Thermal Stress Leads to Behavioral Shifts in two Species of Slippersnail, *Crepidula fornicata* and *Crepidula plana*.

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Anthropogenic climate change, while not only increasing overall ocean temperatures, has also increased the variability in temperature that intertidal organisms experience. These organisms are put under increasing stress every year and need to adapt to these changes. In order to better understand this response of intertidal organisms, we studied the response to thermal stress of two species in the slipper snail genus, *Crepidula*. Using *C. fornicata* and *C. plana* collected from Beverly, MA, we labeled and analyzed the movement and feeding patterns of the *Crepidula* in three separate tanks: cold, control, and hot. All tanks started at 18℃ and the control tank remained constant throughout the experiment, while the cold and hot system temperatures were changed daily, reaching 6℃ and 26℃, respectively. We found that both species had a significant increase in feeding rate in the hot system, yet rates in the control and cold systems had no difference. In terms of movement, we also found that *C. fornicata* were significantly more likely to move out of their petri dish than *C. plana*. Temperature also affected movement, with cold treatment individuals moving significantly less than the other treatments. The increasing number of very cold days could affect the ability of *Crepidula* to consume the food they need to survive, and could limit their ability to move to a more favorable area. Understanding how *Crepidula* respond to variable temperatures is important in predicting how marine life, in general, will respond to rising ocean temperatures and how organisms will have to adapt in order to survive.

Introduction

Anthropogenic climate change has had profound impacts on marine ecosystems over the past two centuries. One of the most pronounced impacts of climate change are the increases in temperature and weather variability in coastal ecosystems, meaning organisms living in these environments have had to rapidly adapt to not only higher temperatures, but also rapidly changing temperatures (He & Silliman, 2019). Because sea and air temperatures can be vastly different simultaneously, intertidal organisms in temperate environments often go through drastic ambient temperature changes when the tide ebbs and organisms are exposed to the air (Diederich, 2013). While the temperature gradient is not always extreme, intertidal organisms also have to adapt to distinct, seasonal temperature changes year round. As climate change continues to increase overall ocean temperatures, marine organisms must acclimate and adapt in response. Global average surface air temperature is continually increasing (He & Silliman, 2019), meaning that in winter while water temperatures can be just above freezing, air temperatures can reach nearly 30°C on warm days. This temperature change with the tidal cycle can be challenging for intertidal organisms (Pechenik et al. 2020).

Understanding how these intertidal organisms will respond to thermal abnormalities in their environment brought about by climate change is critical; if these hardy organisms are shown to succumb to heat stress with rising temperatures, more sensitive organisms can be assumed to be at

an even higher risk of extinction. Also, specifically with the known invasive species *C. fornicata*, rising temperatures may allow them to expand their range and drive out more sensitive native species (Diederich, 2013), so understanding behavioral changes in response to temperature changes can better help us predict future range extensions of invasive populations.

Intertidal organisms are typically well adapted to rapid and dramatic changes in abiotic factors such as temperature and salinity (Diederich, 2013). Sessile organisms especially must be able to withstand large temperature swings as they are alternately exposed and submerged by the tides. Intertidal organisms, especially sessile populations like *Crepidula*, are often at higher risk for climate-change caused mortality (Burge et al, 2014), though *Crepidula fornicata* is also expanding its range as an introduced or invasive species in many parts of the world (Diederich, 2013). The ability of intertidal organisms to adapt to thermal stress is an important area of study as the mechanisms by which these species adapt are still relatively unknown. While many studies have focused on increased temperatures, intertidal species in New England are also faced with cold winter temperatures which can become more extreme in the face of increased winter storms due to arctic warming (Francis et al, 2017). Our two subject species, *Crepidula plana* and *Crepidula fornicata*, have overlapping niches along the eastern coast of North America (Boisvert, 2014), however only *C. fornicata* has become a successful invasive species in Europe. Contrasting stress response behavior between these two similar species might help disentangle the factors that allow *C. fornicata* to successfully invade European waters while *C. plana* has not.

In this study, *Crepidula fornicata* and *Crepidula plana* were studied in order to see their responses to temperature extremes. Both species are small, sessile, shell bearing molluscs that colonize the intertidal and subtidal zones and are generally found five meters below low tide up to the low intertidal zone (Diederich, 2013). Because of this variability in habitats, these organisms have adapted to a wide range of temperatures and are able to tolerate prolonged periods of exposure to sunlight and atmospheric conditions (Diederich, 2013). Both species are native to the eastern coast of North America and live on hard substrates such as rocks, clam shells, and wood pilings (Hoagland, 1978).

Thermal tolerance could also vary with *Crepidula* gender, as males and females are morphologically different. *Crepidula* are sequential hermaphrodites, meaning they can change sex based on their surrounding environmental conditions, and being protandrous they start life as males and change to female based on chemical signals from their surrounding conspecifics (Cahill et al, 2015). Males, which can be identified by the black penis protruding from the base of their neck, often form stacks on top of the larger females, where they tend to stay for a long period of time (Broquet et al, 2015). Once these stacks have formed the *Crepidula* rarely move as they utilize suspension feeding to take in food through their gill into their mouth. This means that they have become more resistant to temperature fluctuations than other more freely moving intertidal species, making them a promising model system for the impacts of climate change on sessile intertidal organisms (Noisette et al, 2014).

Here, we sampled from a single community of *Crepidula* and exposed them to gradual thermal stress to gradually acclimate them to extreme hot and cold temperatures they would experience in situ. In order to quantify behavioral changes both inter and intraspecies, we measured daily movement, and differences in individual

feeding rates between the treatments after the 14 day experimental period. We hypothesize that individuals exposed to increased temperatures will exhibit increased movement when compared to those in the control and cold treatment. As the temperature approaches the extremes of 4°C and 32°C, both species of *Crepidula* will see decreased movement rates. Males will be more likely to move than females as they attempt to find a mating stack. In addition, previous research has shown that not only do juvenile invertebrate marine species have greater upper thermal tolerance (Peck et al. 2013), but smaller species in general do as well (Peck et al, 2009). Thus, we hypothesize that based on their smaller size compared to their female conspecifics, males will have lower levels of mortality and higher rates of movement at the higher temperatures due to their higher thermal tolerance. Individuals at temperature extremes will exhibit lower rates of feeding, as they will not be comfortable at these temperatures, and may shut down bodily function. Understanding behavioral responses to temperature change in these intertidal organisms is important for understanding the future of sessile marine organisms under changing ocean environments, and will help us better predict range expansion of the invasive *C. fornicata*.

Materials and Methods

Crepidula collection and experimental setup

Crepidula fornicata and *Crepidula plana* were collected on 14 November 2020 from Lynch Park, Beverly, MA (Figure 1). Specimens were returned to the Boston University Marine Program Lab, Boston, MA, and maintained at 18°C and 34 ppt salinity for 10 days until the beginning of the experiment.

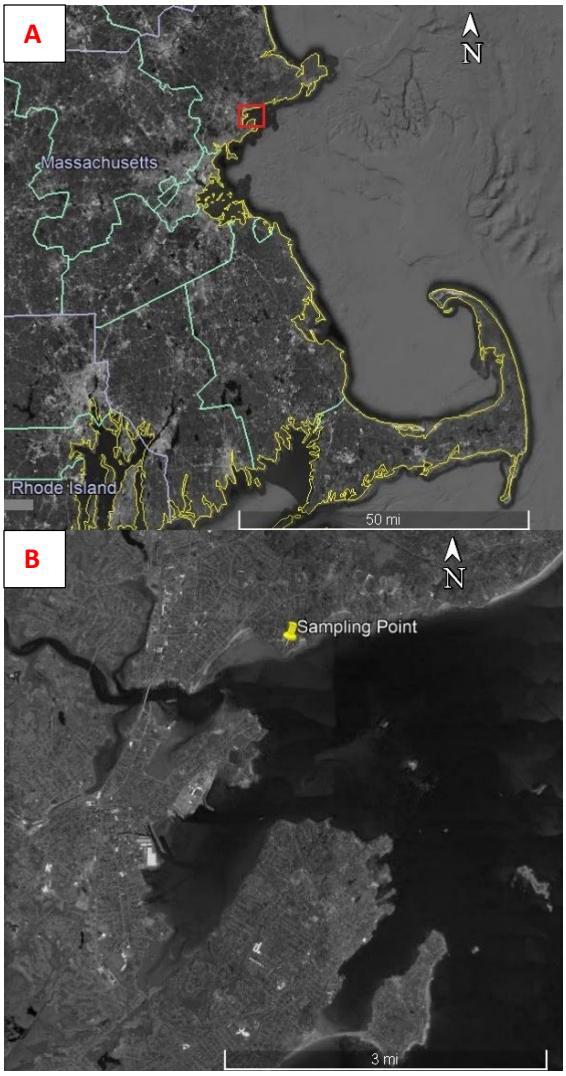

Figure 1. Map of *Crepidula* collection site. A) Map of Southern New England coast with inset in red. B) Inset map of Beverly, Massachusetts with sampling site: Lynch Park, Beverly, MA. *Crepidula* specimens retrieved November 14th, 2020.

Crepidula were carefully separated from their clam shell substrate using flat ended dental excavators, a paint scraper, and a dulled scalpel. Tools were wedged in between the foot and the substrate at the posterior of the shell to gently release the *Crepidula*. *Crepidula* were then placed onto clear, open-faced, plastic petri dishes and allowed 2 hours to reattach before the initial feeding. Each *Crepidula* was measured along its longest axis to determine the initial length,

and any *Crepidula* smaller than 5mm or larger than 35mm were discarded from the group that we used for feeding trials. While separating *Crepidula* we documented which individuals were previously attached to each other to use these data in our behavior analysis.

In order to keep track of the *Crepidula*, we labelled each one. 60 individuals–30 from each species–were used in individual feeding trials and were chosen at random by blindly removing them from a bin containing all specimens. For each species, we chose 15 *Crepidula* <20mm to be the "small" size class and 15 *Crepidula* >20mm to be the "large" size class. Once removed, nail polish was applied to the outer shell surface and allowed to dry before being replaced in water. Using a key of all polish color combinations and their respective specimen length, we tracked their size, sex, and feeding habits to determine if there was a significant difference in feeding or movement across treatment groups. 60 additional *Crepidula* (30 specimens per species), were labeled with a species identifier (F for fornicata and P for plana) and number engraved on their respective petri dishes to collect data on their size, sex, and movement, but these *Crepidula* were not put through feeding trials.

Thermal Stress Experimental Design

Our total number of specimens (n=120) were divided into three temperature treatments (Figure 2). Twenty *Crepidula* of each species were randomly assigned to each system. All systems started at a temperature of 18℃, which was the temperature of the control system for the experimental period. In the cold system, the temperature decreased 1℃ daily, reaching a minimum of 6℃ on day 14. In the hot system, the temperature was increased 1℃ daily, but ended up leveling off at 26-27℃ on day 10 due to an issue with the heating system (Figure 3).

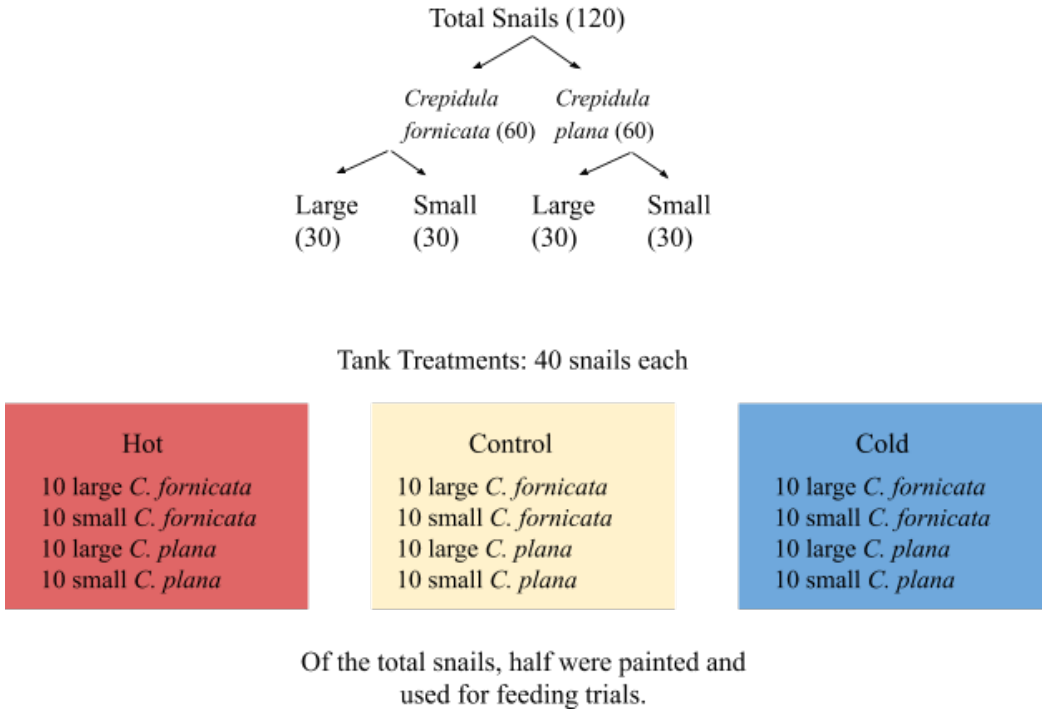

Figure 2. Tank experimental setup. 60 *Crepidula fornicata* and 60 *Crepidula plana* were evenly split into large (>20mm) and small (<20mm) size classes evenly and then distributed as shown into the treatment tanks. 5/10 *Crepidula* from each size class were used for feeding trials.

Tank upkeep and Crepidula feeding

Salinity and temperature were tested multiple times per day and tracked over the experimental period. Salinity was kept at approximately 35ppt over the course of the experiment, although, due to an issue with the tank system, it did drop as low as 17ppt around the middle of the experimental period. To feed the *Crepidula*, on days in which we did not do individual feeding trials, approximately 4.5L of water was removed at each feeding from each treatment tank (replacing it with 34/35ppt salt water to maintain tank equilibrium). Then, 1mL of dry weight 8% Shellfish Diet 1800 was added to each feeding bin. Before the *Crepidula* were added, we mixed the solution to ensure the same density of food per water area. All specimens were fed daily and remained in their feeding tanks for one hour before being rinsed in fresh room temperature seawater and replaced in their treatment tank.

Individual Feeding Trials

To determine the influence of thermal stress on feeding activity, we conducted individual *Dunaliella salina* (DUN) feeding trials on day 14 to analyze the rate at which these *Crepidula* fed. Half of the *Crepidula* underwent individual DUN trials (30 *C. fornicata* and 30 *C. plana*). Of the *Crepidula* that were to undergo individual feeding trials, 15 were in the large size class and 15 were in the small size class for each species (Figure 2). To conduct these trials, we took 60 individual jars, to which we added 20mL of DUN algal culture and 80mL of seawater taken from the same system as each *Crepidula* to dilute the algal culture and keep temperature consistent with what they experience in the system. We took 1 mL aliquots from 3 of the jars before placing the *Crepidula* in them, and then preserved the cells with Lugol's iodine, in order to get an average initial algae concentration. *Crepidula* were then placed in each jar, and left to feed for 1 hour. At the end of the feeding period, 1mL aliquots were taken from each jar, and 50 µL of Lugol's iodine was added to preserve the cells. In order to quantify the number of algal cells, 10 µL of the preserved cell solution was placed on a hemocytometer, and the number of cells in the center square (1mm^2) was counted using a microscope under 10X magnification. This procedure was done three times for each sample to determine the average number of cells in the sample. The three samples taken before feeding were used to determine the average number of cells in all jars before feeding, and the samples taken after feeding were used to determine how much each

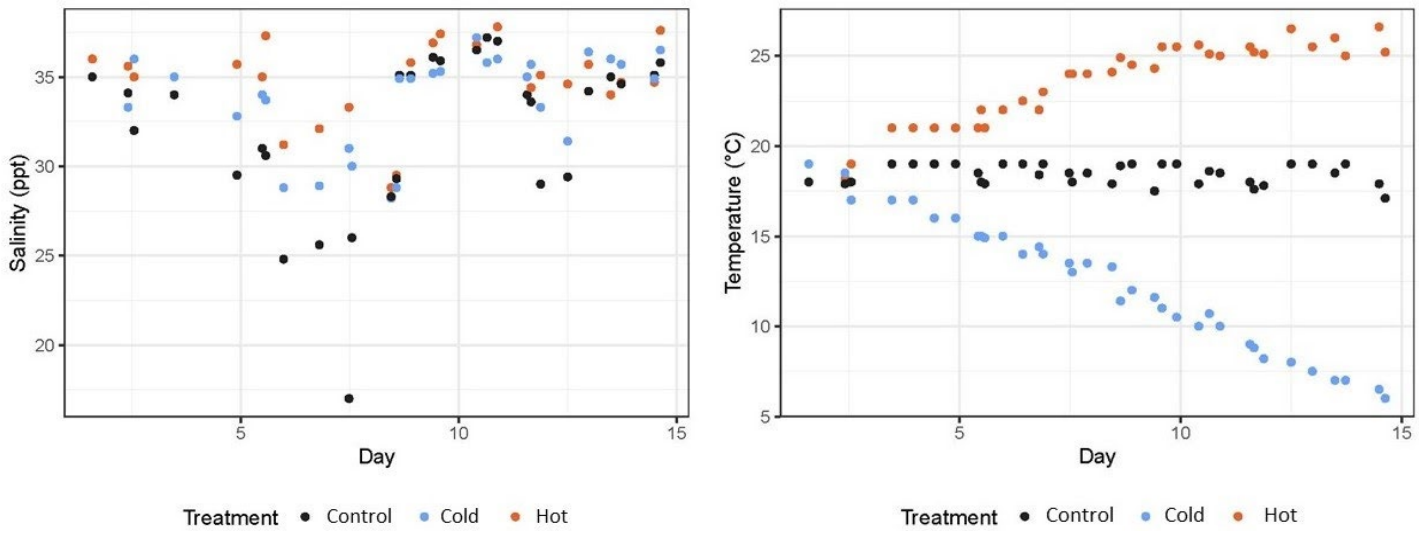

Figure 3. Water quality variables in each treatment group. A. Salinity trends, water was kept around 35ppt, although system errors caused salinity to dip mid-experiment. **B.** Water temperature over the experimental period. Control system kept around 18℃, the hot system reached a high of 26.6℃, and then plateaued due to issues with heating, and the cold system reached a low of 6℃.

Crepidula ate. In order to determine the total number of cells the *Crepidula* ate, the number of cells counted was multiplied by 1.05 to account for the dilution of iodine, and then 10^4 to convert this number into cells/mL. This was then multiplied by 100mL to get the total number of cells in the jar. To determine how many cells each *Crepidula* ate, the number of cells in the jar after feeding was subtracted from the average number of cells in the jars before feeding. Because the pre-feed counts were just an average of the jars and not a complete sample, some of the post-feed counts ended up being higher than the average, giving a negative number for the total cells eaten. Because of this, it is assumed that any *Crepidula* with a negative number of cells eaten had little to none feeding activity.

Statistical analysis

All statistical analysis was done in Rstudio (Rstudio Team, 2020). For analysis of feeding data, we used a 1-way ANOVA and Tukey's HSD test to determine any significance between independent variables. In order to quantify movement data, we used Fisher's exact test to determine if there were any significant differences between how each treatment affected movement. We also used a chi-square test to see if there were significant differences in movement between species. All plots were created in Rstudio.

Results

Crepidula exhibit lower feeding rates under cold stress

While there was no difference between feeding rates of the *Crepidula* in the cold and control treatments ($p = 0.63$), *Crepidula* in the hot treatment ate significantly more than *Crepidula* in the cold and control treatments ($p < 0.0001$). When size class was added to the analysis, it was determined that larger *Crepidula* ate significantly more than smaller *Crepidula* ($p < 0.01$), but there was no significant interaction between size class and treatment ($p = 0.15$). However we did see that the small *Crepidula* in the hot treatment ate significantly more than the small *Crepidula* in the cold and control treatment ($p < 0.001$), while the large *Crepidula* in the hot treatment only ate significantly more than the large *Crepidula* in the cold treatment ($p < 0.05$), and not the control ($p = 0.22$). When the species were compared, one species did not eat significantly more ($p = 0.43$), but the interaction of species and treatment did have a significant effect on feeding rate ($p < 0.05$), as there was more difference in the feeding rates at each treatment for *C. plana* than *C. fornicata* (Figure 4).

Crepidula move less frequently under cold stress

A chi-square test determined that there exists a significant difference between the actual and expected distribution of *Crepidula* that moved on the final day, when non-control temperatures were at their extreme ($df = 2$, $p < 0.0005$), implying that there is a relationship between movement and temperature. We also conducted Fisher's exact test and determined if there were significant differences between any 2 of the treatments. There were significant differences between the hot and cold treatments ($p < .0001$) as well as the cold and neutral treatments ($p < .0001$) on the final day, but no significant difference ($p = 1$) between the amount of movement in the hot and neutral treatments (Figure 5). Additionally, each system only had 2 mortality events during the experimental period.

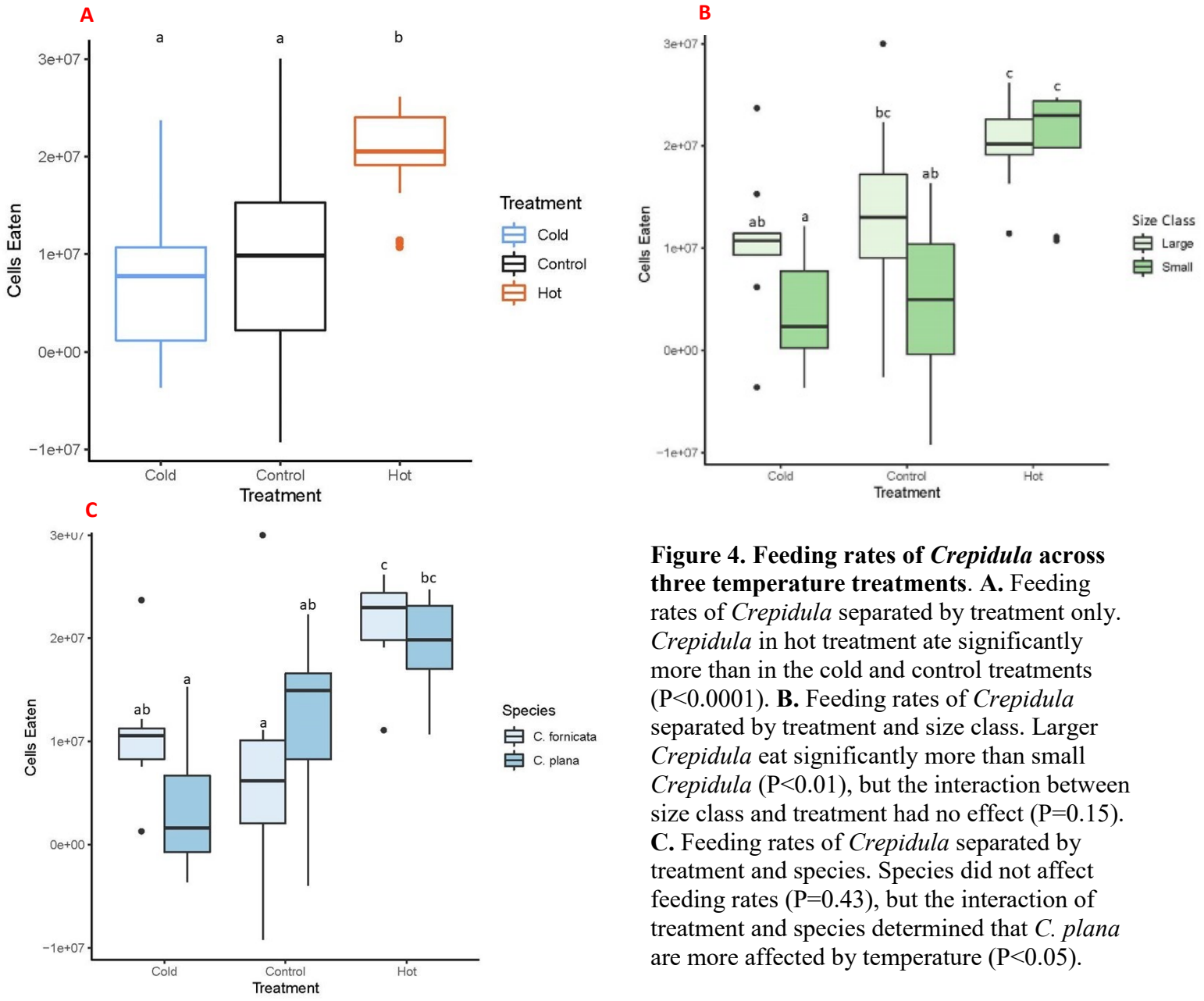

Figure 4. Feeding rates of *Crepidula* across three temperature treatments. **A.** Feeding rates of *Crepidula* separated by treatment only. *Crepidula* in hot treatment ate significantly more than in the cold and control treatments ($P<0.0001$). **B.** Feeding rates of *Crepidula* separated by treatment and size class. Larger *Crepidula* eat significantly more than small *Crepidula* ($P<0.01$), but the interaction between size class and treatment had no effect ($P=0.15$). **C.** Feeding rates of *Crepidula* separated by treatment and species. Species did not affect feeding rates ($P=0.43$), but the interaction of treatment and species determined that *C. plana* are more affected by temperature ($P<0.05$).

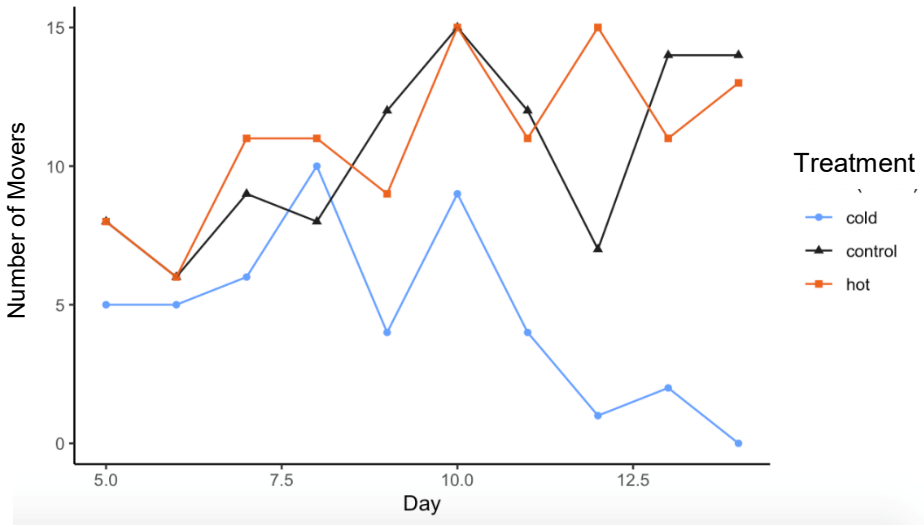

Figure 5. *Crepidula* movement across experimental period. Number of *Crepidula* that moved out of their respective petri dish on days 5-14 of the experiment across all treatments. Hot and control systems were not different from each other, but were both significantly higher than the cold system ($p<0.0001$).

C. fornicata are more likely to move

When looking at patterns of movement between the two species of *Crepidula,* over the course of the experiment, significantly more *C. fornicata* moved than *C. plana* ($df = 1$, $p < 0.0001$) (Figure 6).
To contextualize these data, 45% of the total *C. fornicata* moved while only 0.8% of the total *C. plana* moved. Additionally, the only *C. plana* that moved were in the hot system.

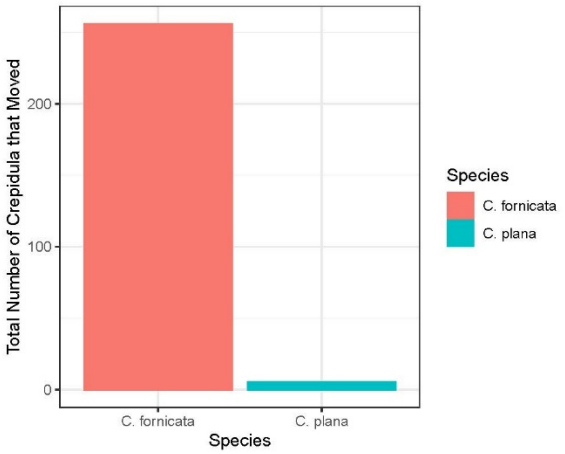

Figure 6. Total movement of each *Crepidula* species. *C. fornicata* moved significantly more than *C. plana* (chi-square, $df = 1$, $p < 0.0001$).

Discussion

C. fornicata in the cold system ate the same amount as C. plana in the control system.

The feeding rates of *C. fornicata* and *C. plana* at the cold treatment are statistically similar, but the trend of the data shows a downward skew in *C. plana* feeding rates; that is, the majority of data points in *C. plana* were found below the lower range of the *C. fornicata* cold feeding data, excluding one outlier. Given the role of *C. fornicata* as an invasive species with a much wider viable range than its more "isolated" congener (Thieltges et al. 2006), this generalist could have a small competitive advantage with regard to its viability at lower temperatures. If *C. fornicata* can feed itself even slightly better than *C. plana* in cold temperatures, it has a better chance of survival. In addition, if such a marginal advantage were to exist, it would explain why the feeding rates are statistically similar at our small sample size. While the ranges of both *C. plana* and *C. fornicata* have a northern limit of about 48°N, *C. plana* is not found south of Georgia (Collin, 2000), and is not known to colonize new environments, while *C. fornicata* can be found as far south as the Gulf of Mexico (Riquet, 2013), and is a known invasive. In the control system, a trend of viability in favor of *C. plana* is seen. As *C. fornicata* fills a similar niche to this species, *C. plana* must have some advantage that allows it to outcompete *C. fornicata* in the area of their ranges that overlap. The control treatment of about 18°C may have mimicked plana's native range in a way that gave it a feeding advantage that *C. fornicata* did not have, but further research would have to be done to determine this. Given that *C. fornicata* is a generalist species (Raven, 2019), its phenotypic plasticity as well as general hardiness gives it the ability to survive in a wide range of environments, so perhaps traits such as its thicker shell, color, or even certain behavioral tendencies allow it to survive while occupying a similar niche in the same range. However, an intertidal species like *C. plana* is a hardy and thermally tolerant organism itself, even compared to *C. fornicata* (Harrelson and Cravens 1986), future research should discern its feeding ability compared to *C. fornicata* at its typical climate to see if one species has an advantage over the other. Given the fact that high variance in feeding rates were consistently found with *C. plana* compared to *C. fornicata*, future experiments could increase the number of trials to find more consistent data. Higher variance in feeding rates were found in *C. plana* compared to *C. fornicata* and in general, the short time frame and small sample size may have contributed to the lack

of statistical power in much of our analysis, so future experimentation in this avenue should increase the number of individuals per treatment and apply them for a longer period of time.

In the hot system, size did not affect feeding rate

Interestingly, larger *Crepidula* ate at statistically similar rates to smaller individuals. This could be explained by the fact that larger *Crepidula* needed only to maintain their mass and energy expenditure while smaller *Crepidula* needed to eat more in order to grow. This would imply that there exists a fixed upper threshold limit that these *Crepidula* adhere to rather than one that is proportionate to their body size. As the treatments went from cold to hot, the medians grew closer and closer together and at the hot treatment they were statistically the same. This lines up with there being an upper limit on feeding, independent of size. A rate of consumption not tied to body size would imply that one of the most important regulators of *Crepidula* consumption is temperature, since it has a direct impact on the speed of biochemical reactions such as feeding and processing food (Clarke, 2004). Many invertebrates have indeterminate growth (Sebens, 1982) but due to their physiology it may not make sense for "greedy" individuals to thrive; if the rate of feeding increased with size out of control, *Crepidula* could grow bigger and bigger until they could no longer function due to the limitations of the invertebrate body plan (Sebens, 1987). The absolute upper limit could represent the most energy a *Crepidula* can use at any point during its life to grow to an appropriate size where it can still function. Maintaining a larger body may require it to devote a disproportionate amount of resources to feeding itself rather than devoting that energy to reproducing. Given that food is abundant in their environment (Orton, 1912), we believe that it's less likely that these organisms need to regulate their consumption to avoid depleting their own food source so perhaps there is less internal selective pressure for more developed systems of food consumption regulation. Using the fact that temperature directly speeds up or slows down biochemical processes, *Crepidula* may have had no need to maintain its diet other than an upper limit so it doesn't grow too much larger. A longer term study that involves tracking the feeding rates of *Crepidula* raised from juvenile to adulthood in a warm climate could determine whether or not such a threshold exists.

Crepidula under cold stress exhibit less movement

By the end of the experimental period, *Crepidula* in the cold system were significantly less likely to move out of their petri dish. There could be many reasons for this, the most obvious being that cold slows down metabolic reactions (Clarke, 2004). This lower metabolic rate would reduce the ability of the *Crepidula* to move. However, if movement was solely temperature dependent, we would expect to see the *Crepidula* in the hot system move more than the *Crepidula* in the control system. This is not the case, so there must be other factors affecting the movement patterns. One possible explanation is that since the *Crepidula* are eating more in the hot treatment, they would have more energy to move around. However, this still does not explain why the *Crepidula* in the control treatment had increased movement too, as they did not eat as much as the *Crepidula* in the hot treatment. The most likely explanation for these differences in movement comes down to the sexual activity of *Crepidula*. In winter, male *Crepidula* become sexually inactive, and can reabsorb their sexual organs (Coe, 1936). Because one of the main reasons that *Crepidula* move is to

mate (Coe, 1938), the *Crepidula* in the cold system would have had less reason to move, as this system was simulating winter water temperatures. The control and hot systems were not experiencing winter temperatures, so the *Crepidula* in those systems were free to mate, and therefore, free to move. The *Crepidula* in the cold system experienced lower metabolic rates and were unable to mate, so movement was much lower in the cold.

C. fornicata move more frequently than C. plana

One of the most immediately obvious differences between *C. plana* and *C. fornicata* is that almost none of the *C. plana* ever moved off of their petri dish, while *C. fornicata* moved off of their petri dish almost half of the time. These data prove that there are distinct behavioral differences between these two species that can dictate movement patterns. This could also explain why *C. fornicata* are an invasive species in Europe (Thieltges et al. 2006), while *C. plana* have not been invasive (Carlton, 1992). It is possible that the increased mobility of *C. fornicata* makes it more likely to move onto vessels traveling across the Atlantic Ocean, and then successfully move off these vessels and find suitable habitat once arrived in Europe. While many other factors can affect the ability of *C. plana* to be invasive, its lower mobility can be a large limiting factor.

Thermal stress does not affect mortality in Crepidula

Because an equal amount of *Crepidula* died in each tank, temperature had no effect on mortality. Most likely these deaths were caused by the constant scraping of these *Crepidula* off the system after they had left their petri dishes. When being scraped off of the system, some shells tended to chip, and it most likely put heavy stress on the foot of the *Crepidula*. It is also expected that no mortality would come from thermal stress, as Diederich & Pechenik (2013) did not observe mortality until *Crepidula* reached above 35℃, and our *Crepidula* only reached 26.6℃. Additionally, winter water temperatures in *Crepidula* habitat routinely dip below 6℃ (NCEI, 2020), so we would not expect mortality from the cold treatment either. These results confirm the hardiness of *Crepidula* and showcase their ability to adapt to a wide range of temperatures.

Conclusion

Crepidula fornicata and *Crepidula plana* both demonstrate impressive tolerance to thermal variation, but thermal stress can still be significant enough to greatly alter behavior. Extreme cold temperatures could reduce viability, while extreme hot temperatures increase the need to feed. As temperature extremes and temperature variations increase in coming years, *Crepidula* will have to adapt to these changing conditions or be forced to find new habitat.

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