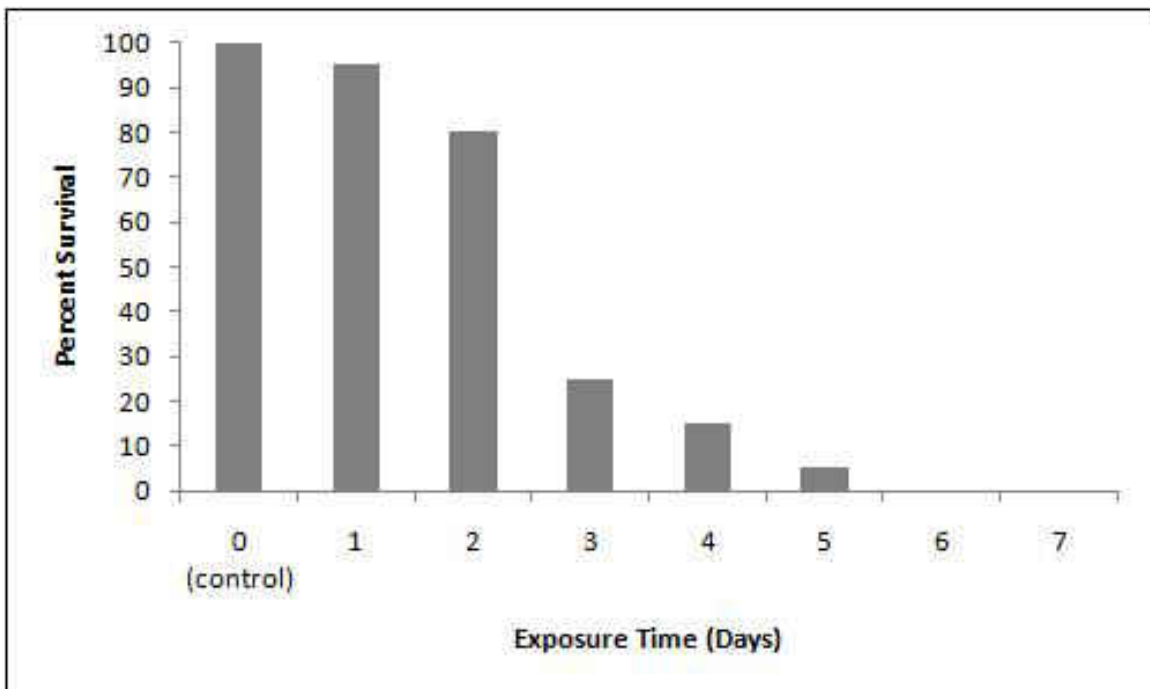
Figure 18: Extended exposure survival across time treatments for large *Perna viridis*. Data shown represent the percentage of replicates that survived in each treatment rather than an average of subsamples so no error bars are shown. The p value for the regression was < 0.0001.
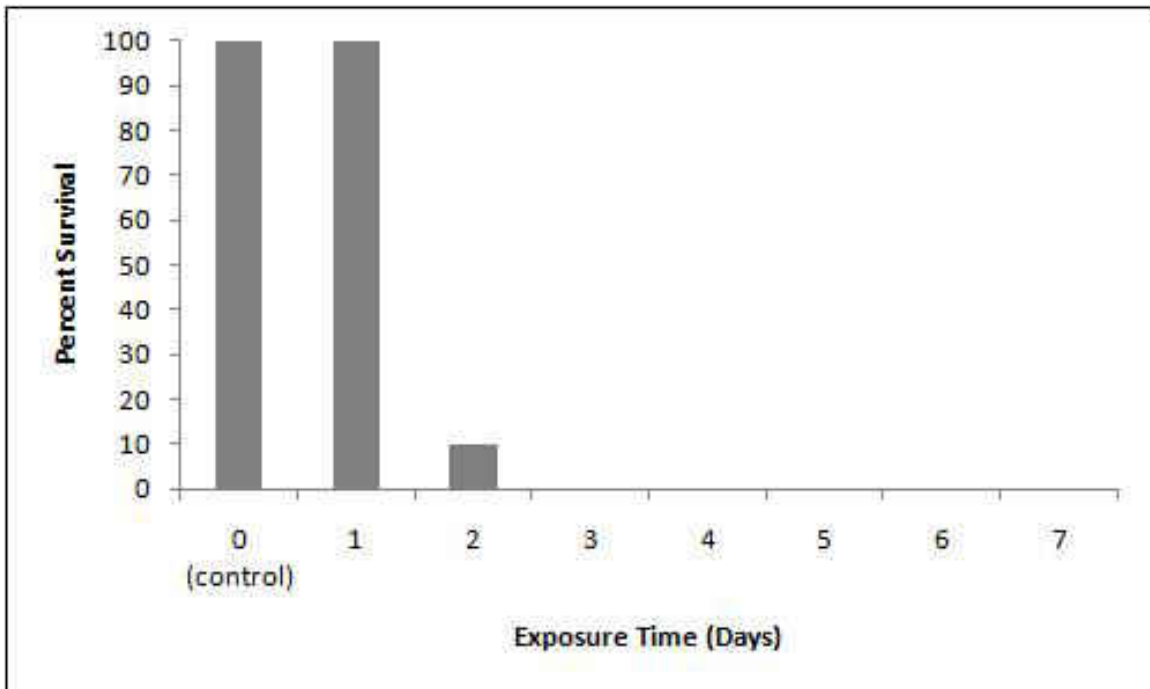
Figure 19: Extended exposure survival across time treatments for small *Perna viridis*. Data shown represent the percentage of replicates that survived in each treatment rather than an average of subsamples so no error bars are shown. The p value for the regression was <0.0001.

Long-term oyster reef temperature data collection

Crassostrea virginica reef temperatures were obtained in summer 2009 in

Mosquito Lagoon for a total of 18 days during the period of 7-24 July. Some data was

lost due to lost or damaged iButton temperature loggers. The data that was recovered for

July 2009 resulted in 3 replicate reference reefs, 4 dead reefs, and 4 restored reefs. Data

points were separated into exposed and submerged periods of time for 12 days of

temperature data. Times chosen for each category were found by locating temperature

peaks or valleys that matched NOAA tide table tidal changes. These times were chosen

if they were determined to be obvious exposed times. Either exposed or submerged times

may have occurred during night or day. Day times versus night times were not separated

as all were simply considered either exposed or submerged. However, July exposed

temperatures typically represented daytime exposures. This was because noticeable

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changes in temperature mostly occurred during daylight hours, while nighttime air

temperatures were often similar to water temperature. Overall average temperature while

reefs were submerged showed that none of the reef types were significantly different (p =

0.767; Table 4). Restored reefs had an average submerged temperature of 30.46° C (±

0.16 S.E.), reference reefs had average of 30.34° C (± 0.28 S.E.), and dead reefs averaged

30.57° C (± 0.05 S.E.) while submerged (Figure 20). Reefs types were different,

however, when it came to temperatures during aerial exposure. For overall average

temperature while exposed to air, the factor of reef type was significant (p = 0.002)

(Table 5). The Tukey test showed that restored reefs which averaged 33.01° C (± 0.77

S.E.) were not significantly different from reference reefs which averaged 34.17° C (±

0.99 S.E.) (p = 0.541), but that both reference (p = 0.014) and restored reefs (p = 0.002)

were significantly different from dead reefs which averaged 38.15° C (± 0.43 S.E.)

(Figure 21).

Significant differences among reef types were found for average high temperature

while exposed to air (p = <0.0001) (Table 6). The average high temperature for dead

reefs was significantly higher than temperatures for either of the other two reef types with

an average of 43.01° C (± 0.85 S.E.). Reference reefs had an average high temperature of

35.52° C (± 1.35 S.E.), while restored reefs had an average high of 34.03° C (± 0.88 S.E)

(Figure 22). These two reef types were found to be statistically similar for mean high

temperature.

Table 4: Analysis of variance table for the test for the affect of reef type on overall average reef temperature while submerged in water

Source	Df	SS	MS	F value	P value
Reef type	2	0.058	0.029	0.274	0.767
Within groups	8	0.854	0.107		

Table 5: Analysis of variance table for the test for the affect of reef type on overall average reef temperature while exposed to air

Source	Df	SS	MS	F value	P value
Reef type	2	57.083	28.542	14.934	0.002
Within groups	8	15.290	1.911		

Table 6: Analysis of variance table for the test for the affect of reef type on average high oyster reef temperature while exposed to air

Source	Df	SS	MS	F value	P value
Reef type	2	180.919	90.459	25.093	<0.0001
Within groups	8	28.839	3.605		
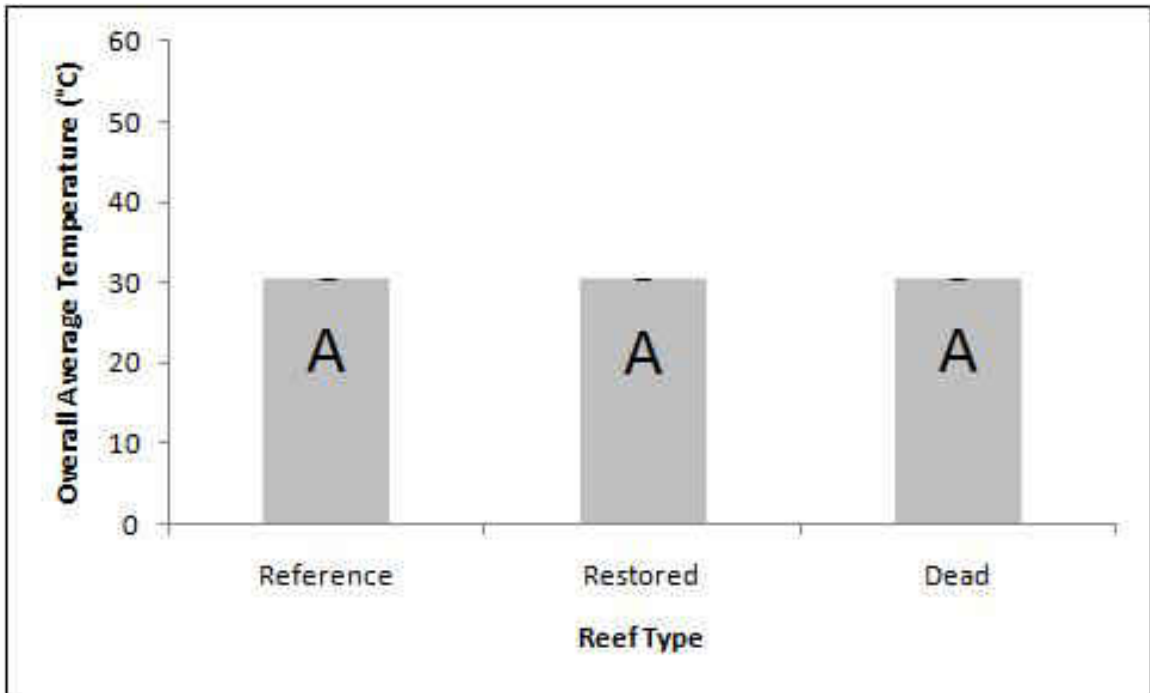
Figure 20: Overall average temperature for each reef type while submerged in water during July 2009. Error bars represent standard error.
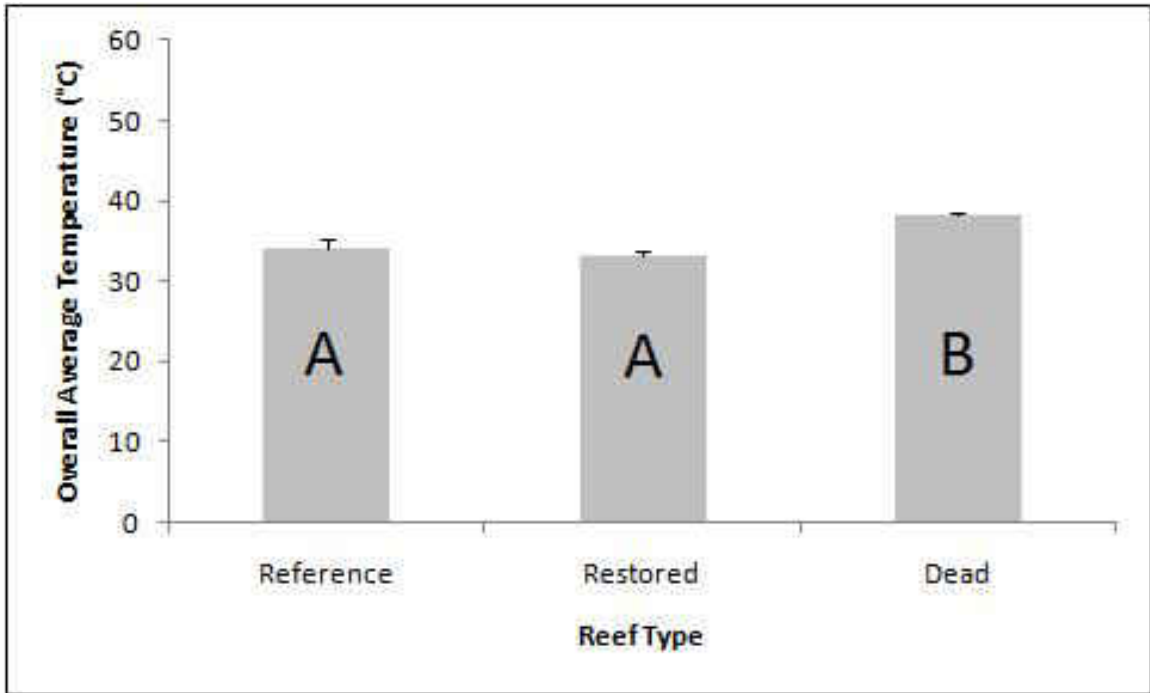
Figure 21: Overall average temperatures for different reef types while exposed to air for times taken from July 2009. Error bars represent standard error and letters on bars represent significant differences in average temperature between reef types.
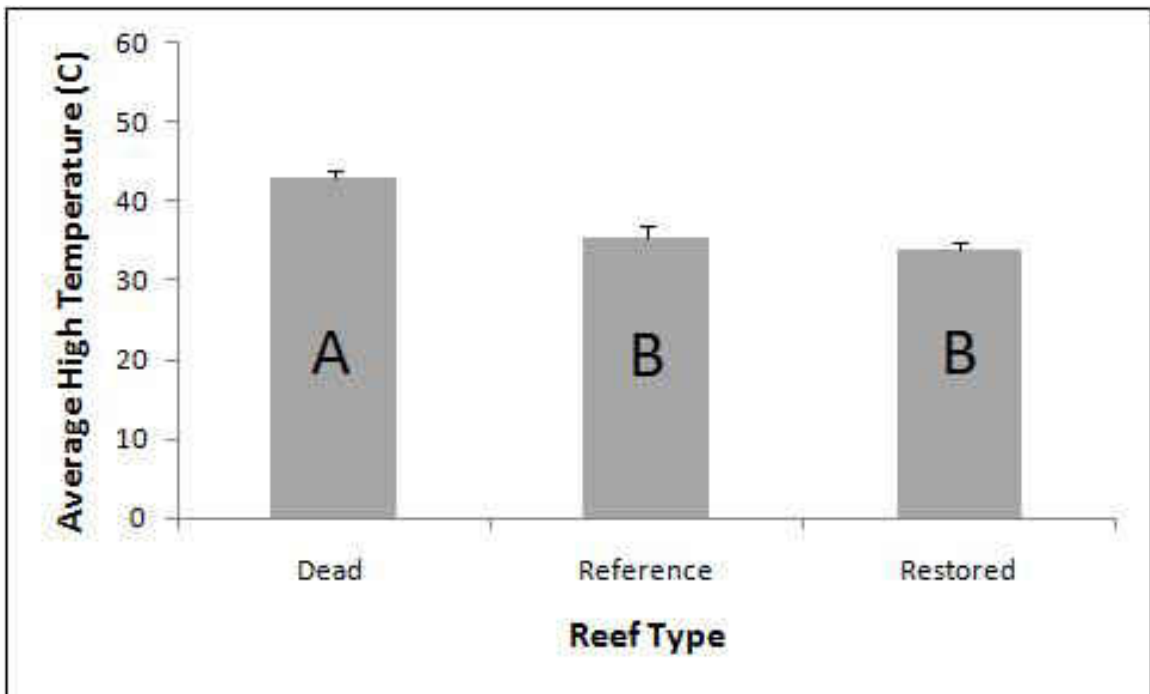
Figure 22: Average maximum temperatures for different reef types at times taken from July 2009. Error bars represent standard error and letters on bars represent significant differences in average maximum temperature among reef types.

Winter reef temperature monitoring resulted in 23 days of temperature data from which clear patterns of aerial exposure and submersion were found. Data that I was able to recover were from 3 replicate reference reefs, 4 restored reefs, and only 1 dead reef. Loggers were lost by iButtons[®] coming loose from cable ties, malfunctioning iButtons[®], or loss of loggers due to shifting of shells on dead reefs which may have covered loggers. Submerged temperatures for reference and restored reefs were not significantly different from one another (p = 0.796) (Table 7) with reference reefs having an average of 13.37° C (± 0.11 S.E.) and restored reefs having an average of 10.50° C (± 0.36 S.E.) (Figure 23). While dead reefs could not be statistically compared, the overall average submerged temperature value for the dead reef was 14.49° C.

When exposed temperatures were tested for significant difference between restored and reference reefs, the t-test yielded a non-significant value of 0.548 (Table 8). While exposed, reference reefs had an average temperature of 10.83° C (± 0.32 S.E.) while restored reefs had an average temperature of 10.50° C (± 0.36 S.E.) (Figure 24). The dead reef measured had an average exposed temperature of 12.45° C. Average minimum values showed a lack of significant difference with p values of 0.850 and 0.496, respectively (Table 8). Average minimum temperatures for reference reefs were 8.49° C (± 0.23 S.E.), restored reefs had an average minimum of 8.44° C (± 0.17 S.E.), and one dead reef, which had an average minimum of 9.63° C (Figure 25).

Table 7: Results for the t-test for significant difference in overall average temperature between reference and restored reefs while submerged in water during January and February 2010.

Test	Df	t-statistic	Sig. (2-Tailed)
Overall average	5	-0.272	0.796

Table 8: Results for two separate t-tests, overall average temperature and average minimum. Tests in this table are for temperatures that occurred during January and February 2010 while reefs were exposed to air.

Test	Df	t-statistic	Sig. (2-Tailed)
Overall average	5	0.644	0.548
Average minimum	5	0.199	0.850
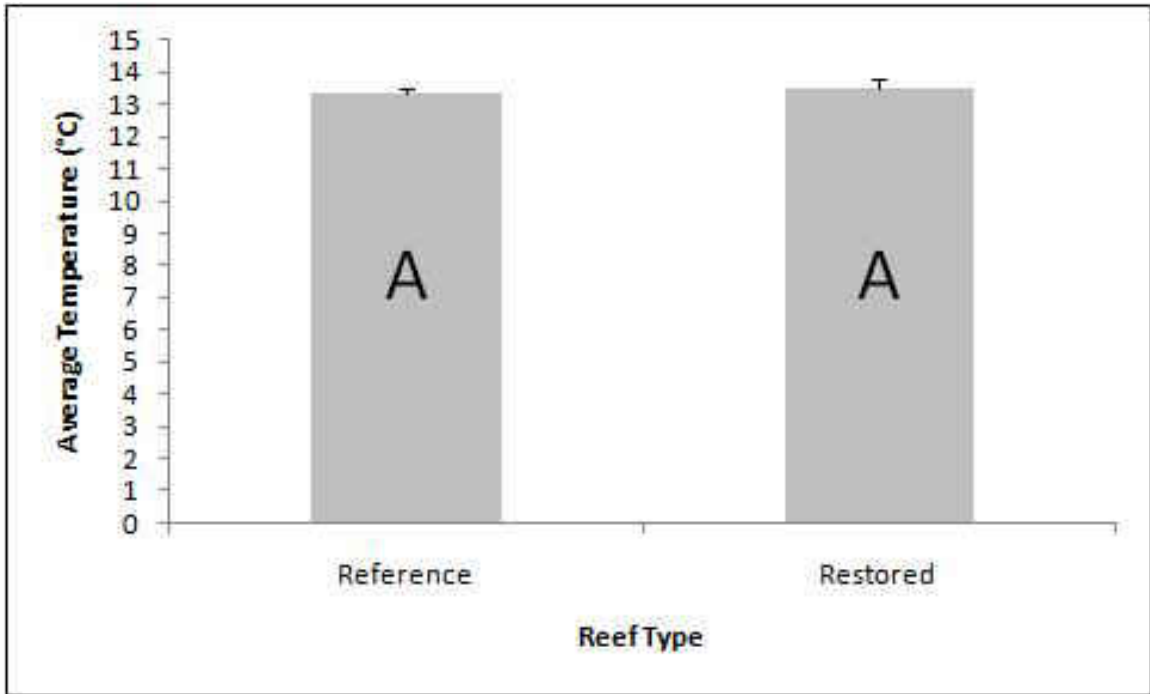
Figure 23: Overall average temperatures for restored versus reference reefs while submerged in water during January and February 2010. Error bars represent standard error.

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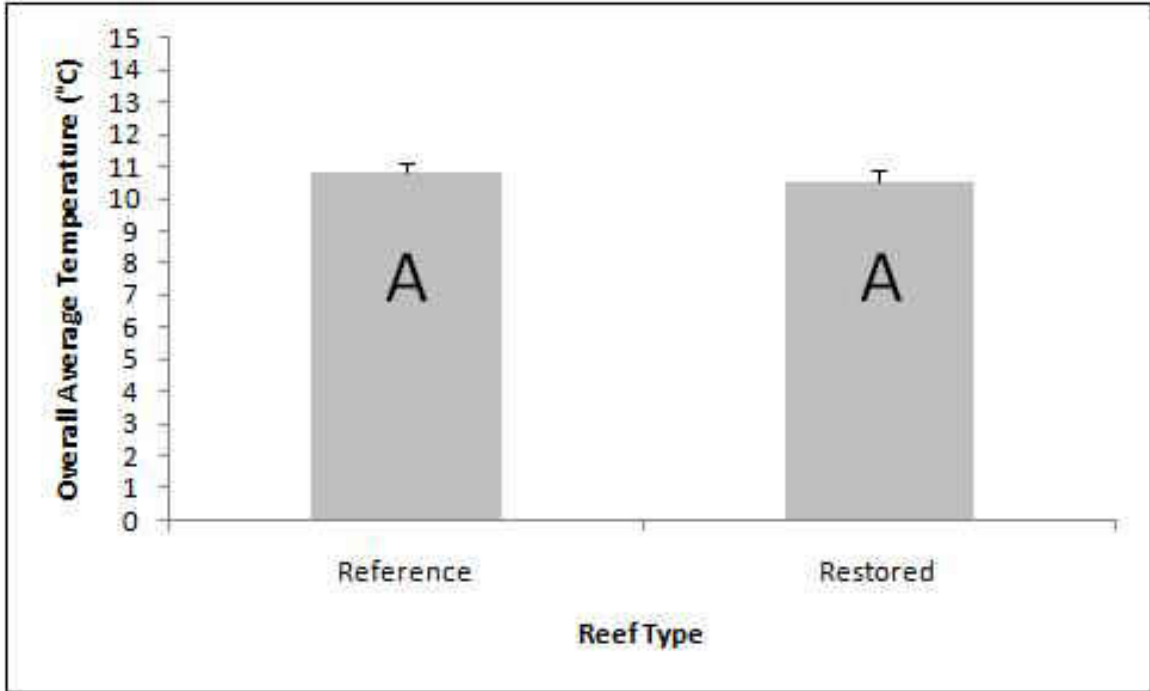
Figure 24: Overall average temperatures for reference and restored reef types while exposed to air during January and February of 2010. Error bars represent standard error.
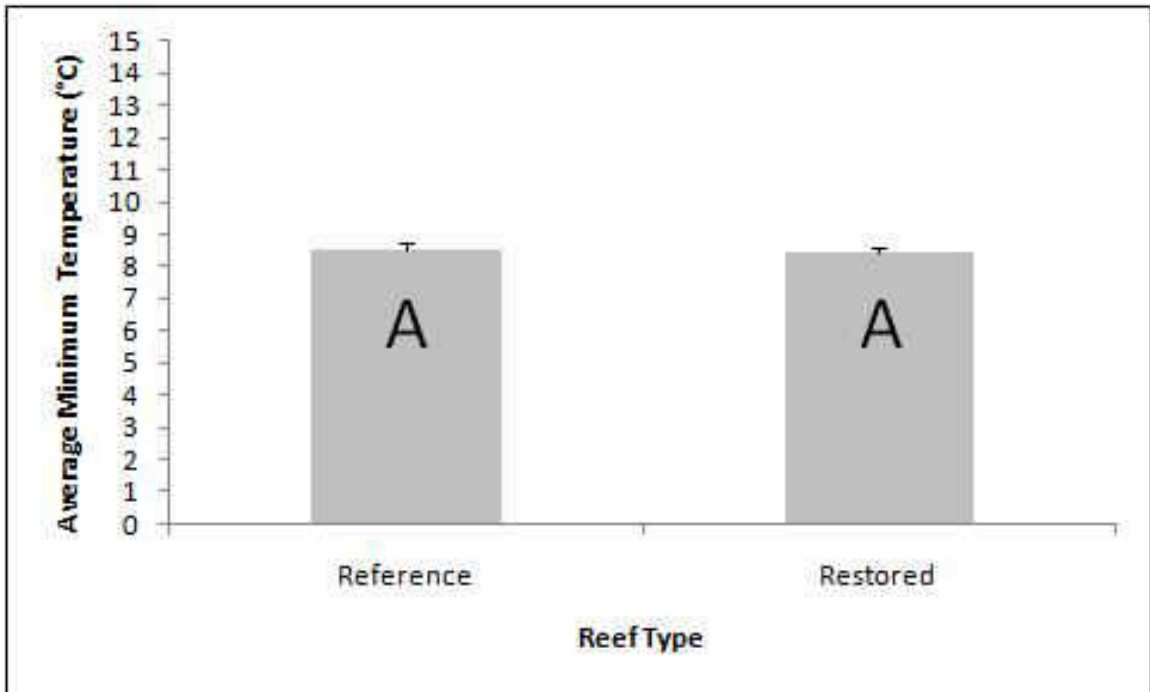
Figure 25: Average minimum temperatures for reference and restored reef types while exposed to air during January and February of 2010. Error bars represent standard error.

Warm temperature tolerance

Results of the ANOVA for warm temperature tolerance found significant p values for the factors of time and temperature with p values of <0.0001 for both factors (Table 9). The blocking factor was also significant (p = 0.0002). For this reason, the results for the warm temperature tolerance experiment will be further discussed as separate blocks. The factors of time and temperature also had an interactive affect (p < 0.0001). Neither time nor temperature interacted with the blocking factor however. Figure 26 shows an interaction plot for the two factors of time and temperature. The line for 25° C crosses the lines for the 29° C and 35° C treatments showing that the main effects of time and temperature cannot be contrasted *post hoc*. For the blocking factor, block two was significantly different from blocks one and three. However, blocks one and three were not significantly different from each other. The blocked, one-way ANOVA for the affect of the factor of combined time and temperature on survival again found that block was significant with the p value of 0.0002 (Table 10). The treatment factor (combined time and temperature) was also significant (p < 0.0001). The 4, 8, and 12 hours at 44° C had the lowest survival but were not significantly different than one another. Twelve hours at 35° C treatment had significantly higher survival but was significantly lower than the 8 hours at 35° C and 12 hours at 29° C treatments, which were not different. Twelve hours at 25° C and 8 hours at 29° C were not significantly different and had significantly higher survival than all the previously mentioned treatments. All treatments had significantly lower survival than the 4 hours at 25, 29, and 35° C as well as the 8 hours at 25° C, which were not significantly different. A graphical representation of the Tukey's test results can be seen in Figure 28. Only the survival results from the seventh day of each block, or the

endpoint of each line in Figure 27, Figure 28, and Figure 29 were included in the

ANOVA. However, it seems clear that number of days of exposure was important in

describing percent survival for all three blocks in some treatments.

Table 9: Results of the two factor, blocked ANOVA for the warm temperature tolerance experiment.

Source	Df	SS	MS	F	P value
Time	2	1.6886	0.8443	308.8709	<0.0001
Temp	3	7.2437	2.4146	883.3307	<0.0001
Block	2	0.1087	0.0544	19.8911	0.0002
Time x Temp	6	1.1574	0.1929	70.5702	<0.0001
Time x Block	4	0.0196	0.0049	1.7926	0.1952
Temp x Block	6	0.0370	0.0062	2.2565	0.1085
Residuals	12	0.0328	0.0027		
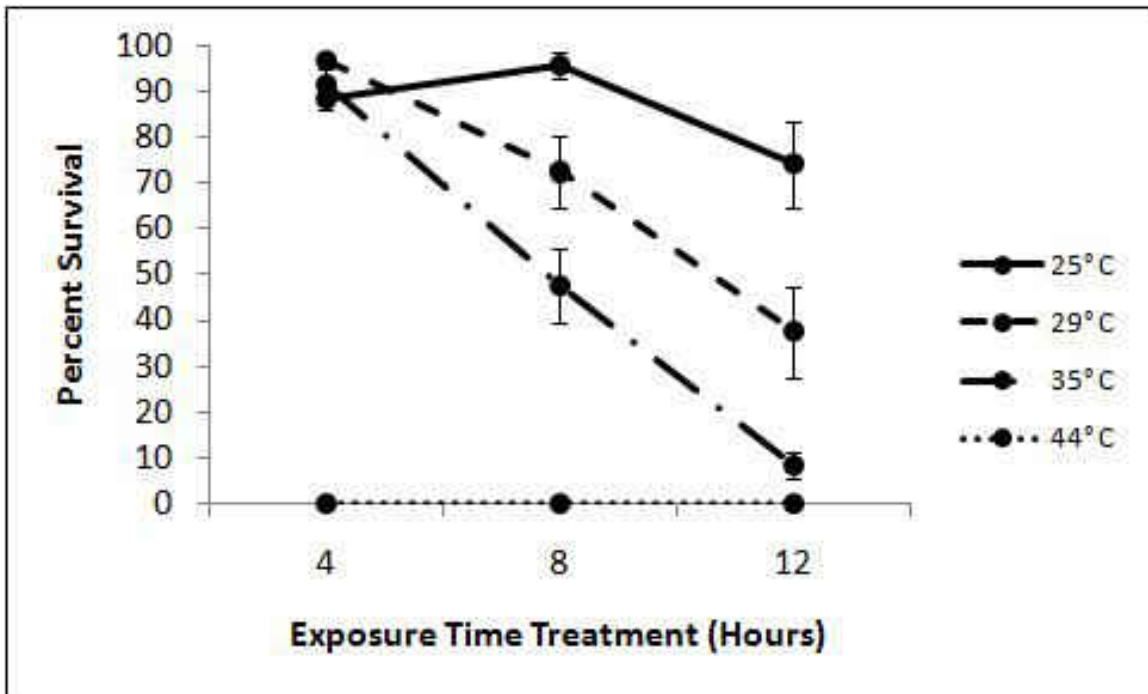
Figure 26: Interaction plot for the factors of time and temperature in the warm temperature tolerance experiment. Lines represent different temperature treatments while time treatments are shown on the x-axis. Error bars represent standard error of the mean at each point. Data shown here represents treatment means across all three blocks for the final survival occurring on the seventh day of the experiment.

Table 10: Analysis of variance table for the blocked one-way ANOVA for the affect of combined air exposure time and temperature treatments for the warm temperature tolerance experiment.

Source	Df	SS	MS	F	P value
Treatment	11	10.0898	0.9173	225.6950	<0.0001
Block	2	0.1087	0.0544	13.3790	0.0002
Residuals	22	0.0894	0.0041		

Results for the first block showed that mussels placed in incubators set at the room temperature treatment (25° C) showed high survival after 7 days. All treatments had at least 85% survival and the first continuously submerged control had 100% survival. The 29° C treatments all had high survival (at least 87.5%) as well except for the 12-hour treatment which had 45% survival (± 7.32 S.E.). The second continuously submerged control had 95% (± 3.27 S.E.) survival. For the 35° C treatments, the 4-hour treatment had 97.5 % (± 2.5 S.E.) survival after 7 days but the 8 and 12-hour treatments had 62.5 (± 10.31 S.E.) and 10% (± 5.35 S.E.) survival, respectively. The third submerged control treatment had 97.5 % (± 2.5 S.E.) survival.   After 7 days, all of the exposed 44° C treatments had 0% survival, while the fourth continuously submerged control treatment had 97.5% (± 2.5 S.E.) survival. Results of the first block are presented graphically in Figure 27.
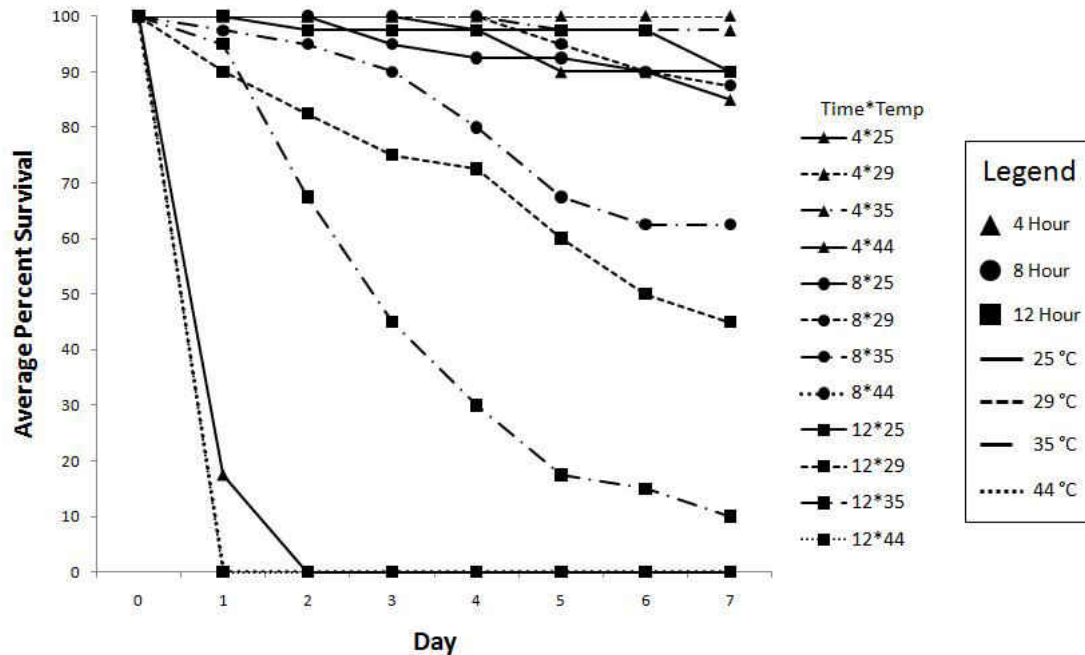
Figure 27: Percent survival over seven repeated days for the combinations of time and temperature treatments in the warm temperature tolerance experiment. Graph represents results for the first block only. The first number in each combination represents time and the second (followed by the "*") represents temperature. Submerged controls are not shown in the graph but all averaged 95% survival or higher after 7 days.

Results of the second block are shown in Figure 28. For the second block of the warm temperature tolerance experiment, 25° C treatments had at least 92.5% survival except for the 12-hour treatment which had 75% (± 10.52 S.E.) survival after 7 days. The first submerged control had 100% survival. After 7 days of repeated exposure, the 29° C had high survival in the 4-hour treatment with a survival percentage of 95% (± 3.27 S.E.) but had 70% (± 7.56 S.E.) survival in the 8-hour exposure treatment. The 12-hour treatment had just 50% (± 8.45 S.E.), survival while the second submerged control had a high survival rate of 95% (± 3.27 S.E.). Thirty-five degree treatment also had high survival after 7 days in the 4 hour treatment with 95% (± 3.27 S.E.) but dropped to 70% (± 7.56 S.E.) for the 8-hour treatment and to only 12.5% (± 5.26 S.E.) for the 12-hour treatment. The third submerged control had 95% (± 3.27 S.E.) survival. The 44° C once

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again had 0% survival for all exposed time treatments after 7 days. The fourth

continuously submerged control had 97.5% survival (± 2.5 S.E.).
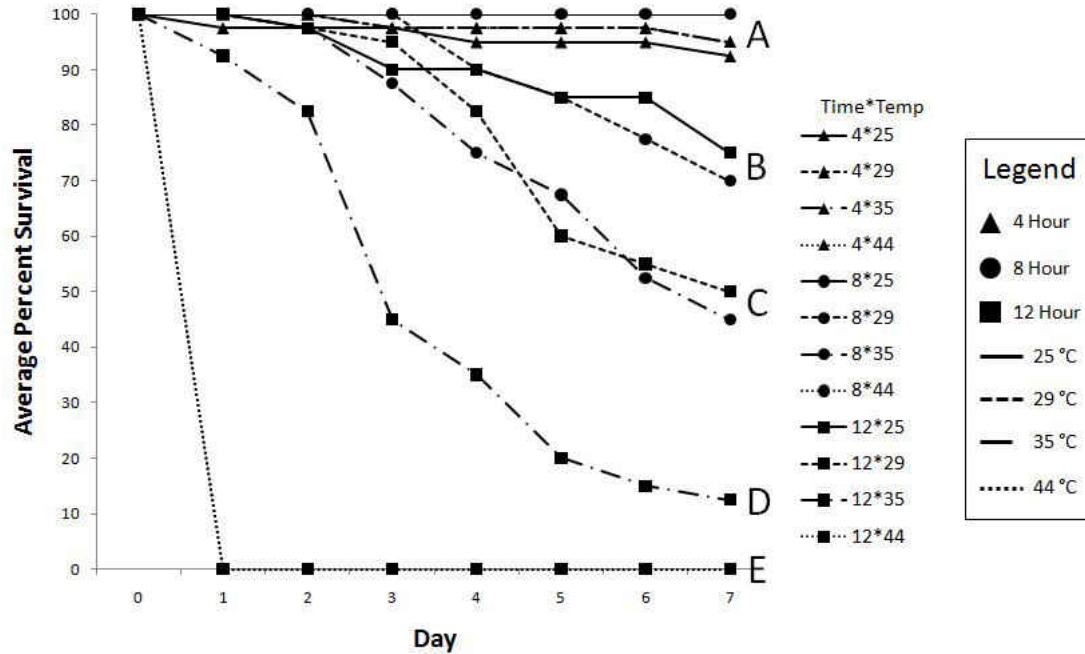
Figure 28: Percent survival over seven repeated days for the combinations of time and temperature treatments in the warm temperature tolerance experiment. Graph represents results for the second block only. The first number in each combination represents time and the second (followed by the "*") represents temperature. Submerged controls are not shown in the graph but all averaged 95% survival or higher after 7 days. Letters show significant differences among treatments found from the one way ANOVA. Data used in the ANOVA included only results from the seventh day (the endpoint of each line in the graph).

The largest differences in the third block, compared to the first and second blocks,

seemed to be in the 29 and 35° C time treatments. The third block was similar to the

other two blocks in that all continuously submerged control treatments had at least 87.5%

survival. After 7 days, the 25° C exposed treatments had high survival (87.5% or better)

in the 4 and 8-hour treatments along with the first submerged control which had 87.5% (±

5.26 S.E.) survival. However, survival dropped to 57.5% (± 4.53 S.E.) in the 12-hour

treatment. Twenty-nine degree treatments had high survival in the 4 hour treatment (95%

± 3.27 S.E.), but had 60% (± 7.56 S.E.) survival in the 8-hour treatment, and only 17.5%

(± 7.01 S.E.) in the 12-hour treatment after 7 days. The 35° C treatments again had fairly

high survival in the 4 hour treatment with 82.5% (± 5.9 S.E.) but dropped to 35% (± 11.8

S.E.) for the 8-hour treatment, and only 2.5% (± 2.5 S.E.) in the 12-hour treatment.

Figure 29: Percent survival over seven repeated days for the combinations of time and temperature treatments in the warm temperature tolerance experiment. Graph represents results for the third block only. The first number in each combination represents time and the second (followed by the "*") represents temperature. Submerged controls are not shown in the graph but all averaged 87.5% survival or higher after 7 days.

Cold temperature tolerance

Results for the cold temperature tolerance experiment showed that both the

factors of time and temperature were significant, both having p values of < 0.0001. The

blocking factor was also significant (p = 0.0140) so further results will be discussed as

separate blocks. Time and temperature did not have a significant interactive effect (p =

0.0990).   This combined with the interaction plot shown in Figure 30 suggest that the

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temperature and time treatments can be compared post hoc with pairwise comparisons.

There was no significant interaction between exposure time and block but temperature

and block did have an interactive effect ($p = 0.0266$). All time treatments were

significantly different (p values of less than 0.05). Within the temperature factor, the 20

and 15° C treatments were not significantly different. However, all other temperature

treatments were significantly different (p values of less than 0.05). Within the blocking

factor, blocks two and three were significantly different while one and three were similar.

Blocks one and two were also statistically similar. Analysis of Variance results are

shown in Table 11. A graph showing significant differences among treatments of the

exposure time and temperature factors is shown in Figure 31. Once again, although only

the data from the last day was statistically tested in the ANOVA, number of repeated

days of exposure seemed to highly affect survival for some treatments.

Table 11: Results of the two factor, blocked ANOVA for the cold temperature tolerance experiment.

Source	Df	SS	MS	F	P value
Time	2	0.9060	0.4530	26.3755	<0.0001
Temp	3	5.5065	1.8355	106.8738	<0.0001
Block	2	0.1403	0.0702	4.0857	0.0443
Time x Temp	6	0.2412	0.0402	2.3407	0.0990
Time x Block	4	0.0427	0.0107	0.6218	0.6557
Temp x Block	6	0.3372	0.0629	3.6604	0.0266
Residuals	12	0.2061	0.0172		
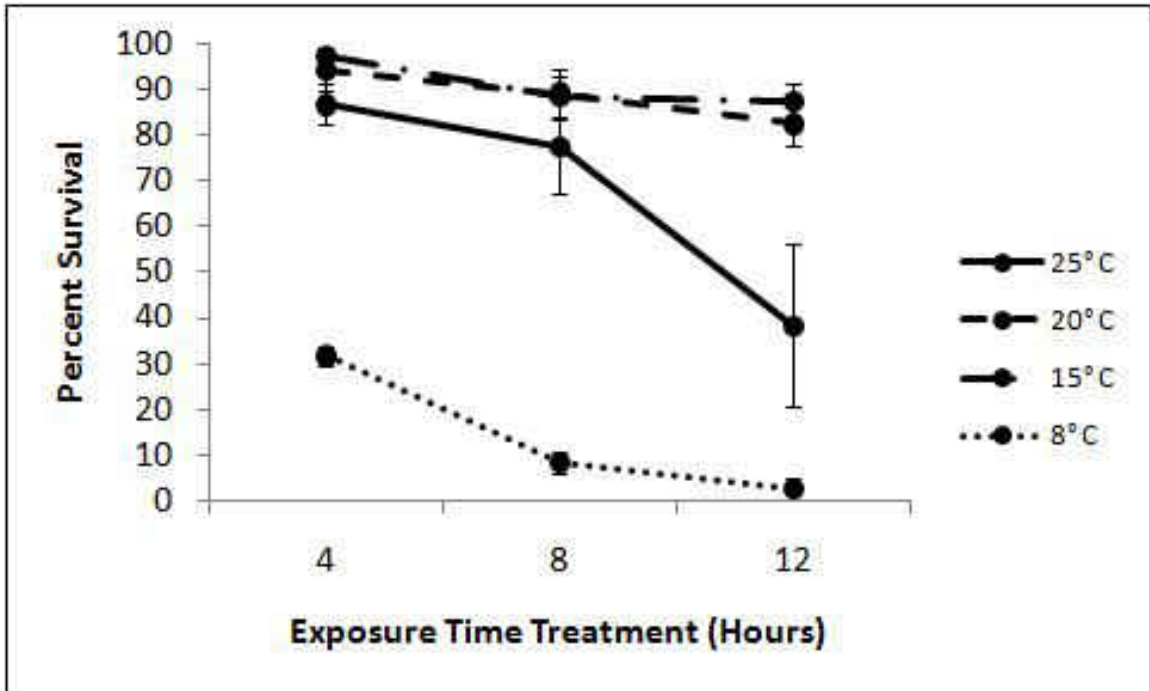
Figure 30: Interaction plot for the factors of time and temperature in the cold temperature tolerance experiment. Lines represent different temperature treatments while exposure time treatments are shown on the x-axis. Error bars represent standard error of the mean at each point. Data shown here represents treatment means across all three blocks for the final survival occurring on the seventh day.
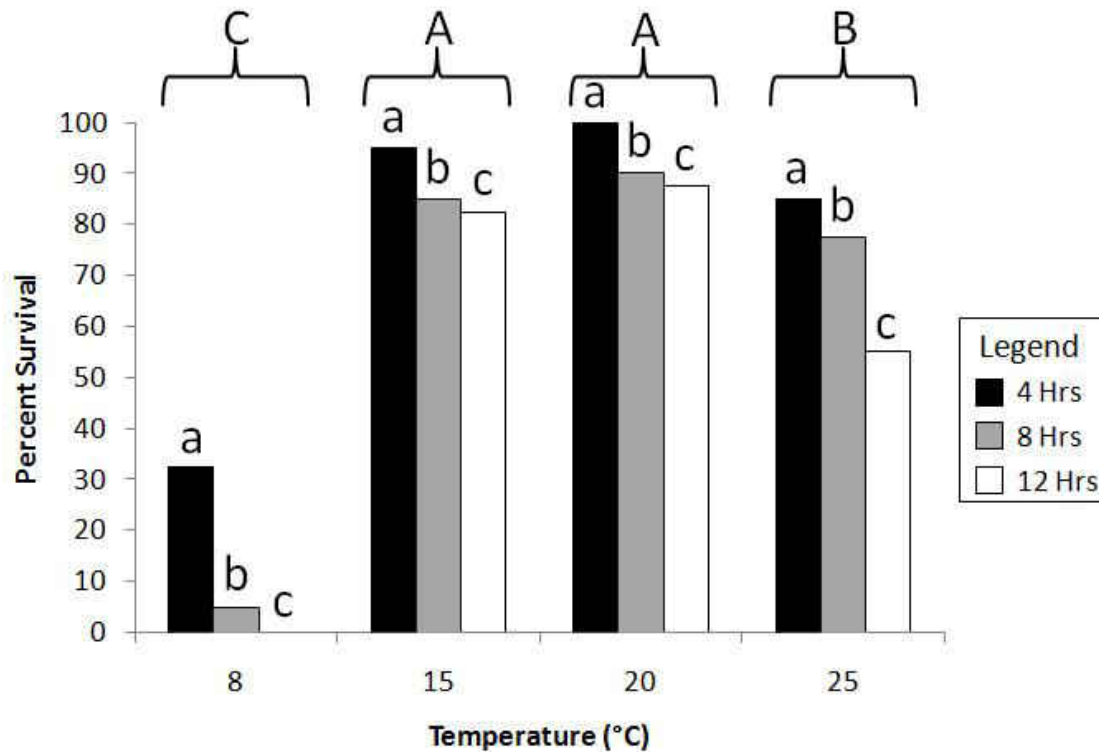
Figure 31: Significant differences among treatments from the cold trial two factor, blocked ANOVA. Upper case letters represent significant differences among temperature treatments. Lower case letters represent significant differences among time treatments. Although the ANOVA included all three blocks, the blocking factor was significant so the graph shown here depicts only block one results as a tool to represent significant differences.

The continuously submerged control treatments all had high survival of at least 95% or better. Results for the 25° C (room temperature) exposed treatments were fairly high after 7 days, as in the warm temperature tolerance experiment, except for the 12-hour treatment which had 55.0% survival. All results for exposed treatments from block 1 can be seen in Figure 32. The 4 and 8-hour treatments set at 25° C had 85.0 and 77.5% survival, respectively. The 20° C treatments all had relatively high survival after 7 days of repeated exposure, with lowest being the 12-hour treatment, which averaged 87.5% survival. The 4 and 8-hour treatments had 100 and 90.0% survival, respectively. Fifteen degree treatments also survived at high rates after 7 days for all treatments. The 4 and 8-hour treatments had 95.0 and 85.0% survival, and the 12-hour treatment had an average

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survival of 82.5%. The 8° C temperature is where survival greatly declined. In the 4-

hour treatment at 8° C, survival was 32.5% and only dropped lower in the 8-hour

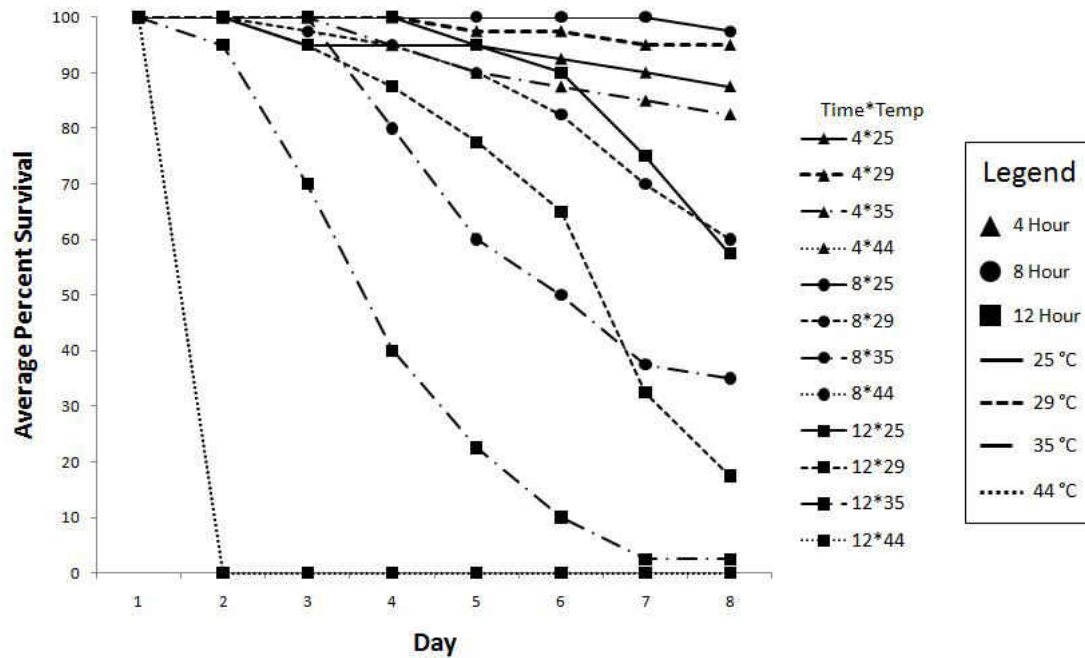treatment, which had 5.0% survival. The 12-hour treatment had 0% survival.
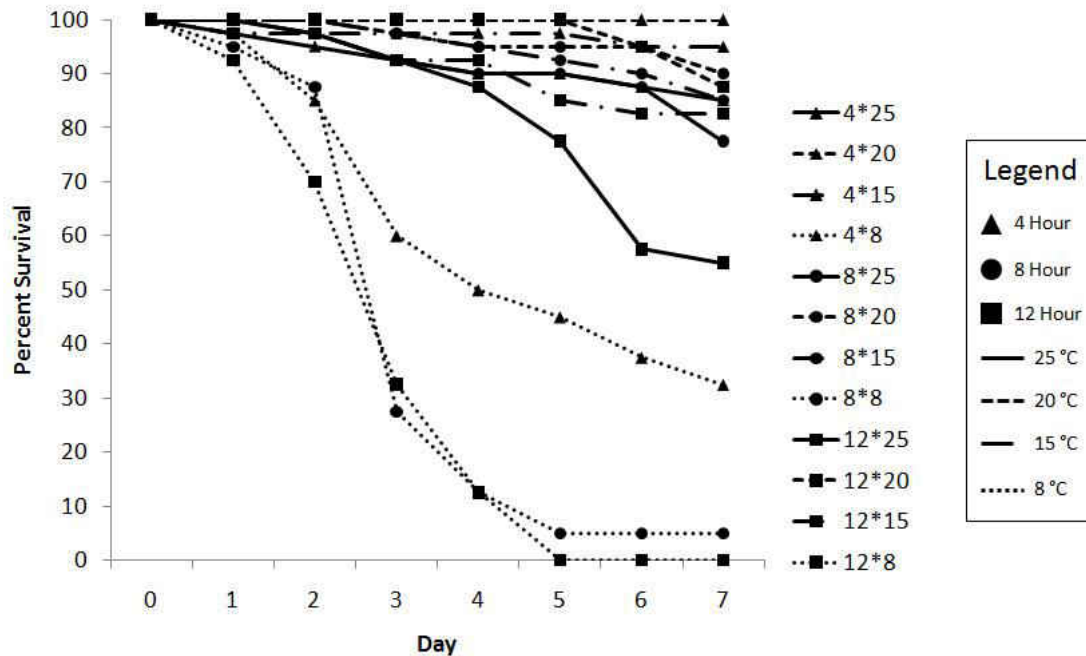
Figure 32: Percent survival over seven repeated days for the combinations of time and temperature treatments in the cold temperature tolerance experiment. Graph represents results for the first block only. The first number in each combination represents time and the second (followed by the "*") represents temperature. Submerged controls are not shown in the graph but all averaged 95% survival or higher after 7 days.

The submerged controls once again had survival of 95% or greater after 7 days.

Figure 33 shows all results for exposed treatments from block 2. Room temperature (25°

C) treatments had high survival at 4 and 8 hours, both having 95% survival after the

seventh day of exposure. However, the 12-hour treatment had just 57.5% survival. In

the 20° C treatments, 4 and 8-hour treatments had 97.5% survival and the 12-hour

treatment dropped to 87.5% survival. Fifteen degree treatments had similar results to

20° C treatments. After 7 days, the 4 hours at 15° C treatment had 97.5% survival as did

the 8-hour treatment, while the 12-hour treatment had 85% survival. All exposed

treatments at 8° C resulted in low survival by the seventh day of repeated exposure. The

4 and 8-hour treatments had 35 and 7.5% survival, respectively and, as with the first

block, the 12-hour treatment had no survival.
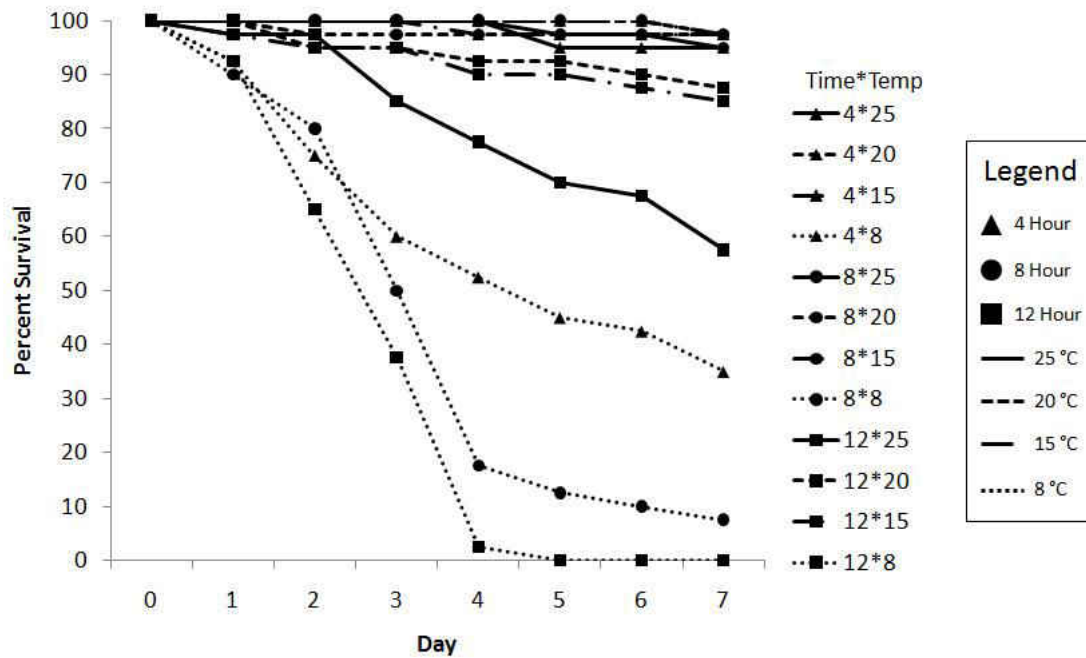
Figure 33: Percent survival over seven repeated days for the combinations of time and temperature treatments in the cold temperature tolerance experiment. Graph represents results for the second block only. The first number in each combination represents time and the second (followed by the "*") represents temperature. Submerged controls are not shown in the graph but all averaged 95% survival or higher after 7 days.

The third block had high survival (90% or better) in the continuously submerged

treatments throughout the 7 days of the experiment. However, the third block had lower

survival in the 25° C treatments than did the first and second blocks, particularly in the 8

and 12-hour treatments which had 60 and 2.5% survival, respectively, after 7 days. The

4-hour treatment had 80% survival. The third block also had lower survival in the 20° C

treatments. After the seventh day of exposure, the 4-hour treatment had 85% survival,

while the 8 and 12-hour treatments had 80 and 72.5% survival. The 15° C treatment had

high survival with 4 and 8-hour treatments having 100 and 82.5% survival, respectively, and the 12-hour treatment having 95% survival. The 8° C treatment again had low survival for all exposed treatments after 7 repeated days of exposure. The 4-hour and the 8-hour treatments had 27.5 and 12.5% survival. The 12-hour treatment had 7.5% survival. Figure 34 shows the results of the third block for all exposed treatments.
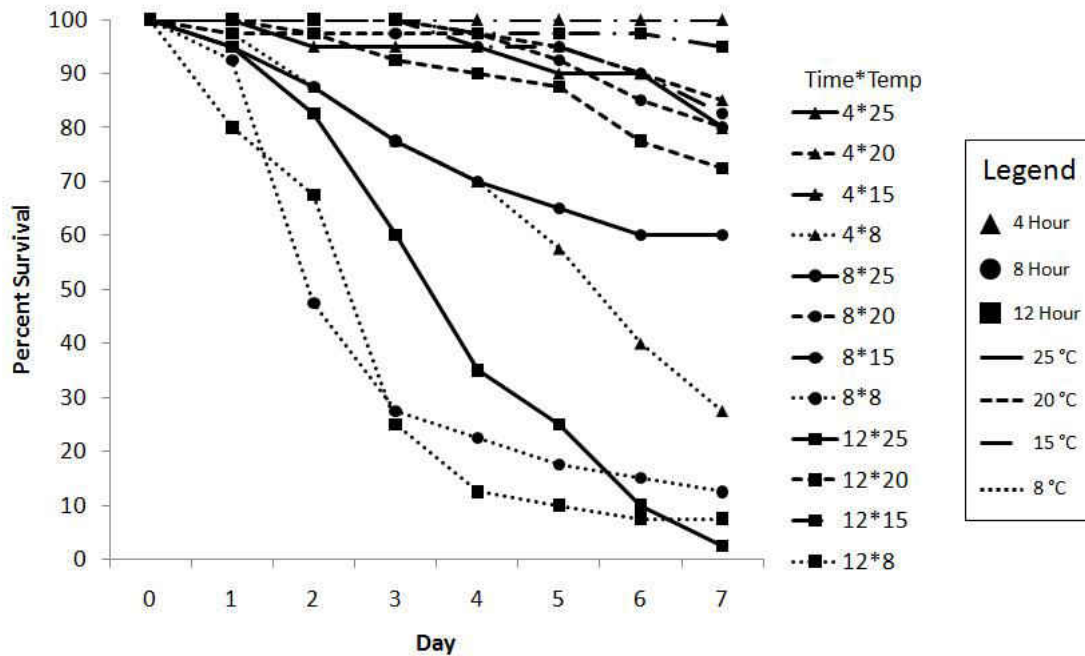
Figure 34: Percent survival over seven repeated days for the combinations of time and temperature treatments in the cold temperature tolerance experiment. Graph represents results for the third block only. The first number in each combination represents time and the second (followed by the "*") represents temperature. Submerged controls are not shown in the graph but all averaged 90% survival or higher after 7 days.

DISCUSSION

<u>Room temperature air exposure experiments</u>

The research presented here provides a test of the ability of anthropogenic disturbance to increase invasibility of reefs of *Crassostrea virginica* by *Mytella charruana* and *Perna viridis*. Several studies (e.g. D'Antonio 2000, Brown and Ford

2002, Minchinton 2002, Schreiber et al. 2003, Marchetti et al. 2004) have suggested that disturbance may aid invasion of novel species, but my work is the first to test this idea on reefs of *C. virginica* disturbed by boat wakes. This is important because *C. virginica* have suffered decline in recent years and studies in Mosquito Lagoon have linked this to recreational boat wake disturbance (Grizzle et al. 2002, Wall et al. 2005, Stiner et al. 2008). My laboratory experiments also represent the first effort to find the aerial temperature tolerance of *M. charruana*. Yuan et al. (2010) explored the thermal tolerance of *M. charruana* while submerged, providing valuable information relatable to possible range expansions. However, thermal tolerance during exposure to air is also important when considering possible invasions of intertidal areas. The importance of understanding air exposure in rocky intertidal and intertidal salt marsh habitats has been cited in works by Helmuth and colleagues (e.g. Schneider and Helmuth 2007, Jost and Helmuth 2007). However, my study differed in that it was also intended to help predict if *M. charruana* would be able to invade and establish on intertidal oyster reefs.

The effects of anthropogenic disturbance on invasibility of oyster reefs could not be fully answered because only two live non-native individuals of *P. viridis* were found on a restored reef and a reference reef in Mosquito Lagoon. However, unattached shells of dead *M. charruana* individuals were found on reference reefs in Mosquito Lagoon during monitoring. Although it is still unclear if boat wake disturbance creates a situation that will increase invasibility of oyster reefs in Mosquito Lagoon, some interesting results were found. Reef survey results did show that the number of live *C. virginica* and the number of live native mussels found on dead reefs was significantly lower than that found on reference reefs. This provides evidence that disturbance on oyster reefs in

Mosquito Lagoon may create more available space for settlement of non-natives. The reef survey results for numbers of live oysters further support the findings of Wall et al. (2005) who showed that survival of *Crassostrea virginica* was significantly lower on dead reefs than reference reefs. Another interesting result was the lack of significant difference between restored and reference reefs for number of live oysters. This is important because it suggests that in Mosquito Lagoon oyster restoration has been successful.

The thermal tolerance experiments provided information regarding *M. charruana*'s ability to successfully invade oyster reefs. Some interesting results can be seen when thermal tolerance data are considered in conjunction with oyster reef temperature results. Summer reef temperatures and the warm temperature tolerance results suggest that extremely warm temperatures (e.g. $\geq 35°$ C for long periods or short periods of $\geq 44°$ C) may lower survival of *Mytella charruana*.

A temperature of 29° C was commonly found on oyster reefs in the mid-intertidal zone during this study in July 2009. Temperature tolerance experiments showed that 8 and 12-hour exposures at 29° C would lower survival for *M. charruana* if they occurred for multiple days. Four hours of air exposure at 35° C was a typical occurrence on oyster reefs in the field and was seen for up to 6 repeated days on dead reefs. Survival was high in the 4 hours at 35° C treatment during the warm tolerance experiment, but if mussels were found higher in the intertidal than where my loggers were placed, they would have been exposed for longer periods of time at this temperature. Longer exposure times could have resulted in lower survival as tolerance results showed that *M. charruana* have significantly less survival when exposed for 8 or 12 hours of exposure at 35° C for 7

repeated days. Therefore, *M. charruana* found higher in the intertidal range might experience lower survival. The 44° C temperature treatments were particularly difficult for *M. charruana* to withstand during the tolerance experiment. Aerial exposure periods of 4 hours resulted in near complete mortality of *M. charruana* after just one occurrence, and were also a good representation of some temperature data collected from the field. All dead reefs reached 44° C. One dead reef reached 44° C or higher for up to 5 consecutive days, but only for 3.5 hours or less each day. One reference reef and one restored reef also reached 44° C, but for time periods only representing one data point (< 1 hour). Even in this microtidal environment, elevation could play an important role in survival. Based on my data, *Mytella charruana* would likely experience conditions on oyster reefs in Mosquito Lagoon during summer that would result in high mortality.

Maximum warm temperatures found on oyster reefs in Mosquito Lagoon during my study were similar to warm body temperatures of *G. demissa* found in salt marshes of South Carolina (Jost and Helmuth 2007). Jost and Helmuth (2007) found average daily maximum temperatures in excess of 40° C during the month of May in mussel mimic loggers placed above ground on well-drained sediment at low tide. Mussel mimic loggers that reached over 40° C included ones in contact with sediment and loggers placed 6 cm above the sediment (Jost and Helmuth 2007). This helps to validate the findings of this study that *M. charruana* on oyster reefs can reach extremely high temperatures. The temperature tolerance results for *G. demissa* found by Jost and Helmuth (2007) were also similar to the results found in this study for *M. charruana*. They found that when average maximum body temperatures reached above 45° C, large decreases in survival resulted, while short periods of exposure to 50° C resulted in 0%

survival. This is a slightly lower tolerance limit than that of *C. virginica* which can survive temperatures of 49.5° C for 3 hours (Ingle et al. 1971). This upper limit found by Ingle et al. (1971) for *C. virginica* was also higher than the limit for *M. charruana* found in this study, although the mussels in this study were exposed for periods of at least 4 hours. This suggests that at extreme temperatures occurring in the middle to high intertidal range, native *C. virginica* may be better adapted to survive than non-native *M. charruana*.

Another interesting finding of this study was that dead reefs reached higher temperatures than reference or restored reefs. Dead reefs consist mostly of dead shells that have been tightly packed into a mound that extends above mean high tide with some live oysters around the lower edges, whereas reference reefs include shells and live oysters intermixed throughout the reef with a sand and mud matrix (Grizzle et al. 2002). It is likely that this difference is a factor that helped result in higher temperatures on dead reefs than on reference or restored reefs during summer. I hypothesize that dead reefs likely drain more quickly at low tide than do reference reefs which contain more mud and sand. This factor combined with the higher proportion of hard shell substrate may combine to increase temperatures.

Cold temperature monitoring and tolerance experiments can also be considered collectively. During thermal tolerance experiments, all 25, 20, and 15° C treatments resulted in high survival. Thus, mild winter temperatures of 15° C or higher should not greatly affect survival of *M. charruana* on oyster reefs in Mosquito Lagoon. Temperatures during January and February field monitoring commonly dipped down to 15° C or below. Temperature tolerance experiments found a 75% survival rate for *M.*

charruana exposed to 4 hours of 8° C aerial exposure for 4 repeated days. Four hour exposure periods at temperatures of 8° C or lower did occur for up to 4 repeated days on 2 reference and 3 restored reefs. However, *M. charruana* could experience longer daily periods of exposure time at low temperatures if found higher in the intertidal. This would likely result in lower survival. It is also important to note that temperatures did drop below 8° C during temperature monitoring. Temperatures dropped below 0° C for 1.5 hours on one night. Temperatures below 8° C were not tested in the laboratory tolerance experiments. However, when one considers that 1 day of exposure for up to 12 hours at 8° C resulted in high survival (88.7%), it seems reasonable that *M. charruana* might withstand one time exposure at temperatures below 8° C. In the literature, aerial exposure tolerance seems to be more frequently studied with respect to warm temperatures than cold, presumably due to interest in desiccation stress at high temperatures. However it has been shown that some bivalves can withstand air temperatures below 0° C while submerged. One example is the cold tolerance limit of *G. demissa*. Murphy and Pierce (1975) found *G. demissa* had a tolerance limit of -13.76° C after being acclimated to winter temperatures. *Perna viridis* have been shown to have a low temperature threshold of between 10 and 13° C (Urian et al. 2010).

Cold temperatures found on oyster reefs in Mosquito Lagoon may not result in complete mortality of *M. charruana* at all locations on reefs based on temperature tolerance results. As with summer temperatures, elevation in the intertidal may have an effect on survival during winter. Mussels high in the intertidal would likely experience near 0% survival, since they would be exposed for longer periods of time than the temperature loggers during my monitoring periods. However, mussels lower in the

intertidal may experience low temperatures for shorter periods of time than found by temperature loggers in this study. Thus, they would likely experience higher survival.

Mytella charruana seem to have a strong tolerance for aerial exposure at cold temperatures if they are exposed during one event rather than over repeated days. A cold tolerance limit for short, one time exposure events was not found during my tolerance experiments. However, 8° C, when occurring repeatedly for seven days, seems to be near a tolerance limit for *M. charruana*. A clear cold tolerance limit during aerial exposure is not known for *C. virginica*. *Crassostrea virginica* has been known to survive being frozen while submerged in shallow water conditions (Loosanoff 1965). It is likely that *C. virginica* can tolerate lower temperatures than *M. charruana* when one considers temperatures that occur in the northern range of *C. virginica* (Nova Scotia).

Reference and restored reefs were not significantly different with regard to temperatures collected during the winter portion of this study. This provides yet more evidence that oyster reef restoration efforts in Mosquito Lagoon have been successful in returning dead reefs to similar thermal regimes of reference reefs. Although dead reefs could not be statistically compared with the other two reef types due to loss of replicates, average values were only slightly higher than averages for reference and restored reefs.

A cold spell with particularly low (well below 8° C) temperatures did occur during the winter 2010 monitoring period. After the winter of 2010, no live *M. charruana* individuals were found along the southeastern U.S. coast during a biannual survey consisting mostly of subtidal locations (L. Walters, E. Hoffman, and K. Schneider unpublished data). Many of the locations monitored in the study were sites where *M. charruana* were found in recent years (L. Walters, E. Hoffman, and K. Schneider

unpublished data). However, the results of the survey were most reflective of harsh

subtidal conditions (L. Walters, E. Hoffman, and K. Schneider unpublished data). My

study suggests that *M. charruana* at lower intertidal levels would likely be able to survive

air exposure on reefs even in the harsh conditions of January and February, 2010. Thus,

M. charruana should be able to survive on oyster reefs during more typical, warmer

winters.

An important factor to consider in conjunction with biological invasions is global

climate change. Much research has focused on predicting future average temperatures as

well as impacts that these changes could have on ecosystems. Some studies have already

begun to examine the possible effects of climate change on biological invasions. For

example, work by Helmuth and colleagues have specifically focused on the effects of

global climate change on intertidal bivalve distributions while including aerial exposure

as an important factor. The study presented here provides a starting point for combining

these ideas using *M. charruana* as a model species. My results also produced a dataset

which can provide a step toward gathering baseline information about the effects of

climate change on species introductions that have not yet become invasive. Lockwood et

al. (2007) suggested a need for greater understanding of the effects of global climate

change on species that have not yet caused problems as well as those not yet established.

Future modeling research using my data could produce an interesting look at predictions

of new areas where *M. charruana* might be a successful invader.

Specifically in Mosquito Lagoon, my results can help provide an idea of whether

global climate change may affect the possibility of invasion of oyster reefs by *M.

charruana*. Global average temperature is projected to rise by 0.6 to 4.0° C by the end of

the 21st century according to various models (IPCC 2007). According to my results, *M. charruana* would seem to be near its tolerance limit on oyster reefs, both during summer and winter extremes. Temperature increases of up to 4° C would likely make it more difficult for *M. charruana* to establish if summer extremes increased. Von Holle et al. (2010) found that the majority of the counties in Florida have experienced colder minimum temperatures in Winter and Spring while reaching higher maximum temperatures in Summer and Fall throughout historical time. Colder minimum temperatures should also make it more difficult for *M. charruana* to establish on oyster reefs in Mosquito Lagoon. Any successful individuals would likely establish at lower intertidal elevations where time of aerial exposure during extreme temperatures would be lessened. More study of *M. charruana*'s reaction to climate change is important as other factors could be involved. For example, Stachowicz et al. (2002) found that three species of ascidians experienced earlier recruitment times and higher total recruitment than native species in correlation with warmer winter temperatures.

Conclusions

Oyster reef surveys did not support or refute the hypothesis that anthropogenic disturbance is aiding invasions of *Mytella charruana*. However, surveys did show a decrease in native species on dead reefs providing possible space for settlement of *M. charruana*. Also, several dead *M. charruana* and *P. viridis* individuals were found on reefs. *Mytella charruana* should not be ignored as a possible future invader on reefs of *Crassostrea virginica* as non-native species may undergo long lag times before becoming invasive. Both *M. charruana* and *P. viridis* can survive aerial exposure for long enough periods at 22° C to survive on reefs of *C. virginica* at this mild temperature. Tolerance

and temperature monitoring results suggest that *M. charruana* found high in the intertidal on oyster reefs would likely experience high mortality. However, I found no evidence to suggest that *M. charruana* could not survive in low to mid-intertidal conditions on reefs of *C. virginica* in Mosquito Lagoon. This study provides a model technique for predicting establishment of non-native species in novel intertidal habitats. The data set for the tolerance of *M. charruana* for aerial exposure at different temperatures should provide a useful tool for others to compare with temperatures collected from other areas. Collection of temperatures from different elevations on oyster reefs would greatly aid in prediction of possible invasion by *M. charruana* in any intertidal habitat as it is likely an important factor in the results of this study. Although temperature is an important factor affecting the distribution of intertidal organisms, there is a need for better understanding of interactions between temperature and other biotic and abiotic factors (Schneider and Helmuth 2007). Future research efforts should investigate the effects of other factors in conjunction with aerial and submerged temperatures. Prediction of invasions into new areas based on environmental tolerance limits may become of great importance in light of future global climate change predictions. This study provides a step toward better understanding of invasion prediction in intertidal systems.

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