The effects of menthol treatment on facultatively symbiotic corals

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Abstract

Climate change is a growing concern in today's society and largely impacts the health and longevity of coral reefs. Not only do coral reefs play an important role in creating a healthy ecosystem for marine organisms, but they also possess an economic value to humans. This study was conducted on *Oculina arbuscula* and *Astrangia poculata* to investigate the photosynthetic abilities in temperate and tropical species of corals with the ability to survive in an aposymbiotic state and how their photosynthetic abilities are affected with the loss of symbionts. This was done by using a menthol-ethanol treatment over the course of fifteen days in order to pseudo-bleach the corals and induce an aposymbiotic state. Photosynthetic efficiency was continuously measured using PAM measurements as the corals became increasingly bleached over time. Symbiont counts, photo color analysis, and chlorophyll analysis were also used to measure symbiont loss and bleaching progress over time in menthol and control groups. The results that were achieved display that for the tropical species *Oculina arbuscula*, this menthol methodology is effective at inducing bleaching, though at different rates depending on the genet.

Introduction

Coral reefs protect coastlines from wave and storm damage while providing homes and nurseries for countless species of marine organisms (Cesar et al. 2003). Reefs are also important components of the ecotourism and fisheries industries. Estimates show that coral reefs bring in nearly $30 billion USD in net benefits in goods and services to the global economy (Cesar et al. 2003). However, coral reefs are being lost at unprecedented rates and these losses can have detrimental effects both to ocean and human life, which makes the mission to save these ecosystems imperative.

The majority of reef-building corals, especially those residing in the tropics and subtropics, have a mutualistic relationship with single-celled photosynthetic algae that live inside their gastrodermis (Pantos and Bythell 2010). These symbionts provide the host with organic carbon in exchange for nutrients and a light exposed environment (Baker 2003). This symbiotic relationship is necessary for the coral hosts - without the symbionts, the coral loses its primary source of carbon (Baker 2003). However, under stress, this relationship is compromised and the symbionts lose their efficiency, which

then leads to the coral hosts ejecting the symbionts (Douglas 2003). The process is known as bleaching, as coral hosts lose the color associated with the algae and therefore appear white. Loss of their photosynthetic symbionts will often lead to starvation and death for coral colonies should the stressor not subside (Douglas 2003). Increasing ocean temperatures due to anthropomorphic driven climate change is the major driver causing bleaching in corals (Douglas 2003).

Manually-induced coral bleaching in laboratory experiments has been previously achieved in many tropical coral species (Douglas 2003, Wang et al. 2012). These studies largely aim to simulate heat stress-induced bleaching events in a controlled setting to evaluate the corals' biological response. For example, one study increased temperature to induce coral bleaching and corals became visually paler and lost algal cell density (Desalvo et al. 2008). This study then identified differentially expressed genes between unbleached and bleached corals, and they found that thermal stress and bleaching in the Caribbean coral *Montastraea faveolata* affects carbon homeostasis, cytoskeletal organization, cell death, metabolism, and many more facilitative processes (Desalvo et al. 2008).

Although this study (and many others) successfully achieved bleaching, the response of the coral host to heat stress cannot be disentangled from the bleaching response itself since these responses are often paired in tropical corals. One avenue to address this issue is to experimentally induce bleaching and then maintain bleached corals long-term by offering them heterotrophic carbon. One way to experimentally stimulate coral bleaching in the absence of heat stress is using menthol (Wang et al. 2012). A protocol-oriented study focusing on the physiological and biochemical performances of menthol bleached corals has served as a blueprint for this protocol (Wang et al. 2012). This study found a negative correlation between symbionts in menthol-treated corals and increased incubation time; most corals lost half of their symbionts in 10 to 48 hours depending on the administered menthol concentration (Wang et al. 2012). A follow-up study from the same authors attempted to compare menthol and DCMU (an inhibitor of photosynthesis) bleaching's effect on corals (Wang et al. 2017). It was found that menthol-treated cells had greater photosynthetic suppression than DCMU-treated cells which influenced our choice to use menthol as our bleaching operant (Wang et al, 2017).

However, all studies leveraging menthol bleaching have been performed on tropical coral species. Here, we perform similar methods to Wang et al. (2012), but conduct these experiments on both tropical and temperate species to broaden the application of experimental bleaching and its effects on coral species. Furthermore, the experiment was carried out on facultative coral species. Facultative corals are valuable to study because while they exist with symbionts, they are also able to thrive in an

aposymbiotic state. This is especially valuable in the midst of climate change because corals are very sensitive to changes in their environment, and with rising ocean surface temperatures, the studies on the efficiency of a coral's ability to photosynthesize with decreased symbiont counts would help scientists understand the true impact heat stress could potentially have on aposymbiotic coral species. The purpose of this study is to observe the effectiveness of menthol-induced bleaching in three different coral species (two facultative and one obligate) under ambient conditions. Maintaining corals in aposymbiotic states will allow for a more comprehensive understanding of how corals respond to thermal stress with and without their symbionts. As corals play a critical role in building and supporting reef ecosystems, the importance of disentangling the coral thermal stress response from that of the bleaching process is a growing need as global warming continues to negatively affect coral reefs on a global scale.

Methods

Coral collection

Orbicella faveolata specimens were collected from the East Flower Garden Banks National Marine Sanctuary offshore of Galveston, Texas in August 2018. They were collected from depths ranging from 19.2 m to 24.1 m, transported to Boston University, fragmented, and maintained in aquaria for 14 months at 26 ºC. *Oculina arbuscula* specimens were collected in August 2018 from Radio Island, North Carolina. Colonies were transported to Boston University, fragmented, attached to ceramic disks, and maintained in aquaria for long term acclimation at 25 ºC.

Figure 1. Locations of coral collection, marked by a red star. a) Woods Hole, MA where *Astrangia poculata* colonies were collected in November 2017. b) Flower Garden Banks National Marine Sanctuary, off the coast of Galveston, TX where *Orbicella faveolata* colonies were collected in August 2018. c) Radio Island, NC where the *Oculina arbuscula* colonies were collected in summer 2018.

Astrangia poculata specimens were collected from Woods Hole, Massachusetts in November 2017. They were transported to Boston University, fragmented, and maintained in aquaria at 16 ºC. Pictured in Figure 1 are the locations where the coral colonies were collected from, marked on maps of the regions with a red star.

Experimental design

Three genotypes of each of the three coral species were used - two samples of each genotype were then randomly assigned as control or menthol treatment group for a total of 18 samples. Coral samples were assigned and placed into labeled 473 mL jars, filled with 200mL of artificial seawater (ASW), then placed into temperature-regulated baths similar to the acclimation temperatures for each species. *Astrangia poculata* fragments were placed in an 18 °C bath and *O. faveolata* and *O. arbuscula* were maintained at 24 °C. Seawater for one coral sample from each genotype was then treated with a 20% menthol solution, which was made using 200mL of ethanol and 40mg of menthol following *Wang et. al.,* (2012). 90.7 µL of the 20% menthol solution was then added to 200 mL of ASW in each jar. Daily water changes were performed on all of coral samples with menthol being inoculated post water change.

Photochemical efficiency

To measure photosynthetic efficiency (Fv/Fm), a JUNIOR-PAM was used, a miniature pulse-amplitude modulated fluorometer (PAM). Corals were dark-acclimated for at least 30 minutes before PAM measurements were taken. The fiber optic cable was placed in each treatment of water for the F offset to be adjusted, then Fv/Fm for each coral was measured until 3 values within 0.1 of each other were recorded. Values at 0.750 or above were disregarded as not real (see Thornhill et al. 2008). PAM data was collected in the morning every other day.

The settings used for PAM were saturation pulse width (0.6), saturation light intensity (12), electronic signal damping (2), electronic signal gain (4), and measuring light intensity (2).

Coral Color Change

Photos of corals were taken daily with an iPhone 11 Pro in relatively similar lighting and setting. Prior to daily water changes, corals were placed on top of a CoralWatch coral health chart in order to determine coral color. After the photo was taken, corals were then placed back into their respective jars to undergo water change.

The photos taken were edited in Adobe Photoshop in order to calibrate the ‘true white’ to the same level in each photo. After this was done, these images were run through a MATLAB code that calculated red, green, and blue values from 10 random points on each coral nubbin (procedure from Winters et al. 2009).

Symbiont loss through time

In order to assess symbiont loss, water from the daily water changes were kept for symbiont and chlorophyll a quantification. Initially, the contents of each 200 mL jar were centrifuged down in 50 mL conicals at a speed of 3000 rpm for five minutes. Excess water was serially decanted until all that remained was pelleted symbionts in 3 mL of seawater. After the sixth experimental day, the centrifuge speed was increased to 5000 rpm to better pellet the symbionts. Vials were then stored in a refrigerator at 4ºC until counting was performed.

Pelleted symbionts from each coral were resuspended using a Vortex Genie 2.10 µL was placed on a hemocytometer with a slide cover and symbiont cells on the hemocytometer were counted using a binocular microscope in three replicates. Average symbiont number was then used to extrapolate the predicted number of total symbionts for the full 200 mL of each jar using the following equation:

$$total\ symbionts = \frac{avg.\ symbiont\ count}{10\ \mu L} \times \frac{1000\ \mu L}{1\ mL} \times 200$$

Chlorophyll was measured using a spectrophotometer to analyze and record the chlorophyll concentration of the symbionts that were previously collected from the coral samples after counting subsamples. The samples were placed in a centrifuge for one minute at 5000 rpm and excess water from symbiont samples were poured off. Until enough samples had been amassed, conicals were kept in a freezer at -20ºC. Samples were then filled with 1mL of 90% acetone, which was made using 90mL of acetone and 10mL of deionized water. Acetone-filled samples were then refrigerated for 24 hours in the dark. After refrigeration for 24 hours, 200 µL of each sample was pipetted into a designated well on a specific plate used for spectrophotometry in triplicate, then run through the spectrophotometer.

Statistical analysis

To analyze the changes in red color intensity (a proxy for coral color), the percent change was calculated by subtracting the final red value from the initial red value and dividing the resulting value by the initial red value. This calculation was computed for each coral nubbin and was multiplied by -100 to get percent loss. Control and menthol groups from each species were tested for significant difference using a two sample t-test. The data was visualized by comparing the medians of control and menthol groups for both species in boxplots (Figure 4).

Symbiont loss was analyzed by totalling the symbionts lost for each nubbin over the course of the menthol exposure and then calculating the percent lost each day from that value. A regression was created plotting the percent symbiont loss from each experimental day (Figure 6). Mean total symbiont loss was calculated and displayed in Figure 7. The mean count of each nubbin was added each day and graphed over time.

Results

For both species of coral, it can be seen that the percent change in photosynthetic efficiency decreases from the control group to the menthol-treated group, and that for the most part, all photosynthetic efficiency

decreases, as shown in Figure 2. For most of the *Astrangia poculata* samples, photosynthetic efficiency decreased with time and the addition of menthol treatment. However, colony T sees a much more dramatic decrease in photosynthetic efficiency in the control nubbin compared to the experimental nubbin, though both decrease in efficiency. For the *Oculina arbuscula,* two colonies in the menthol treatment ended with higher photosynthetic efficiency than their control group counterparts, and all but one control nubbin see a decrease in photosyntethic efficiency.

Figure 3 also shows the correlation between photosynthetic efficiency over time with the treatment of menthol. A two sample t-test was conducted and there is no significant difference between the menthol and control groups ($p>0.05$). The rate of photosynthetic efficiency was also plotted against time with each colony represented by whether they were a control group or a menthol-treated group (Figure 5). There is no statistically significant trend in the rate of photosynthetic efficiency with the administering of the menthol treatment.

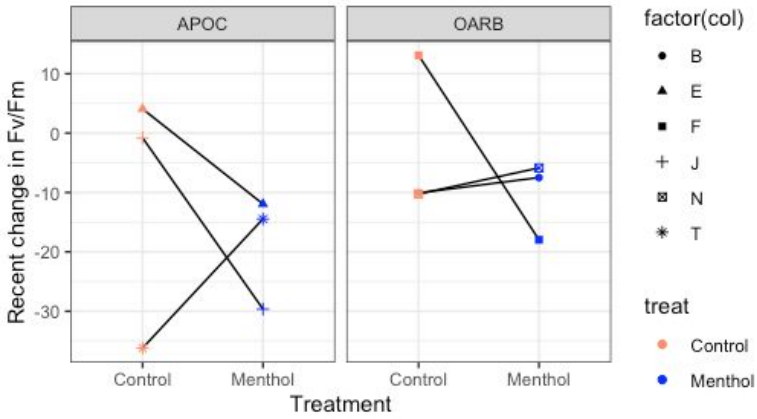

Figure 2. Percent change in photosynthetic efficiency. Determined through R. The y-axis demonstrates the percent change while the x-axis indicates the group. Each symbol represents a different genet. a) Change measured in *Astrangia poculata.* b) Change measured in *Oculina arbuscula*.

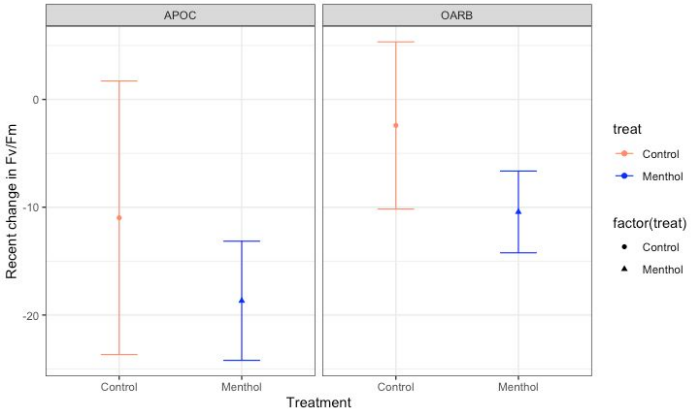

Figure 3. The percent change in photosynthetic efficiency from the beginning to the end of the experiment separated by species and treatment.

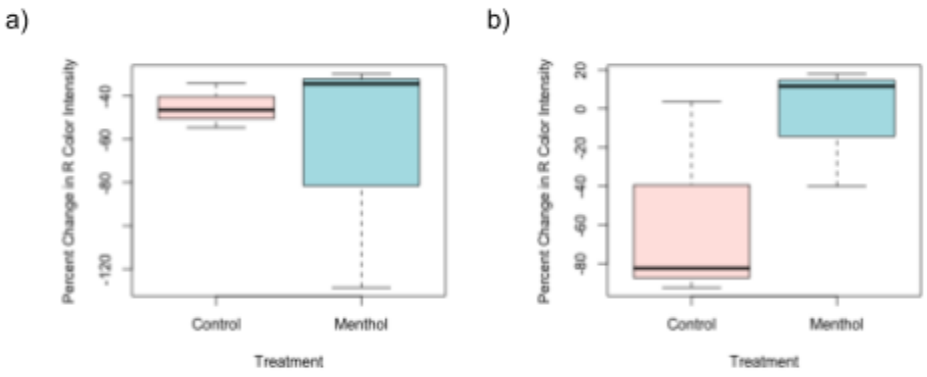

Figure 4. Percent change in red color intensity. a) Change measured in *Oculina arbuscula*. b) Change measured in *Astrangia poculata.*

Percent of R value (red) color intensity is shown by Figure 4, and demonstrates the percent change in red color intensity within both species of corals. The menthol-treated group for *O. arbuscula* has a larger variation in percentage of color change solely in the menthol group, with a relatively small variation in the red color of the control group. *A. poculata* has larger variations in the color of both the control and menthol groups. Neither the red color change in the *O. arbuscula* nor the *A. poculata* are statistically significant using a two sample t-test ($p>0.05$).

The percent of symbionts lost per day in each species for both control and experimental treatments is visualized by Figure 6. There is a clear increase in percent lost per day as the experiment progresses, with the greatest percent of total loss occurring past the eighth experimental day. The total loss of symbionts for each treatment/species over time is shown in Figure 7. The greatest symbiont loss occurred in the *O. arbuscula* experimental group, with the second highest lost being from the *O. arbuscula* control group. There was not much overall symbiont loss observed in either the control or the treated groups of the *A. poculata*.

The percent chlorophyll a concentration loss from the symbionts that occurred over time is shown by the measurement of chlorophyll a, as shown in Figure 8. The chlorophyll a lost over time does not mimic the symbiont loss, as expected, but is quite stochastic.

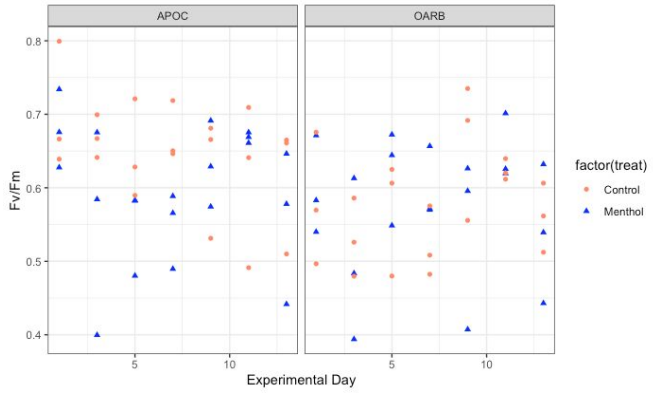

Figure 5. The photosynthetic efficiency of the menthol-treated groups versus the control groups. Each colony is represented for each day, though not specified. The pink circles represent the control groups while the blue triangles represent the menthol-treated groups.

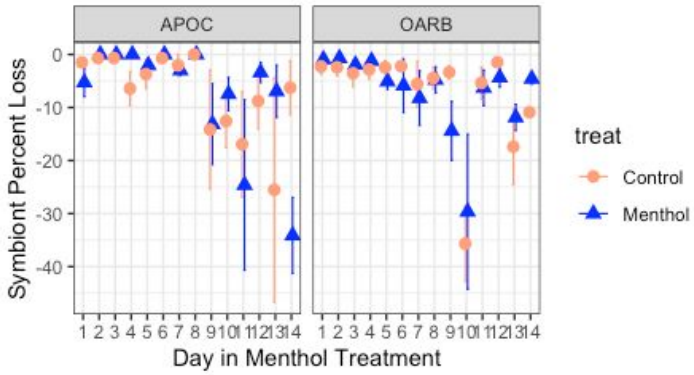

Figure 6. The percent of symbionts lost each experimental day from the total loss for each experimental species over time. The pink circles represent the control groups while the blue triangles represent the menthol-treated groups.

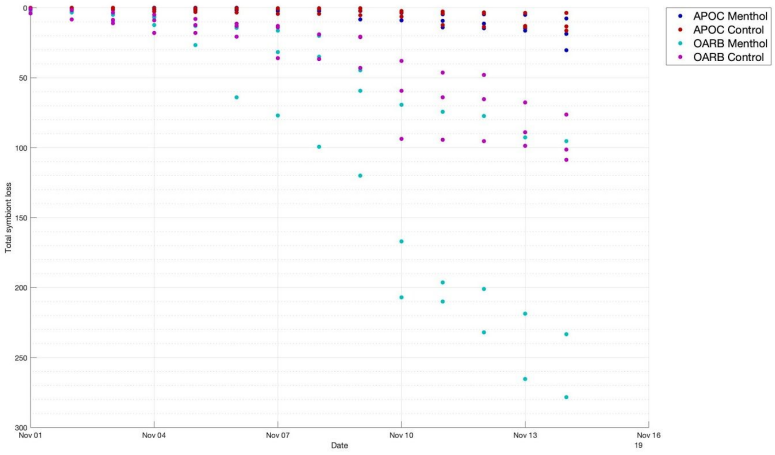

Figure 7. Total loss of symbionts per treatment and species over time. Each treatment and species has 3 points each day for the 3 nubbins of each treatment/species. APOC is used as an abbreviation for *Astrangia poculata* and OARB is used as an abbreviation for *Oculina arbuscula*.

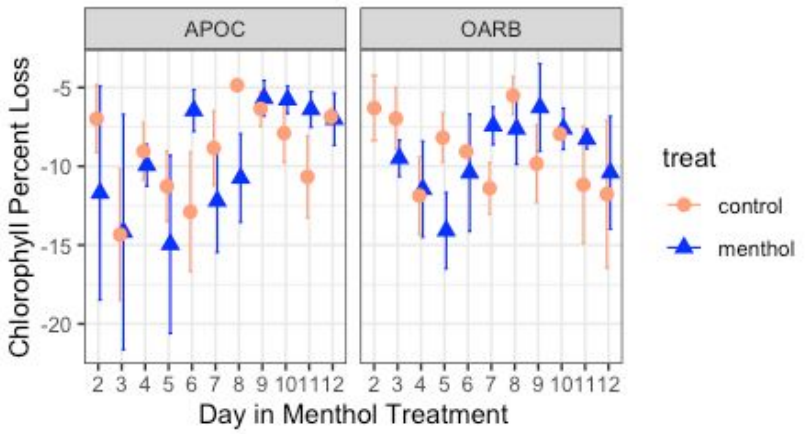

Figure 8. The percent of chlorophyll a lost each experimental day from the total loss for each species over time. The pink circles represents the control nubbins while the blue triangles represents the menthol-treated nubbins.

Discussion

The loss of symbionts can be accounted for in *A. poculata* due to the poor health of the coral samples, which was not necessarily caused by the menthol treatment, but rather due to factors outside of the experimental control. When the nubbins were brushed on experimental day 12, there was some evidence in bleaching in the remaining tissue, but there is no way to tell if this bleaching was from the menthol or from whatever was killing the corals and their stress response to that.

There is no clear trend nor any statistical significance in Figure 5; the photosynthetic efficiency of both the treated and control groups neither increase nor decrease as time goes on. All menthol treated individual colonies of both *A. poculata* and *O. arbuscula* had net decrease in photosynthetic efficiency, thought ⅔ of the control nubbins of both species also saw a decrease in efficiency. The sharp decline of the efficiency in the control colony T of *A. poculata* may be due to tissue death or potentially dead algae, as alage cover could have contributed to the photosynthetic efficiency measured by the JUNIOR-PAM. This algae cover would explain the more normal values of the E and J control colonies.

The expected result of the R value color intensity (Figure 4) was that the control groups for all species would stay consistent while the menthol-treated groups would slowly lose their color over time, consistently with the rate of symbionts being expelled. The change in the percent value in both control and experimental groups is most likely due to the algae growth on the surface of the coral, rather than loss of symbionts. There was heavy algae cover on all six *A. poculata* nubbins by the end of the experiment, which may have contributed to the higher value for color intensity, especially for *A. poculata*. This heavily influenced color analysis and most likely influenced other polyp-based analyses as well.

The exponential increase of the percent of symbionts lost can most likely be attributed to the prolonged exposure to stressful conditions all of the corals were subject to every day when being handled for water changes and removed from water for pictures. For *A. poculata*, it was observed that the coenosarc of both the control and treated corals had developed bacterial mats, which in turn caused the entire nubbin to decay. This happened to all three genets in *A. poculata*, which contributed to the bleaching effect that was not seen. Rather than observing bleached nubbins, what we observed were hypoxic bacterial mats where the coenosarc of the coral had been, which were also white in color but a different shade of white than usually accompanies coral bleaching. It was seen that a control group of *O. Faveolata* also experienced an unexpected number of symbiont loss. This can be attributed to the damage from the airstone, which caused the polyps of the coral to die but not as a result of menthol bleaching.

It is clear from both looking at the number of total symbionts lost as well as the change in red intensity from the photo analysis (Figure 4) that bleaching *O. arbuscula* using menthol is a successful methodology, which holds true to the previous methodology of Wang et al.'s 2012 paper on *Isopora palifera* and *Stylophora pistillata*, two tropical species from the Indo Pacific. This methodology for bleaching using menthol was not proven as successful for *A. poculata* from this experiment, though the lack of success is most likely due to experimental conditions outside the control of the research team rather than a lack of menthol effectiveness. The lack of success may be attributed to the methodology of menthol bleaching not being an effective method to pseudo-bleach temperate species. This may be explained by the hypothesis that temperate corals are more tolerant to environmental stressors due to the wide range in temperature they experience and need to acclimate to throughout the year (Obura 2005).

While the control group of *A. poculata* experienced a higher amount of symbiont loss over the course of the experiment, neither group experienced a noteworthy loss of symbionts. Damage caused by the airstones and the death of coenosarc most likely caused the control groups to expel their symbionts at a much higher rate than was expected, as they were expected to not expel symbionts (Figure 8). Potentially, a less light intensive environment or an aquaria environment with less movement might have allowed these coral nubbins to thrive. More frequent and regular gentle cleaning of the corals and their jars may have also allowed the corals to remain healthier.

Limitations

This experiment was heavily limited by the time frame. With only 14 experimental days, there was not much room for correction if experimental issues came up, nor was there time to continue the experiment to full aposymbiosis. More experimental time would have allowed the *O. arbuscula*

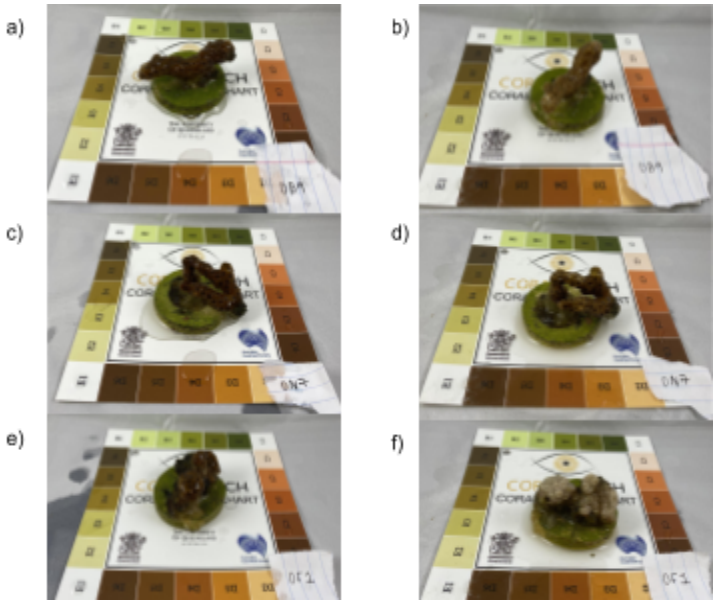

Figure 9. Photos of experimental *Oculina arbuscula* nubbins on days 0 (left column) and 14 (right column) of the experiment. Each row is one nubbin: OB9 (a and b), ON7 (c and d), and OF1 (e and f).

experimental samples to fully bleach and reach an aposymbiotic state, which would have allowed us to better monitor how aposymbiosis affects the rate at which photosynthesis is carried out.

An increased number of replicates of each genet and colony could have given us a better understanding within species if certain genets performed better under menthol-induced stress than others. Within the experimental group for *O. arbuscula*, one of the coral genets bleached much more quickly and evenly than the other 2 genets of the same species. By the end of the experiment, the rate of bleaching was different in every genet; the rate of bleaching was not constant, even within the same species. Figure 9 shows the inconsistencies visually between bleached genets of *O. arbuscula*. Nubbin OF1 began to bleach fairly early into the experiment, and was nearly transparent by the conclusion of the experiment. Nubbin ON7 had bleached the least by day 14, which indicates that there are most likely genet dependent tolerances to stressors like perceived cold/menthol related to coral genetics.

The limited aquaria setup heavily impacted the growth in our experiment; water changes and damage to corals were the primary victims. Our hypothesis explaining the poor health of the coenosarc of *A. poculata* is that the air stones placed in the jars with the coral nubbins disturbed the environment by causing too much movement or by providing too much oxygen. The air stones also caused some damage to a sample of *O. faveolata* when the airstone was left leaning against the nubbin overnight, killing the polyps it touched. Another possibility is that not

enough nutrients were delivered to the coral samples, which caused them to starve. The overall state of the corals were greatly affected due to the stressful conditions the samples were already under. A larger aquaria set up would have allowed us to create and regulate an environment more suited for the nubbins and prevent unintentional damage. *A. poculata* were brushed gently with a toothbrush on November 12, 2019 (experimental day 12) to remove the thick layer of algae growing on the surface of the coral.

Another limitation in this study was the species used. Originally, the subjects of this experiment were *Astrangia poculata, Oculina arbuscula,* and *Orbicella faveolata*. *O. faveolata* was cut from the experiment on November 11, 2019 (day 11) due to the ineffectiveness of the menthol treatment - the treated group was still identical in color to those in the control group, and very few symbionts were found. It was established that the *O. faveolata*, which is an endangered species of coral, would not bleach during the time frame of this experiment, and so were removed from the experimental treatments rather than further attempting to induce bleaching. Experimental *O. faveolata* nubbins were kept in pure artificial seawater for 3 days to detox any menthol from their tissues before being returned to their main aquaria.

Sources of error and bias

During the centrifuging process, while transferring the water with the symbionts into 50mL conical vials, some of the water spilled, which could have caused a lower count in symbionts than was actually in the water. There were possible incidents when samples were poured in the wrong conical when sample IDs were misread.

Due to human error, water quality measurements were not taken in the afternoon a few times. Water quality before and after the missed check time were used to estimate water quality in between.

Subsamples for symbiont counts had clumping of symbiont cells, leading to bias in counts when clumps did or did not make it into the subsample entirely. Vortexing samples to resuspend centrifuged pellets before counting was not always effective in breaking up chunks.

After the 1 mL of 90% acetone was added to the symbiont samples, the lids were not always replaced tightly enough, which led to a few samples having acetone leakage when placed upside down in the dark box to go in the fridge. There was no consistent loss among samples, and it was not noted which samples lost their acetone.

Conclusion

Over the course of the 2.5 weeks this experiment took place in, we discovered that the Wang et al. 2012 methodology of inducing bleaching in corals using menthol translates well to other tropical coral species, thought the rates of bleaching differ. The nubbins of *O. arbuscula* bleached unevenly between genets and at different paces than reported in Wang et al. 2012.

Overall symbiont loss increased exponentially over time, with the vast majority of symbiont loss occurring well into the second week of the experiment. Overall, *O. arbuscula* experienced a higher percentage of symbionts lost over the course of the experiment than *A. poculata* did. This could be a result of *A. poculata* being temperate species, and thus harboring less sensitivity to environmental stressors than tropical species due to being exposed to a larger range in temperature (Dimond and Carrington 2007).

There was no increase in symbiont ejection despite the decline in the health of *A. poculata*, but this could be due to the polyps digesting their symbionts rather than ejecting them, which corals are known to do (Fujise et al. 2014).

Acknowledgements

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Supplemental Figures

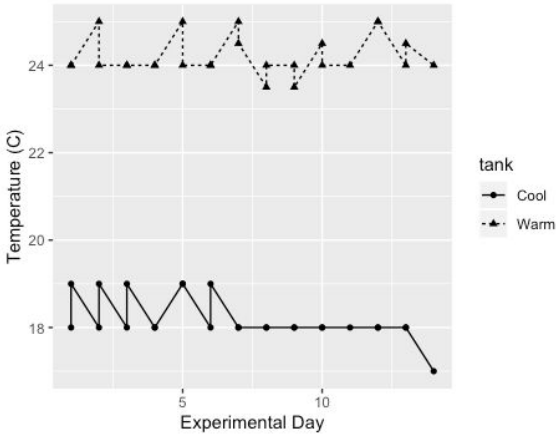

Figure 10. Temperature of stock water placed in the same bath as the coral nubbin jars over time. The temperature of the cool tank is represented by the solid line while the temperature of the tropical tank is represented by the dotted line.