4-4-2022

Physiological and Biochemical Thermal Stress Conditions of the Ribbed Mussel, Geukensia demissa, from Exposed and Less Exposed Areas in the Intertidal Salt Marsh on Tybee Island, Georgia.

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An Honors Thesis submitted in partial fulfillment of the requirements for Honors in the *Department of Chemistry and Biochemistry.*

By
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Under the Mentorship of *Dr. Worlanyo Eric Gato* and *Dr. Sophie B. George*

ABSTRACT

Geukensia demissa, the ribbed mussel, is a keystone species of Georgia's coastline that is at risk of experiencing detrimental thermal stress due to climate change. *G. demissa* interacts positively with a species of salt marsh cordgrass, *Spartina alterniflora*. Mussels form aggregates beneath salt marsh cordgrass stems where they are shaded and less exposed to sun rays. However, some mussels end up in areas which lack cordgrass and are directly exposed to sun rays. Body temperatures of mussels from exposed areas were found to be higher than mussels from less exposed areas. Thermal stress levels of mussels can be indicated using heart rate and heat shock proteins (HSPs). If exposed mussels acclimate to higher temperatures than less exposed mussels, then they may exhibit a higher maximum temperature tolerance point and lower heart rates at an elevated temperature. Exposed mussels were found to maintain lower heart rates than less exposed mussels after elevated temperature subjection. Mussels collected from less exposed areas had greater expression of most HSPs and responded to heat exposure with stronger upregulation of HSPs than those from exposed areas. This study supports our hypothesis that the presence of *S. alterniflora* in the intertidal salt marsh is important for the regulation of thermal stress conditions of *G. demissa*. Continuing to study *G. demissa* and other keystone intertidal species is incredibly important for understanding how the health of intertidal ecosystems can be maintained, despite climate change, for the good of both people and the planet.

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April 2022
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Acknowledgements

Thank you to the College Office of Undergraduate Research for funding this project. Thank you to Dr. Worlanyo Eric Gato for mentoring me and teaching me so much about the research process and execution. Thank you to Dr. Sophie George for the opportunity to work on this incredible project and her invaluable mentoring. She introduced me to this research and taught me how to be an effective researcher. Thank you to Ashlyn Smith and Asia Watson for helping me learn the ropes and assisting in my project. Thank you so much to Awa Cisse for helping me in the field and lab and being extremely supportive and encouraging. This interdisciplinary project would not have been possible without these wonderful people helping me and teaching me along the way, so I am incredibly thankful for them all.

Introduction

Climate change is regarded as a significant concern to many environmental systems. One environmental system at particular risk of detrimental changes due to climate change is marine intertidal ecosystems such as salt marshes (Harley et al., 2006). Marine intertidal ecosystems are extremely important, both ecologically and economically. The intertidal zones provide food and materials and function as a buffer protecting inland areas from storms (Harley et al., 2006; Leonardi et al., 2018). The organisms in intertidal zones already experience high baseline stress due to daily changes in conditions such as water level, salinity, and temperature (Helmuth, 1998). Additional changes in climate due to human impact can have a variety of negative effects on the health of intertidal ecosystems. Temperature changes in coastal intertidal areas may be detrimental to the organisms inhabiting them, especially sessile organisms. Sessile organisms such as mussels are at particular risk because of their inability to escape from unsurvivable conditions. Mussel populations on the West coast have been in decline over the past several decades due to changes in the climate (Smith et al., 2006; Harley et al., 2006). On the South-eastern coast, similar effects may be at play. For example, *Geukensia demissa*, also known as the ribbed mussel, is one species that is potentially at risk of experiencing detrimental heat stress due to increasing temperatures.

Ribbed mussels are a habitat-building, keystone species in Georgia's salt marshes (Angelini et al., 2015). Despite covering a very small percentage of the salt marsh surface, they enhance five ecosystem functions: water infiltration, decomposition, substrate stabilization, cordgrass biomass, and species diversity. Monitoring their condition is important for understanding how the mussel population and the overall

health of the salt marsh is changing with increasing temperatures. There is limited research on the thermal stress conditions of *Geukensia demissa* on the southeastern coast of the United States and the impact of interactions between *G. demissa* with other species such as salt marsh grasses on their thermal stress status.

Spartina alterniflora, a common salt marsh grass also known as cordgrass, is another extremely important species in intertidal communities along the Atlantic coast (Altieri et al., 2007; Angelini et al., 2015). Cordgrass and ribbed mussels have a mutualistic relationship. Cordgrass provides shading from sun rays, while ribbed mussels provide substrate stabilization and nutrients. These two species coexist in raised areas called mounds. Ribbed mussels tend to cluster in the center of mounds and aggregate beneath patches of cordgrass; however, some mussels end up in areas of the mound near the edge which lack cordgrass. The mussels located directly beneath a patch of cordgrass are shaded and therefore *less exposed* to direct sun rays. Mussels that end up in an area without cordgrass do not have this shading and are therefore directly *exposed* to sun rays. Facilitative interactions between cordgrass and ribbed mussels are well known, but whether the shading provided by cordgrass has direct effects on the thermal stress conditions of ribbed mussels of this species is less understood.

When organisms experience atypical environmental demands such as elevated temperatures, they have the ability to respond or acclimate to these stressors. As such, mussels can acclimate to varying temperatures over time. Heart rate has often been used as a tool to analyze the physiological condition of mussels and other sessile organisms (Burnett et al., 2013). Heart rate can be used to analyze the thermal stress condition of mussels, as the heart rate of mussels increases with increasing temperatures until the

temperature reaches the maximum tolerance point for the animal (Braby and Somero, 2006). Some species of mussels which are acclimated to higher temperatures can tolerate higher temperatures than those acclimated to lower temperatures (Braby and Somero, 2006; Galbraith et al., 2012). High temperature acclimation allows mussels to maintain lower heart rates at higher temperatures (Braby and Somero, 2006).

The ability of mussels to acclimate to increased temperatures and thermal stress stems from the upregulation of heat shock genes (Snyder et al., 2001). Heat shock protein induction is used by mussels to respond to a variety of stressful conditions (Liu & Chen, 2013). At elevated temperatures, proteins can fold incorrectly after translation and existing proteins can begin to denature. Heat shock proteins (HSPs) are molecular chaperones which help proteins fold properly. Denatured and incorrectly folded proteins can cause harm to the organism (Liu & Chen, 2013). When organisms such as the ribbed mussel are exposed to high temperatures, they increase the transcription of HSP genes to produce more HSP proteins (Liu & Chen, 2013). Constitutively expressed heat shock cognates (HSCs), which also function as molecular chaperones, are also upregulated as temperatures increase during the summer (Franzellitti & Fabbri, 2005; Liu & Chen, 2013). Elevated HSP and HSC levels can increase the mussel's maximum temperature tolerance point (Hamdoun et al., 2003).

The expression of a highly conserved family of heat shock proteins including HSP70 and HSC70 can be analyzed to evaluate the thermal stress conditions and acclimation of a wide range of organisms (Hassan et al., 2019). During periods of low stress, the baseline expression of HSC70 is higher than HSP70 (Franzellitti & Fabbri, 2005). Increased expression of HSP70 in response to environmental changes such as

acute heat stress has been observed in the mussel species *Mytilus galloprovincialis* (Franzellitti & Fabbri, 2005). In contrast, HSC70 expression remains relatively constant even after exposure to acute heat stress (Franzellitti & Fabbri, 2005). Overall, HSP70 expression is inducible by subjection to acute heat stress, while HSC70 is probably not inducible by subjection to acute heat stress (Liu & Chen, 2013; Ali et al., 2003; Clark et al., 2008; Franzellitti & Fabbri, 2005).

The focus of this study is to determine whether the shading provided by *S. alterniflora* impacts the thermal stress conditions of *G. demissa* and discuss whether these interactions could impact the survivability of mussels as temperatures increase. Less exposed mussels are those located directly under a dense patch of cordgrass, while exposed mussels are not. The thermal stress condition of less exposed and exposed mussels will be analyzed using field body temperatures, heart rate after exposure to an elevated temperature in the lab, and heat shock gene expression. These variables will be used as proxies of thermal stress related to exposure.

We hypothesized that exposed mussels would have higher body temperatures than less exposed mussels in the field. Furthermore, if exposed mussels acclimate to higher temperatures than less exposed mussels, then they may exhibit a higher maximum temperature tolerance point and thus lower heart rates at an elevated temperature. Mussels collected from exposed and less exposed areas will also be analyzed for their HSP70 and HSC70 gene expression with RT-qPCR. Expression of HSP70 and HSC70 genes under thermal stress in *G. demissa* is expected to be similar to other species of bivalves. Half of the mussels will be subjected to an elevated temperature in the lab prior to dissection. Control mussels are those which are not subjected to an elevated

temperature in the lab. Control mussels from exposed areas are expected to have higher expression of HSP70 and HSC70 compared to mussels from less exposed areas. The change in expression of HSP70 and HSC70 genes between the control mussels and mussels subjected to an elevated temperature will be compared for exposed and less exposed mussels. If mussels in exposed areas of the salt marsh are acclimated to elevated temperatures, their change in expression of HSP70 genes after subjection to an elevated temperature is expected to be lower compared to less exposed mussels. The level of expression of the HSC70 gene is not expected to change between control and high temperature-subjected mussels. Overall, if these predictions are supported it would suggest that *S. alterniflora* plays a significant role in the regulation of the thermal stress conditions of *G. demissa*.

Methods

1. Description of sites

This study was conducted from March 2019 to August 2021 on eight mussel mounds located in the intertidal, mid-marsh zone of Tybee Island, Georgia (32° 1' 2.348" N 80° 51' 38.693" W). The mounds were located off of Old Tybee Road (Figure 1). The intertidal, mid-marsh zone is characterized by regular tidal flooding, medium height (33-71 cm) *Spartina alterniflora* (cordgrass), and the presence of mussel mounds. Our 8 mounds ranged from 140 cm to 318 cm in maximum diameter, across a transect line of about 52 meters (Figure 2). Mussel mounds are raised areas of the marsh that contain large clusters of mussels, known as aggregates, that can contain over 200 mussels. These mussel aggregates are located around the clustered stems of cordgrass. Within each

mound there is variation in the density of cordgrass stems. Mussels in areas of the mound with very low or no cordgrass density are more directly exposed to sun rays, while mussels in areas of the mound with a higher density of cordgrass are less exposed to sun rays. In other words, cordgrass provides shading to the mussels located directly under their stems.

The area of the salt marsh which was utilized for this experiment was nearby a tributary of Tybee Creek, making the side of the site closer to the tributary wetter and muddier than the side located further from the tributary (Appendix 1). The conditions of the exposed versus less exposed areas of each mound depended on the mound's location relative to the tributary. In particular, the difference in conditions between mound 7 and mound 8 were quite pronounced over the course of this experiment. A comparison between these two mounds is used to demonstrate how the distance from the tributary affected the mussel conditions because mound 7 was the closest to the tributary while mound 8 was the farthest away. Over the course of this experiment, mound 7 had a consistently higher water level and cordgrass density. The center of the mound which was abundant with mussels and cordgrass was clearly distinguishable from the edges of the mound which had very little cordgrass or mussels. Mound 8 on the other hand had consistently lower water levels and cordgrass density. The overall mussel population was significantly lower on mound 8. The center of the mound versus the edges were significantly less distinguishable compared to on mound 7.

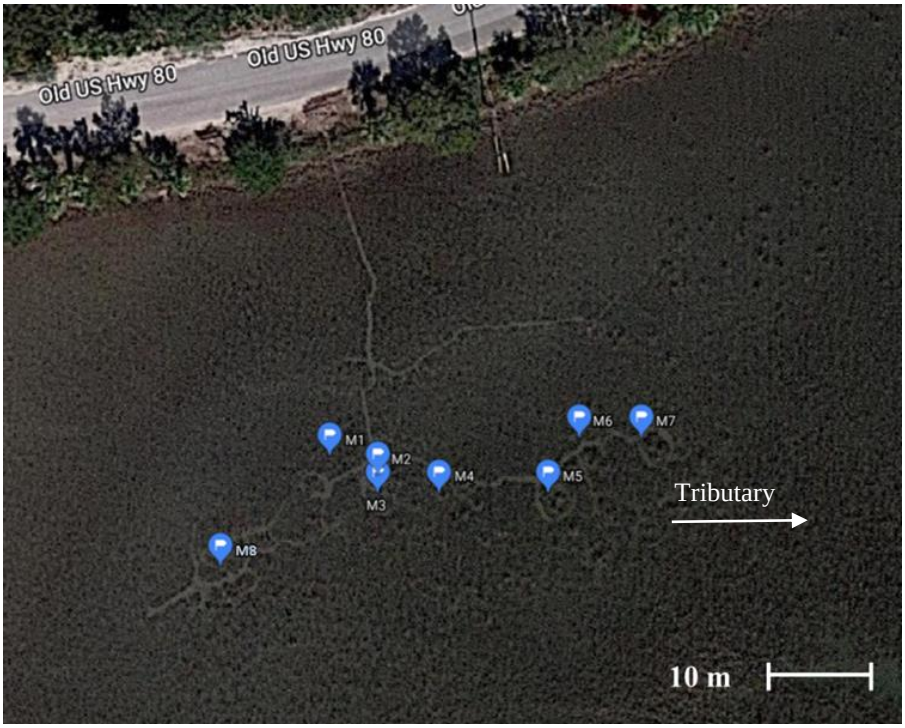

Figure 1. Satellite image of mound locations off Old US Hwy 80, Tybee Island.

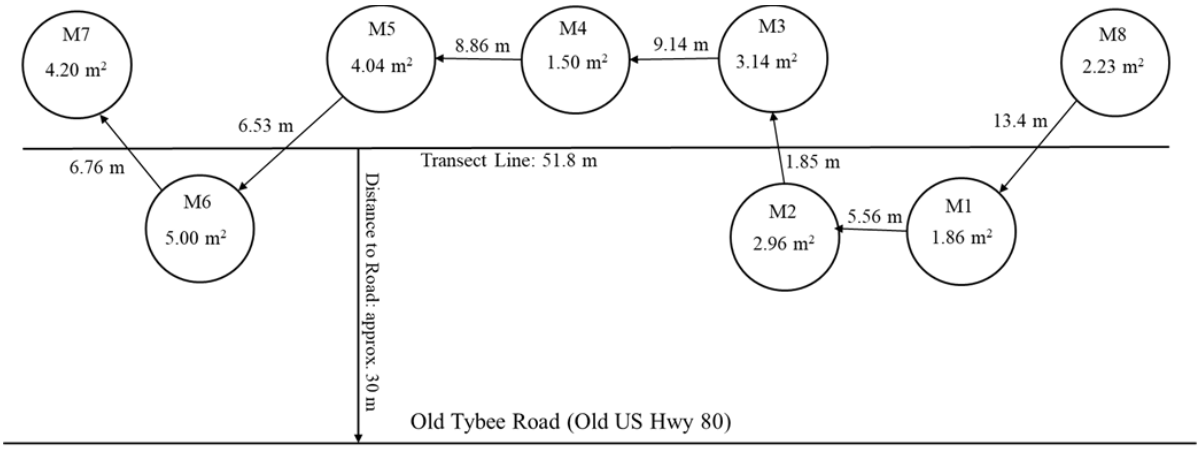

Figure 2. Mounds on a transect line with distance between mounds, mound areas, and approximate distance to the road.

2. Body Temperature Measurements

In order to determine whether exposed mussels had higher body temperatures than less exposed mussels, we recorded the temperatures of 10 exposed and 10 less exposed randomly-chosen mussels from each mound. Mussel body temperatures were measured using a FLUKE 62 MAX infrared temperature gun. These data were collected in August 2019 on each of the 8 mounds and in May 2021 on mounds 7 and 8 during low tide. In August 2019, the weather conditions were cloudy and with maximum air temperatures of 31°C, and in May 2021, the weather was fair with maximum air temperatures of 31°C. To analyze this data, a two-way nested analysis of variance (ANOVA) was utilized with mound and exposure as main factors.

3. Mussel Care

Mussels brought in from the field were placed in 8 plastic tanks, 6 measuring 34 cm x 18 cm wide and 2 measuring 33 cm x 19 cm, each with 5 mussels and ~ 3 L of water. The tanks were aerated with air bubblers. The water level and salinity were kept consistent for the duration of time the mussels were kept in the lab. The mussels were fed 2.8 mL per tank of Shellfish Diet 1800™ from Reed Mariculture three times per week. The tanks were cleared of waste two times per week. Room temperatures were between 18-20°C.

4. Heart Rate Measurements

To determine whether the heart rate of less exposed mussels was higher than the heart rate of exposed mussels when exposed to a high temperature (36 °C) in the lab, less

exposed mussels were collected from 4 mounds (2, 5, 6, and 7) with the lowest overall body temperatures and exposed mussels were collected from 4 mounds (1, 3, 4, and 8) with the highest overall body temperatures. In August and September 2019, we collected 5 similarly sized mussels from each mound (either exposed or less exposed). The mussels collected in August were 10.0 ± 0.6 cm in length (n = 40), and the mussels collected in September were 10.1 ± 0.6 cm in length (n = 40). In August, the weather conditions were cloudy and with maximum air temperatures of 31°C, and in September, the weather was clear, sunny, with maximum air temperatures of 29 °C. In September, we returned the mussels collected in August and collected 5 new mussels from each mound (n = 40) to repeat the lab experiment. All but 8 of these mussels were returned in mid-October.

Due to inclement weather, heart rate measurements were performed two weeks after collecting the mussels from the field instead of the planned one week. The system used to measure the heart rates, designed by Burnett et al. (2013), consists of an infrared sensor placed directly on the mussel's shell, an amplifier to make the electrical signal readable and reduce electrical noise, a Picoscope to convert the analog signal to a digital signal, and a computer with the Picoscope program to display the signal (Figure 3).

Mussels were placed into a water bath in groups by mound with a starting temperature of 22 °C. The temperature of the water bath was gradually increased from 22°C to 36°C over a period of 1 hour. The water temperature was maintained at 36°C for 30 minutes before heart rate measurements were taken. The temperature 36°C was chosen because it was the maximum recorded mussel body temperature from August 2019. The water level in the water bath was lowered to reveal the top layer of mussels, so the mussels in the lower levels were maintained in 36°C water until their heart rates were

measured. The IR sensor was placed on the shell of each mussel at the location of the heart (Figure 4), and the heart rate was recorded for 60 to 80 seconds. Each Picoscope file was then saved to the computer to review and calculate heart rates later. Heart rates were calculated using the Picoscope files by manually counting the oscillations in the signal (Figure 5). To determine whether the heart rates of less exposed mussels were higher than the heart rates of exposed mussels a mixed model three-way ANOVA with exposure, mound, and month as the main factors was used.

Figure 3. Set-up for heart rate measurements; mussel, IR sensor, amplifier, Picoscope, and computer (right to left).

Figure 4. Correct location of IR sensor on ribbed mussel shell to measure heart rate (red star).

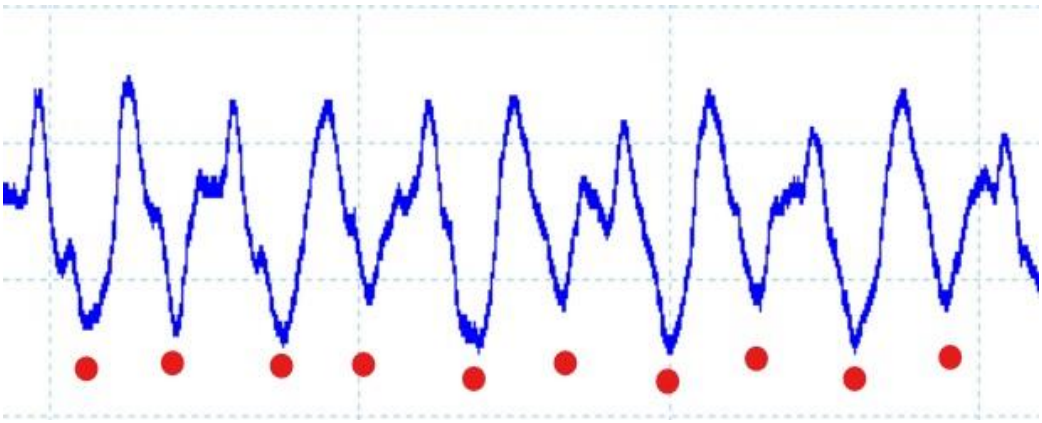

Figure 5. Example of how heart rate is counted in Picoscope software. Each red dot represents one heartbeat.

5. Heat Shock Gene Expression

In May 2021, 8 mussels were collected to measure their heat shock gene expression (Table 1, Figure 6). The weather was fair with maximum temperatures of 31°C. Four mussels were collected from 2 separate mounds (M7 and M8). Mussels from M7 and M8 were collected to represent the two extremes in site conditions as they were the closest and farthest away from the tributary respectively. From each of these mounds, two mussels from less exposed areas and two from exposed areas were collected (Figure 6). However, the two exposed mussels from M8 were found to be dead, so they were not included in the analysis. One mussel from each location/exposure level was subjected to a temperature of 36°C for an hour and a half in the laboratory following procedures used in section 4. In July 2021, an additional 8 mussels were collected. The weather was partly cloudy with maximum temperatures of 31°C. In order to avoid inadvertently collecting dead mussels, all 8 were collected from mound 7 (M7). Four of them were from less exposed areas and four from exposed areas of the mound (Figure 6). Two mussels from

each exposure level were also subjected to a 36°C for an hour and a half in the laboratory. All of the mussels were then sacrificed, and their gill tissues stored at -80°C until ready for analysis.

For analysis of gene expression, first total RNA isolation was completed using the RNeasy Mini by Qiagen. Approximately 20 to 30 mg of each tissue sample was added to 900 µL of QIAzol lysis reagent for homogenization and bonded to the RNA spin column. The purity, via 260/280 ratio, and concentration of the total RNA of each sample was examined with a Nanodrop (Thermo Scientific Nanodrop, 2000/2000c Spectrophotometer) nucleic acid spectrophotometer. According to a technical bulletin for the Nanodrop 2000, a 260/280 ratio of about 2.0 is generally considered a pure RNA sample. Ratios slightly higher than 2 are generally not a concern but may be due to a low-quality blank.

The expression of HSP70 and HSC70 genes was determined in duplicate using real-time quantitative PCR. The selected genes and their associated accession numbers were obtained from prior studies on heat shock genes in mussels (Franzellitti et al., 2020; Kourtidis et al., 2006) (Table 2). FASTA mRNA sequences from each mRNA transcript were derived on the National Center for Biological Information (NCBI) database. Forward and reverse primers needed for each gene were generated using the Integrated DNA Technologies (IDT) Inc.. Bio-Rad iScript Reverse Transcription Supermix for RT-qPCR was used to synthesize cDNAs from total RNA. Primers and SsoFast EvaGreen Supermix were combined with the cDNAs for the real-time quantitative PCR (RT-qPCR). Bio-Rad CFX96 Rt-PCR system was used to measure the combination according to the manufacturer's guidelines (Bio-Rad Laboratories Inc.).

Table 1. Collected mussel sample identities and conditions including mound location, exposure level (exposed or less exposed), and whether the mussel was subjected to 36 ℃ in the lab for 1.5 hours. Mussels were collected in May 2021 (S1-S6, SX, SY) and July 2021 (S7-S14) on Tybee Island, GA and their gill tissues used to determine heat shock gene expression. SX and SY were found to be dead, so no analyses were performed.

Sample #	Mound	Exposure Level	Subjected to 36℃
S1	M7	Less Exposed	No
S2	M7	Less Exposed	Yes
S3	M7	Exposed	No
S4	M7	Exposed	Yes
S5	M8	Less Exposed	No
S6	M8	Less Exposed	Yes
SX	M8	Exposed	N/A
SY	M8	Exposed	N/A
S7	M7	Less Exposed	No
S8	M7	Less Exposed	No
S9	M7	Less Exposed	Yes
S10	M7	Less Exposed	Yes
S11	M7	Exposed	No
S12	M7	Exposed	No
S13	M7	Exposed	Yes
S14	M7	Exposed	Yes

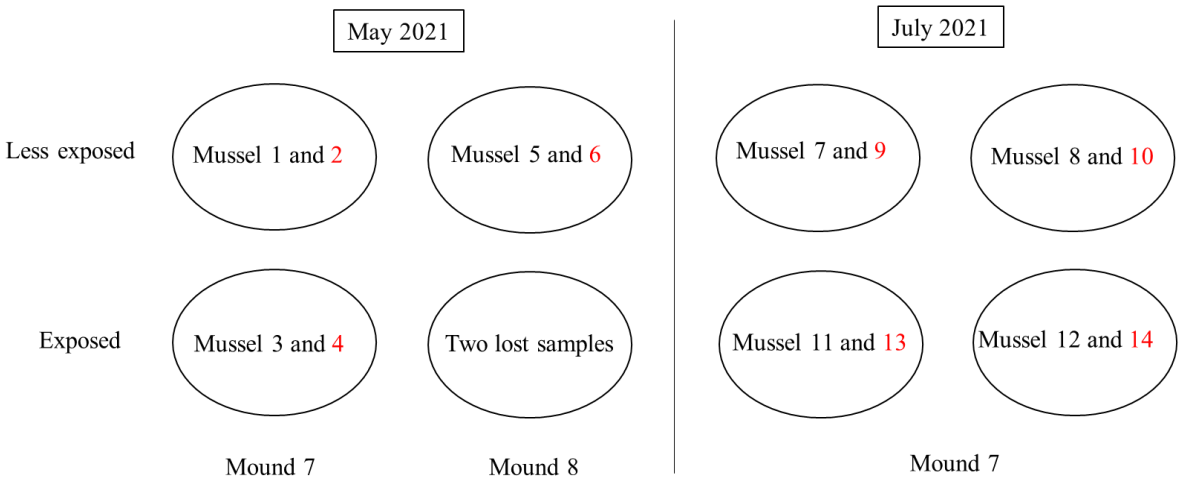

Figure 6. Experimental design used to determine whether mussels collected from less exposed and exposed sites in a salt marsh differ in HSP70 expression and whether these mussels will express higher levels when exposed to 36°C in the lab. Numbers in red indicate mussels that were subjected to 36°C.

Table 2. Accession number and forward and reverse primer sequences for selected heat shock genes (Franzellitti et al., 2020; Kourtidis et al., 2006).

Gene	Accession #	Forward Primer	Reverse Primer
HSP70	AY861684	CTGCTTGTGAAAGGGCAAAG	CTCTTGGTGCTGGAGGTATTC
HSP70-2	AJ783711	CTCTTGGTGCTGGAGGTATTC	GCTTTGTCCTCCTGTGCTATTT
HSP70-3	AJ783712	GCTCCTTTGTCCCTTGGTATT	GAGTCTTCCTCTGTCATTGGTG
HSP70-4	AJ783713	TCCGTTGTCCCTTGGTATTG	CATGACGGGCGTACATACTT
HSC70	AJ783715	CTGCTTGTGAAAGGGCAAAG	GTGGAAACCGCGAATGAATG

Results

1. Body Temperature Measurements

For the mussel body temperature measurements recorded using a FLUKE 62 MAX infrared temperature gun in August 2019, there was a significant difference between the body temperatures of exposed and less exposed mussels ($p < 0.0001$) (Figure 7). The average body temperature for exposed mussels (30.1 ± 1.8 °C) was significantly higher than the average body temperature for less exposed mussels (28.4 ± 1.0 °C). For the mussel body temperature measurements taken in May 2021, there was also a significant difference between the exposed and less exposed body temperatures ($p < 0.0001$) (Figure 8). The average body temperature for exposed mussels (33.5 ± 2.7 °C) was significantly higher than the average body temperature for less exposed mussels (26.7 ± 2.1 °C). There was also an observed larger difference between exposed and less exposed temperatures for the mound 7 mussels compared to the mound 8 mussels.

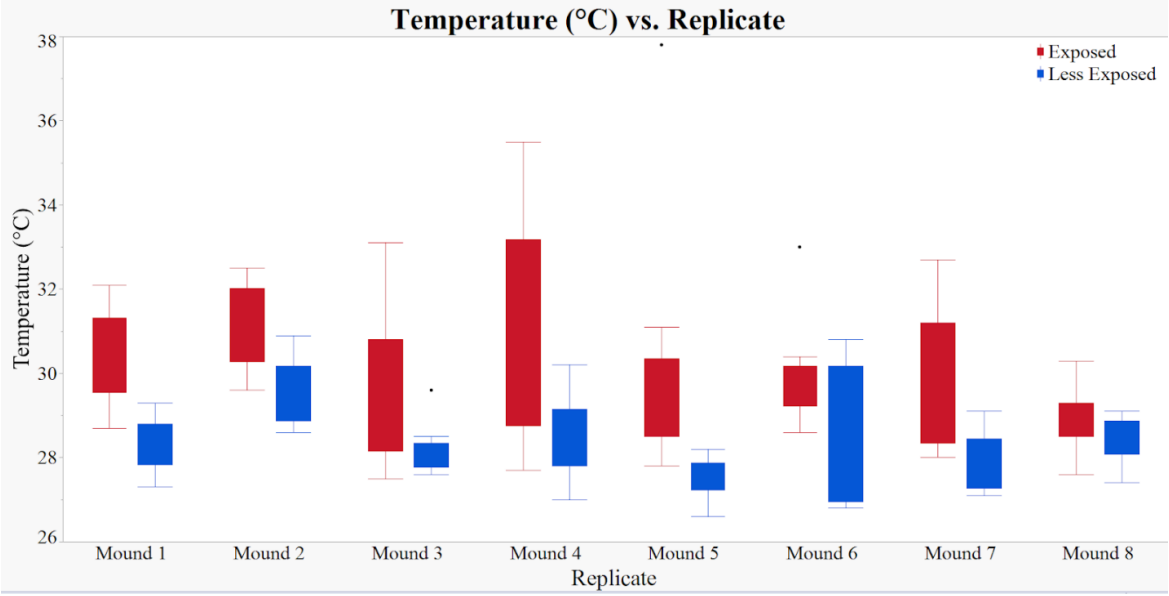

Figure 7. Box plots of body temperatures of 10 exposed and 10 less exposed ribbed mussels in each mound off Old Tybee Island, Georgia on August 30th, 2019 (n = 160). Maximum air temperature was 31°C. Temperatures were recorded using a FLUKE 62 MAX infrared temperature gun. The length of the box is the interquartile range; the bottom and top of the box are the 25th and 75th quartiles, respectively; the upper and lower whiskers extend to the maximum and minimum values.

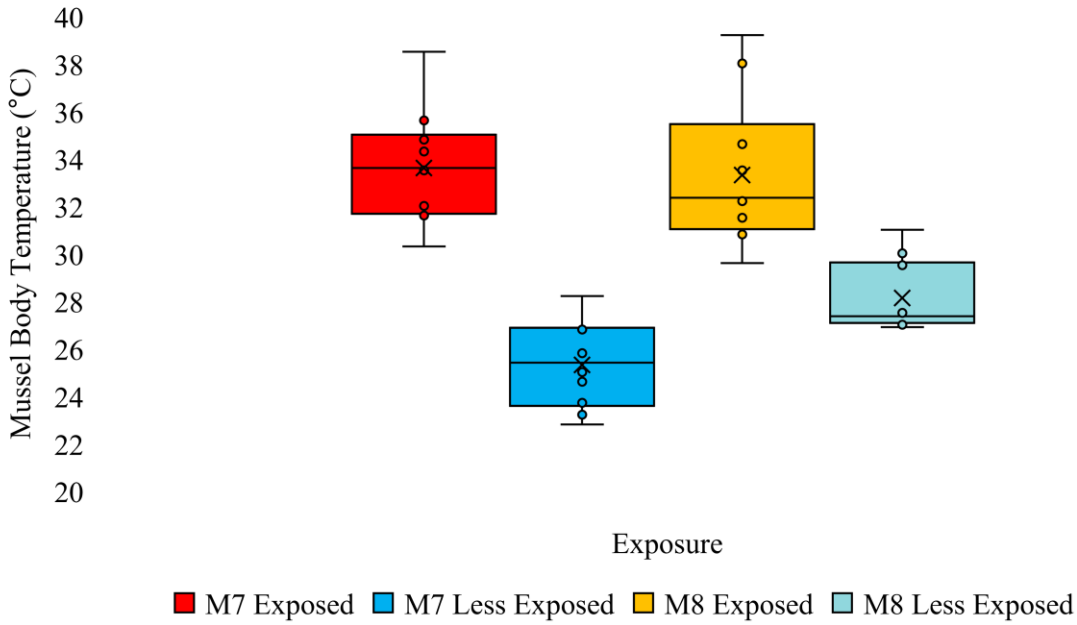

Figure 8. Box plots of mussel body temperatures of 10 exposed and 10 less exposed ribbed mussels on mounds 7 (M7) and 8 (M8) off Old Tybee Island, Georgia on May 24th, 2021 (n = 40). Maximum air temperature was 31°C. Temperatures were recorded using a FLUKE 62 MAX infrared temperature gun. The length of the box is the interquartile range, the horizontal line in the box is the median; the bottom and top of the box are the 25th and 75th quartiles, respectively; the upper and lower whiskers extend to the maximum and minimum values.

2. Heart Rate Measurements

In the laboratory experiment, mussels from less exposed areas of the mound had significantly higher heart rates (66.2 ± 8.5 bpm) than mussels from exposed areas (53.5 ± 10.8 bpm, $p < 0.0001$) (Figure 9). Mound 7 had especially high heart rates in both August and September, while mound 2 had the lowest heart rates of all the less exposed mussels over the course of the experiment. Mound 4 had the highest heart rates of the exposed mussels in both August and September. There was no significant difference in overall heart rates between August and September for both exposed and less exposed mussels ($p > 0.1$).

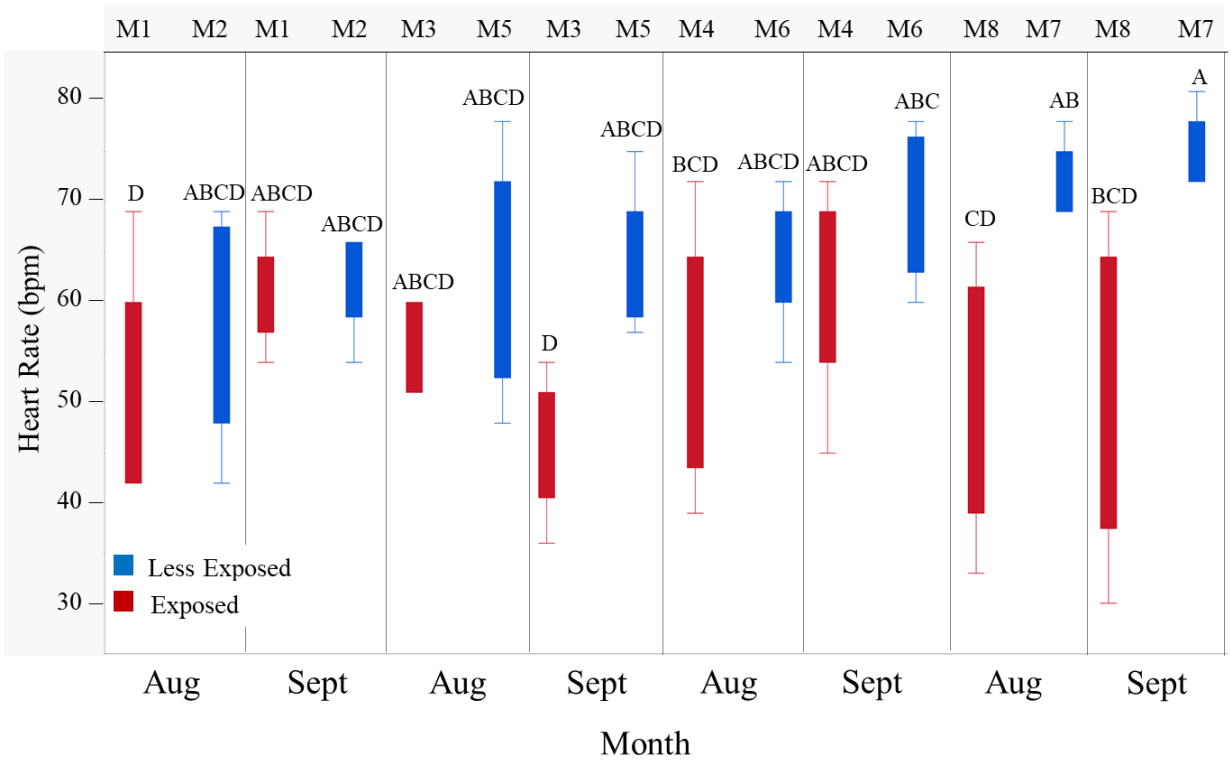

Figure 9. Box plots of heart rates in beats per minute (bpm) of 5 exposed and 5 less exposed ribbed mussels collected from 8 mounds (M1-8) off of Old Tybee Island,

Georgia in August and September 2019 and exposed to 36 °C in the laboratory (n = 80). The length of the box is the interquartile range; the bottom and top of the box are the 25th and 75th quartiles, respectively; the upper and lower whiskers extend to the maximum and minimum values.

3. RNA Concentrations and 260/280 Ratios

The concentration and purity of the RNA isolated from the mussel gill tissues was determined using a Nanodrop 2000 UV/Visible spectrophotometer. The 260/280 ratios were 2.13 ± 0.04, n = 14) which represented good RNA purity.

In May, mussels subjected to 36°C had a higher average RNA concentration (125 ± 52 ng/µL; n = 3) than mussels not subjected to 36°C (50 ± 23 ng/µL; n = 3) (Table 3a). The lowest RNA concentrations were observed for mussels from less exposed areas of mounds, not subjected to 36°C in the lab (34.1 and 40.1 respectively). The highest RNA concentration was also from a mussel collected from a less exposed area of mound 7 (172.3 ng/µL, Table 3a). A similar pattern was observed for mussels collected in July, but RNA concentrations were in most cases 9 to over 20-fold higher than in May. In July, RNA concentrations for mussels subjected to 36°C in the lab (843 ± 139 ng/µL; n = 4) was greater than for mussels not subjected to 36°C (770 ± 472 ng/µL; n = 4) (Table 3b). In July, the highest RNA concentration (1359.6 ng/µL), was observed for samples from the 12th mussel and the lowest (281.2 ng/µL) for samples from the 11th mussel. Both these mussels were not subjected to 36°C in the lab.

Table 3a. RNA concentration measurements and 260/280 ratios for RNA purity analysis for 6 mussels collected in May (S1 to S6) using a Nanodrop 2000.

Mussel Sample	Exposure Level	Subjected to 36°C	RNA Concentration (ng/μL)	260/280 Ratio
S1	Less Exposed	No	40.1	2.13
S2	Less Exposed	Yes	172.3	2.13
S3	Exposed	No	76.2	2.05
S4	Exposed	Yes	132.3	2.11
S5	Less Exposed	No	34.1	2.18
S6	Less Exposed	Yes	69.3	2.09

Table 3b. RNA concentration measurements and 260/280 ratios for RNA purity analysis for 8 mussels collected in July (S7 to S14) using a Nanodrop 2000.

Mussel Sample	Exposure Level	Subjected to 36°C	RNA Concentration (ng/μL)	260/280 Ratio
S7	Less Exposed	No	522.7	2.09
S8	Less Exposed	No	916.5	2.16
S9	Less Exposed	Yes	996.6	2.16
S10	Less Exposed	Yes	946.7	2.19
S11	Exposed	No	281.2	2.13
S12	Exposed	No	1359.6	2.14
S13	Exposed	Yes	866.3	2.17
S14	Exposed	Yes	565.1	2.12

4. Heat Shock Gene Expression

Expression of HSC70 in mussel gill tissues was analyzed using RT-qPCR. Results from May showed that mussels from less exposed areas of mound 7 had greater expression than mussels from exposed areas of mound 7 (Figure 10a). Mussels collected in July from exposed and less exposed areas of mound 7 had comparably very low expression of HSC70 except for S14. HSC70 values for mussels collected in July were considerably lower than from mussels collected in May. Except for S14, expression of HSC70 was similar for control mussels and mussels subjected to 36°C regardless of whether mussels were from less exposed or exposed areas of mound 7.

Expression of HSP70 was detected in all samples (Figure 10b). In both May and July, except for S13, HSP70 expression was higher for mussels subjected to 36°C than for the control mussels. In July, control mussels from exposed areas had lower expression than control mussels from less exposed areas. For mound 7, overall expression appeared to be higher in July than in May. High HSP70 expression seemed correlated with low HSC70 expression and vice versa.

For HSP70-2, expression was detected in 1 mussel in May, but in 6 mussels in July (Figure 10c). The data was inconsistent with significant variation among samples. Less exposed mussels appeared to have slightly greater expression than exposed mussels. Less exposed mussels had comparable expression regardless of subjection to 36°C. For mussels from exposed areas, control mussels had higher expression than mussels subjected to 36°C, but data to support this is limited.

For HSP70-3, 11 of 14 mussels had detected expression but no clear patterns emerged (Figure 10d). In both May and July, all mussels subjected to 36°C in the lab

expressed HSP70-3 while no HSP 70-3 was detected for 3 control mussels, 2 from exposed areas and 1 from a less exposed area of mound 7 (Figure 10d).

For HSP70-4, expression was inconsistently detected but some pattern can be observed (Figure 10e). Expression levels for mussels from less exposed areas subjected to 36°C were higher than control mussels regardless of month. In mound 7, mussels from less exposed areas subjected to 36°C had higher expression than mussels from exposed areas.

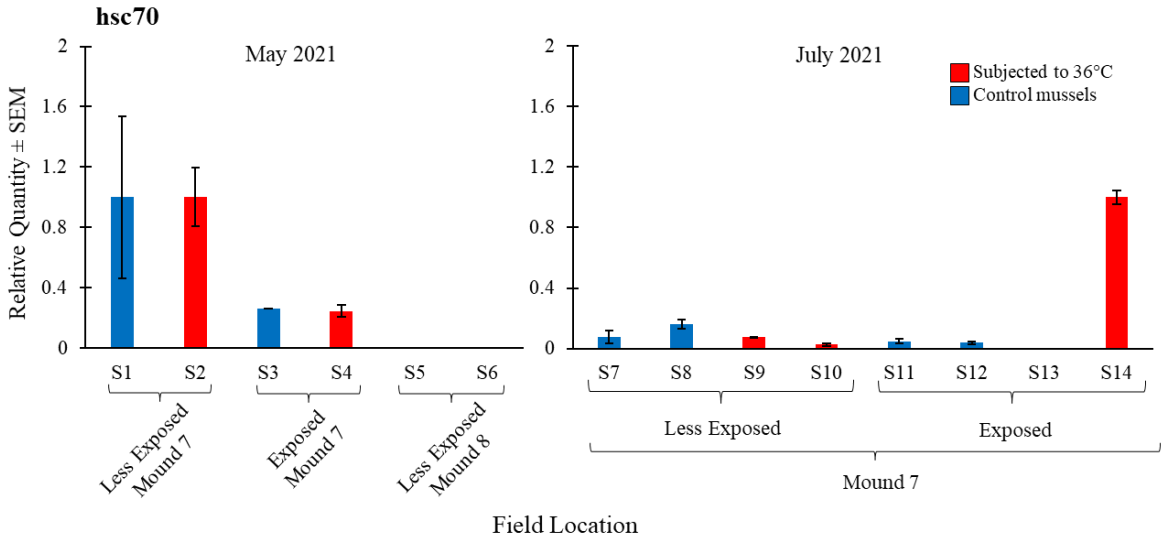

Figure 10a. Expression of HSC70 mRNA relative to zero for mussel gill tissue samples collected in May and July 2021 (total number of mussels used = 14). Each sample (S1-S14) was measured in duplicate using RT-qPCR. Values are expressed as the mean ± standard error of the mean (SEM). X-axis defines the exposure level and mound location of each sample. Bar color defines whether the sample was subjected to 36°C. No bar indicates expression was undetected.

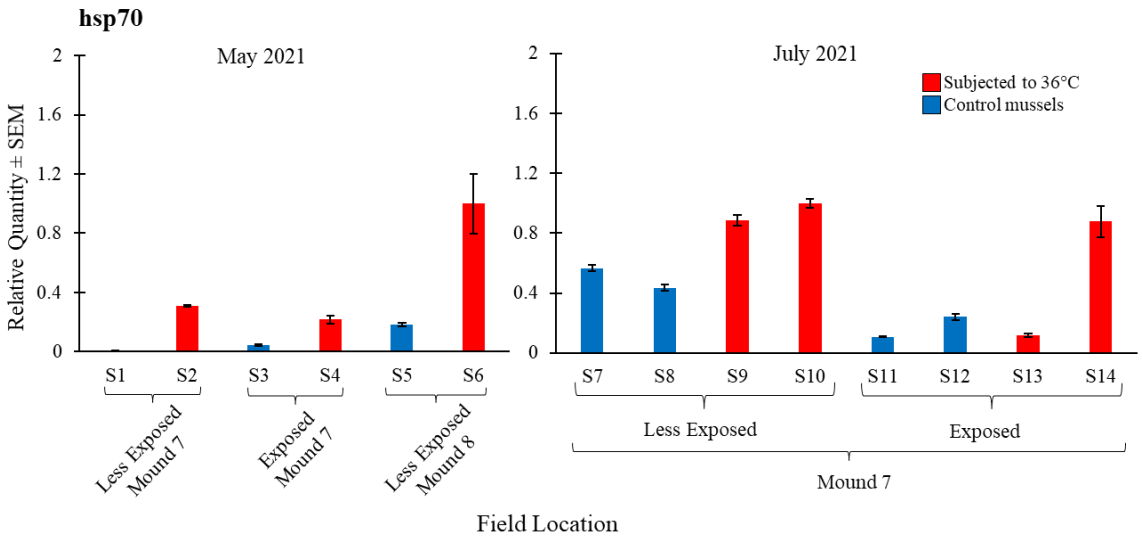

Figure 10b. Expression of HSP70 mRNA relative to zero for mussel gill tissue samples collected in May and July 2021 (total number of mussels used = 14). Each sample (S1-S14) was measured in duplicate using RT-qPCR. Error bars represent standard error of the mean (SEM). X-axis defines the exposure level and mound location of each sample. Bar color defines whether the sample was subjected to 36°C. No bar indicates expression was undetected.

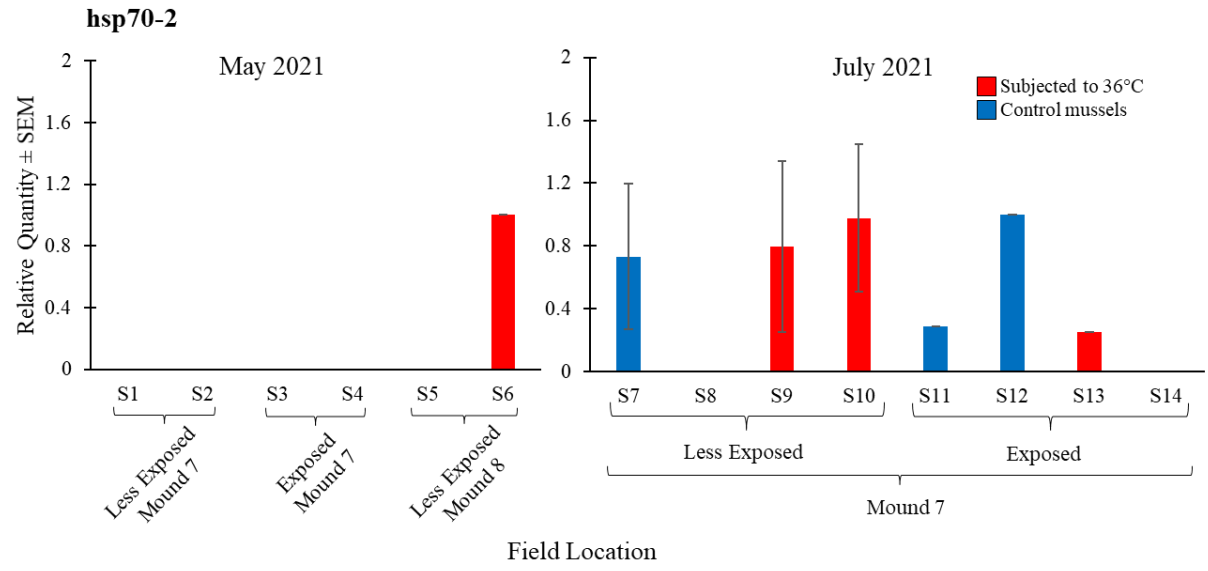

Figure 10c. Expression of HSP70-2 mRNA relative to zero for mussel gill tissue samples collected in May and July 2021 (total number of mussels used = 14). Each sample (S1-S14) was measured in duplicate using RT-qPCR. Error bars represent standard error of the mean (SEM). X-axis defines the exposure level and mound location of each sample. Bar color defines whether the sample was subjected to 36°C. No bar indicates expression was undetected.

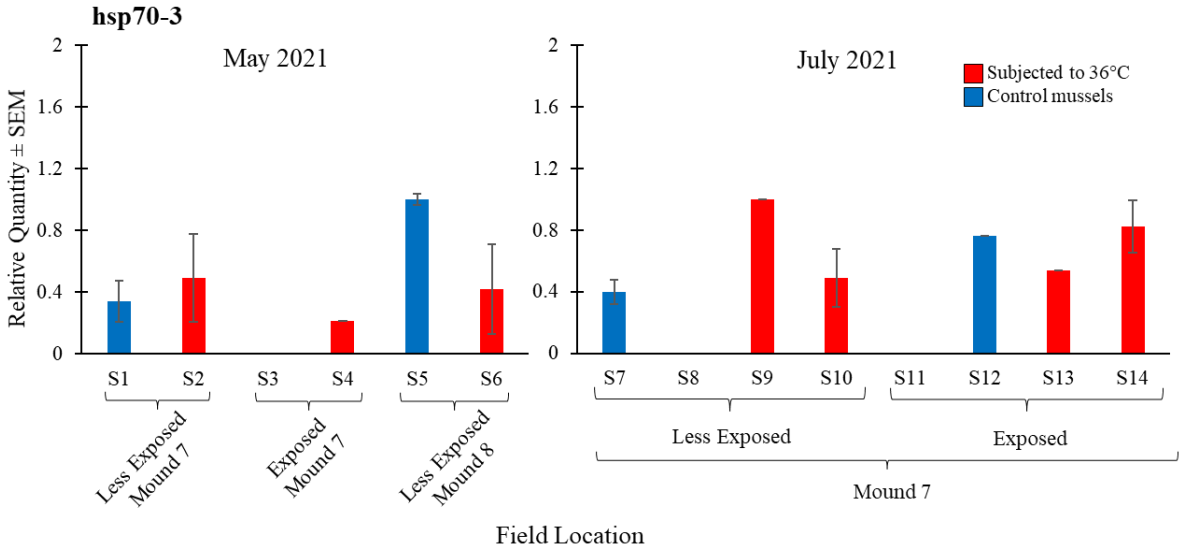

Figure 10d. Expression of HSP70-3 mRNA relative to zero for mussel gill tissue samples collected in May and July 2021 (total number of mussels used = 14). Each sample (S1-S14) was measured in duplicate using RT-qPCR. Error bars represent standard error of the mean (SEM). X-axis defines the exposure level and mound location of each sample. Bar color defines whether the sample was subjected to 36°C. No bar indicates expression was undetected.

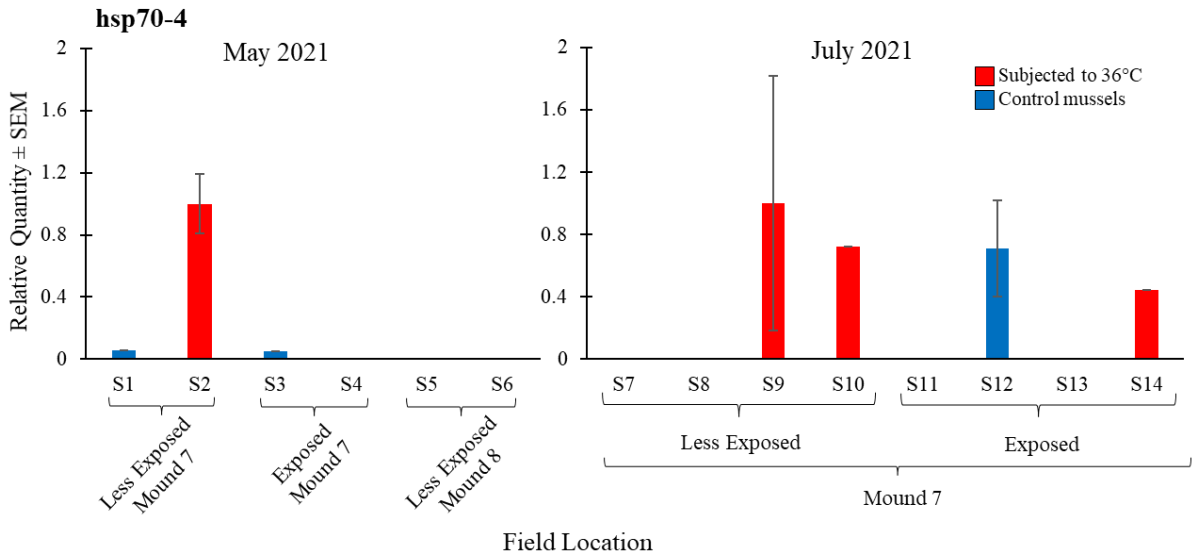

Figure 10e. Expression of HSP70-3 mRNA relative to zero for mussel gill tissue samples collected in May and July 2021 (total number of mussels used = 14). Each sample (S1-S14) was measured in duplicate using RT-qPCR. Error bars represent standard error of the mean (SEM). X-axis defines the exposure level and mound location of each sample. Bar color defines whether the sample was subjected to 36°C. No bar indicates expression was undetected.

Discussion

To our knowledge, this is the first study that provides insight into the possible effects of rising temperatures on heart rate, HSC70 and HSP70 expression in the ribbed mussel *Geukensia demissa* found in salt marshes along the southeastern United States.

1. Body Temperature Measurements

The body temperature data collected over the course of this study suggest that shading provided by *S. alterniflora* does have an impact on the body temperatures of the

ribbed mussel. Data collected on two different dates almost two-years apart showed very similar body temperature patterns between mussels in exposed and less exposed areas. This indicates a long-term effect on the mussels living in exposed versus less exposed areas of mounds in the salt marsh. In addition, one collection date was in early summer while the other was in late summer. This suggests that this temperature differential persists over a significant portion of the year.

Another interesting observation was that for the May 2021 data, mound 7 (M7) and mound 8 (M8) mussel body temperature differential between exposed and less exposed groups were very different. The difference in body temperatures between exposed and less exposed groups was greater for M7 than M8. The body temperatures for exposed mussels in both M7 and M8 were about the same, but the body temperatures for M7 less exposed mussels were significantly lower than for less exposed M8 mussels. A likely explanation for this pattern was that since M7 was located significantly closer to the tributary, it had higher water levels and greater cordgrass density in less exposed areas leading to lower mussel temperatures (Appendix 1).

Besides the presence of cordgrass, there may be another factor at play which impacted the lower body temperatures of mussels in less exposed areas. These mussels, located near the center of the mound, are not only covered by denser cordgrass but are also surrounded by an aggregate of other mussels. Being located in an aggregate of mussels changes the thermal inertia of the mussel, making them less sensitive to daily temperature fluctuations (Helmuth, 1998). During the heat of the day, sessile organisms living in an aggregation have been found to have significantly lower temperatures than ones not in an aggregate. While this study focused on the effects of cordgrass on mussel

temperatures in the field, this other factor must also be considered as a contributing factor to mussels in less exposed areas having lower body temperatures.

2. Heart Rate Measurements

The heart rate data collected for less exposed and exposed mussels indicated that exposed mussels had significantly lower heart rates after exposure to temperatures of 36°C in the lab. We expected mussels from exposed areas of the mound to have lower heart rates because they are frequently exposed to much higher temperatures in the field than less exposed mussels which requires them to acclimate in response. In comparison, less exposed mussels, which are thermally protected by cordgrass shading and potentially also mussel clustering, are not as used to experiencing extreme temperatures. This is likely why their heart rates were higher than mussels from exposed areas after a high temperature subjection in the lab.

The necessity of acclimation in the exposed mussels indicates that the temperatures the mussels are exposed to are stressful. Exposed mussels were able to maintain lower heart rates under stressful conditions which may give them an advantage if temperatures spike for a short period of time. Meanwhile, less exposed mussels may be dependent on other factors such as cordgrass shading and large mussel aggregates to buffer them from extreme temperature conditions.

3. RNA Concentrations

While RNA concentrations alone cannot be used to draw any conclusions about which genes and proteins are being expressed, the overall level of gene and protein

expression can be compared using these values. The data showed fairly consistently that mussels subjected to 36°C in the lab had greater RNA concentrations than non-subjected mussels. This pattern is likely because these mussels were subjected to a high level of acute stress which may have led to them responding by expressing various compensatory molecules and proteins. This result suggests that the temperature conditions and length of exposure time used were stressful enough to trigger compensatory pathways and an increase in protein expression in the subjected mussels.

4. Heat Shock Gene Expression

The expression of multiple HSP70 genes and HSC70 were analyzed using RT-qPCR to evaluate the thermal stress conditions of the ribbed mussel in exposed and less exposed areas of the salt marsh after subjection to a high temperature in the lab.

We did not expect HSC70 expression to differ between control and high temperature-subjected mussels because prior studies for another bivalve, *Mytilus galloprovincialis*, indicate that HSC70 levels are not affected by acute heat shock (Franzellitti & Fabbri, 2005). HSC70 is a constitutively expressed isoform which plays a role as a molecular chaperone even in unstressed mussels (Hoffman & Somero, 1995). They noted that expression of HSC70 does not change significantly in response to short-term exposure to thermal stress. As expected, HSC70 expression was unaltered for mussels subjected to an elevated temperature of 36°C in the lab. This was quite evident for mussels collected from the salt marsh in May, irrespective of whether they were from exposed or less exposed areas of a mound. However, basal expression of HSC70 was higher in mussels from less exposed areas than from exposed areas, particularly in May.

In addition, overall expression of HSC70 was lower in July compared to May. This is in contrast to a study on the Pacific oyster (*Crassostrea gigas*), where long-term, seasonal increases in temperature were associated with higher HSC77 and HSC72 expression (Hamdoun et al., 2003). The differences observed may reflect species-specific differences and differences in environmental conditions and the specific genes studied. The low HSC70 values in July may be a result of the prioritization of HSP70 expression due to increased thermal stress. To our knowledge this is the first time that HSC70 expression has been observed for *G. demissa*. Duplicating the study with a larger sample size would provide additional evidence to support the present findings.

Based on past studies, we predicted that mussels from exposed areas of the salt marsh would have higher expression of HSP70. In addition, less exposed mussels were expected to exhibit a stronger response to subjection to 36°C with greater induction of HSP70 compared to exposed mussels. Our data only partially supported our predictions. Contrary to what we had expected, our results indicate that mussels from exposed areas had lower expression of HSP70 genes. A possible explanation for the lower expression of HSP70 in exposed mussels is that at extreme temperatures the biochemical machinery required for a compensatory response to thermal stress may be compromised or less efficient (Piano et al., 2004). Expression levels may have been higher if we waited 24 hours for mussels to recover before collecting the gill tissue or a temperature of 36°C was not high enough to induce high HSP70 expression for mussels from exposed sites. For example, Hamdoun et al. (2003) showed that the level of HSP70 for the pacific oyster *Crassostrea gigas* from high tidal levels, increased significantly after heat shock at 40 and 43°C but not after heat shock at 33 and 37°C. They concluded that this might be

because the threshold temperature for the induction of HSPs may be associated with the level of environmental thermal stress that oysters are exposed to. More studies will reveal whether the threshold temperature required for HSP70 induction is higher for *G. demissa* living in exposed areas of the salt marsh.

As expected, exposure to high temperatures in the lab always led to an increase in HSP70 expression. Mussels from less exposed areas had a more consistent and stronger increase in expression of HSP70. For HSP70-2, HSP70-3, and HSP70-4, this pattern was less evident, but it was consistent for mussels from less exposed areas of mound 7. This agrees with the Hamdoun et al. (2003) study in that the threshold temperature for mussels from the less exposed sites might be lower than for mussels from exposed sites. Mussels from exposed areas and mussels from mound 8 sometimes decreased expression of HSP70-2, HSP70-3, and HSP70-4 after subjection to a high temperature.

An interesting observation in the present study is that we observed higher HSP70 values in July than in May, especially for mussels from less exposed sites. This is in agreement with studies by Hoffman and Somero (1995) and Hamdoun et al. (2003). Both of these studies indicate significantly higher HSP70 expression in the summer months than in winter or spring. These results indicate that HSP70 is induced in ribbed mussels after subjection to heat shock (36°C in the lab) and that less exposed mussels tend to respond more strongly. This is the first time that these observations have been made and supports the prediction that mussels from exposed areas are more acclimated to elevated temperatures than mussels from less exposed areas. Furthermore, the shading provided by cordgrass in less exposed areas of a mound reduces mussel thermal stress and perhaps the necessity of acclimation to high temperatures.

5. Future directions

Future studies should focus on the expression of HSC70 and HSP70, as the inconsistency in detection of HSP70-2, HSP70-3, and HSP70-4 make them difficult to draw conclusions from. These sequences were found in Mediterranean mussels (*Mytilus galloprovincialis*), not in the species analyzed in this study, *Geukensia demissa* (Franzellitti et al., 2020; Kourtidis et al., 2006). The reason for this was a lack of prior research on heat shock gene expression in *G. demissa*. Although the HSP70 family is highly conserved, future work may include sequencing the genome of *G. demissa* to determine the exact sequences for HSP70, HSC70, and other HSPs such as HSP90. In addition to sequencing, increasing sample size would better allow for the observation of a pattern of expression in the different groups.

Direct analysis of HSP70 protein expression could be performed using ELISA. This would provide more direct information about the levels of transcribed HSP70 protein in the tissues of *G. demissa*. A limitation for this method would be the detection sensitivity of HSP70 ELISA kits developed for use in other species. If the proteins are different enough where overlap in sensitivity does not occur, a custom ELISA system would need to be designed or another method of specific protein detection could be utilized. Another potential experimental indicator of temperature acclimation which could be analyzed in the future is acetylcholinesterase activity. Acetylcholinesterase plays an important role in the nervous system, and a few studies have correlated elevated temperature acclimation and increased acetylcholinesterase activity (Pfeifer et al., 2004; Radhakrishnaiah, 1984). Ideally, future experimentation will expand upon both the gene and protein expression and other potential markers of heat stress for *G. demissa*.

Conclusion

The purpose of this study was to explore the thermal stress conditions of the ribbed mussel, *G. demissa*, based on location relative to the mutualistic species, *S. alterniflora*, in the context of changing climate conditions in intertidal zones. Shading provided by *S. alterniflora* had a significant impact on the body temperatures of *G. demissa* during low tide, reducing the body temperatures of mussels in less exposed areas. The temperature differential may force mussels in exposed areas to be more resilient to higher temperatures through acclimation. This study showed that when mussels are not shaded by cordgrass, they acclimate to the increased stress by maintaining lower heart rates at elevated temperatures. The effect of cordgrass shading on heat shock gene expression showed that mussels from exposed areas often have lower levels of HSP70 and HSC70 expression than mussels from less exposed areas. This may be due to a compromised thermal stress compensatory response due to the extreme body temperatures of mussels from exposed areas. Mussels from exposed areas also had weaker induction of HSP70 after subjected to an elevated temperature in the lab, possibly due to prior acclimation to high temperatures in the field.

Acclimated mussels are able to conserve energy under stressful conditions and increase maximum temperature tolerance point by maintaining a lower heart rate and increasing expression of heat shock proteins. Regardless of this acclimation, if temperatures continue to rise the mortality rate of mussels may increase as molecular compensatory mechanisms become compromised. Furthermore, if cordgrass coverage decreases in the intertidal zone, the survivability of many mussels may be reduced. When unacclimated mussels from less exposed areas are no longer shaded by cordgrass, they

may not be able to survive the increase in temperature. This study supports the idea that the presence of cordgrass in the mid-marsh intertidal zone is extremely important in the regulation of thermal stress conditions of *G. demissa*. Continuing to study *G. demissa* and other keystone intertidal species, especially during this period of climate change and increasing environmental degradation, is incredibly important for understanding how the health of intertidal ecosystems can be maintained over time for the good of both people and the planet.

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Appendix

Appendix 1. Satellite photo of mounds with tributary visible.