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Abstract

Coral bleaching—the breakdown of the cnidarian–algal symbiosis—is a major cause of reef decline. The sea anemone *Exaiptasia diaphana*, commonly known as Aiptasia, is used as a model to study cnidarian-algal symbiosis in laboratory settings. Aiptasia can live with or without symbionts, which allows scientists to study the host combined and separate from the influence of the symbionts. Scientists are able to trigger the breakdown of the symbiosis using heat or cold stress. Cold stress is more commonly used to render aposymbiotic Aiptasia because it seems to be less harmful to the host than bleaching under heat stress. Is cold really less harmful than heat stress? We compared hot and cold stress responses to different stress regiments: a gradual temperature change, a gradual temperature change followed by a sudden temperature change, and a sudden temperature change from ambient conditions. We explored multiple physiological responses of the anemones to determine their level of stress response. We measured mortality and algal density in the host, as well as carbohydrate in the host and symbiont fractions. We also measured peroxide production in algal cultures exposed to the same treatment regimes. After repeating the experiment twice, we found that anemones had different responses, which emphasizes the necessity for repeated experiments in research conducted with live subjects.

INTRODUCTION

While corals face many environmental and anthropogenic threats, such as ocean acidification and harmful fishing practices, the most serious problem facing coral reefs now is coral bleaching (Lesser 2011; Reaka 2001). Bleaching occurs when the symbiotic dinoflagellates that live inside the cnidarian host tissues are expelled as a stress response, which is detrimental to coral health because the host relies on its symbionts for nutrients (Weis 2008). Past studies have predicted there will be widespread coral loss due to bleaching by 2050 (DeFilippo et al. 2022). Understanding coral bleaching allows us to better mitigate the impacts of climate change on coral and to develop the tools necessary to preserve these key ecosystems.

While there are many hypothesized ways in which symbionts may be expelled from their host organism, there is still uncertainty about the actual mechanisms used by the coral in this stress response (Bieri 2016; Weis 2008). Coral bleaching can be the result of many different environmental stressors, but research demonstrates that mass bleaching events are the result of a change in water temperature—which may be an increase or decrease from ambient temperatures—and an increase in solar radiation (Weis 2008). Past experiments have demonstrated that heat stress is more detrimental to the symbiosis than cold stress over a longer period of time, although cold stress has a more immediate negative impact on coral growth (Roth et al. 2012). One potential form of bleaching mitigation still being researched is the possibility to mix and match symbionts and hosts that have greater thermal tolerance to create more heat-tolerant pairings (Weis 2008).

The anemone species *Exaiptasia diaphana* can be used as a model organism for studies of the coral-algal symbiosis because they are also cnidarians and contain the same host-algal relationship (Bellis & Denver 2017). Using a model organism such as *Exaiptasia* has been important in coral studies because coral is difficult to maintain in a laboratory setting and cannot survive for long periods of time without its symbionts (Baumgarten et al. 2015, Bellis & Denver 2017). *Exaiptasia*, however, can be maintained aposymbiotically for an infinite period of time (Baumgarten et al. 2015). In addition, *Exaiptasia* is able to reproduce both asexually via cloning and sexually, which means scientists can maintain clonal lines in the lab (Baumgarten et al. 2015).

To bleach the anemones in the lab, Aiptasia are placed in a tank of artificial saltwater at 4℃ and then kept in a 4℃ refrigerator for four hours to initiate the bleaching process (Pringle Lab 2018). After that time period, anemones are allowed to recover in room temperature saltwater, which is repeatedly changed to remove symbionts from the water, and then they are kept in complete darkness (Pringle Lab 2018). Anemones are kept in complete darkness and water is changed frequently until anemones are fully bleached (Pringle Lab 2018). If it's necessary to bleach anemones quickly, DCMU artificial saltwater can be used to speed the bleaching process (Pringle Lab 2018). Bleached anemones must be fed at least once per week, as they no longer have symbionts to provide them with nutrients (Pringle Lab 2018). Anemones are checked for symbionts using fluorescence microscopy, which can begin about a month after the initial bleaching (Pringle Lab 2018).

A past experiment analyzing the impact of thermal stress on Aiptasia identified differences in the response of the anemones and symbionts to heat stress, but not to cold stress (Bellis & Denver 2017). In this experiment, anemone genets with an origin in Hawaii, which had symbiont strain B1, exhibited a significantly higher bleaching response than other genets which came from Florida (Bellis & Denver 2017). Symbiont types A4 and B2 exhibited less bleaching than symbiont type B1 (Bellis & Denver 2017). Researchers conducting this experiment also observed shorter tentacles in anemones under thermal stress (Bellis & Denver 2017). Aiptasia's response to heat stress was recorded through the experiment by Bellis and Denver, but the response to cold stress is less well documented.

METHODS

Animal rearing and algal culturing

Anemones were reared in saltwater that was kept at approximately 34 ppt and were fed *Artemia salina* at least once a week. Prior to the start of the first rendition of the experiment, anemones were being fed three times a week, and prior to the start of the second rendition they were being fed one to two times a week. Anemones were kept on a 12 hr lights on and 12 hr lights off schedule.

Algal samples were isolated from a single anemone from Carolina Biological Supply. These algae were cultured in sterile f/2 culture media (Bigelow) at 24–25°C under white incubator LED lights at ~12 PAR.

Temperature challenge experimental design

Three experiments were conducted with three temperature treatments. The three experiments were then repeated with similar conditions to determine whether similar results were obtained. In the second rendition of the experiment, only mortality data was collected. All physiological assays were performed with samples from the first rendition of the experiment.

In the first iteration of the experiment, four genets of *Exaiptasia diaphana* were placed into individual wells on a 6-well plate: CC7, H2, VWB, and Bulk/CBS. In addition, the sixth well was filled with *Symbiodineaceae* of origin CBS clone 6. Bulk/CBS anemones are a mix of possible genets from the Carolina Biological Supply with unknown symbiont status. CBS anemones were expected to be similar to genet CC7, but have been found to be behaviorally different. Symbiont status is unconfirmed for the other genets as well, though CC7 typically has symbiont type SSA01, H2 symbiont type B1, and VWB (anemones from the lab of Virginia Weis) symbiont type B1.

The fifth well was filled with artificial saltwater but was left without organisms in it to provide water of the correct temperature for topping off wells after peroxide measurements were performed. In the second rendition of the experiment, a separate conical tube of artificial salt water was stored in each incubator and the control tank to provide water for topping off tanks, as all six wells of the plates contained anemones. In the second rendition of the experiment, four genets of *Exaiptasia diaphana* were placed into individual wells: CC7, H2, VWB, and Bulk/CBS. In addition, the fifth and sixth wells contained anemones of the genets CC7 and Bulk/CBS respectively that were symbiont depleted.

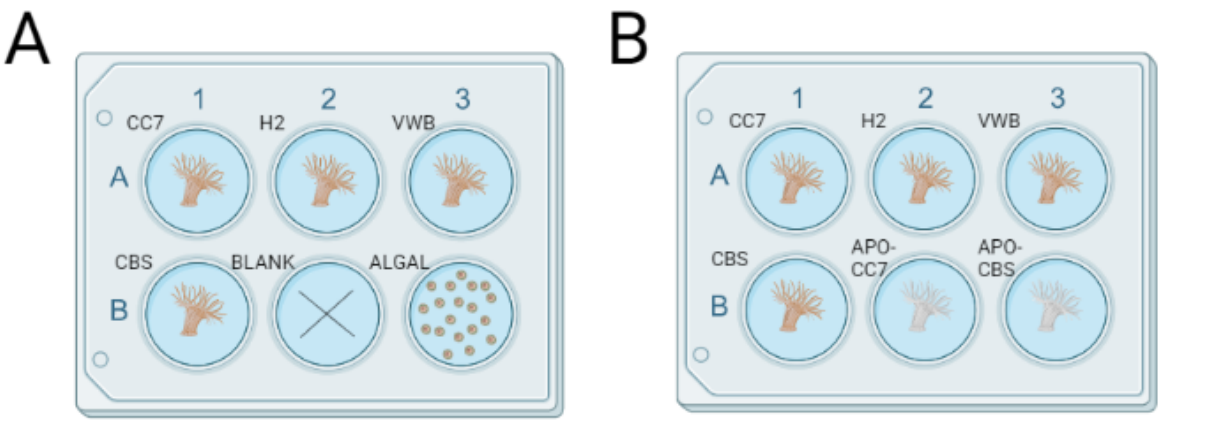

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Figure 1: A) Plate setup for rendition one of the experiment. B) Plate setup for rendition two of the experiment.

10 mL of artificial seawater (ASW) was placed in each well containing anemones, and 5 mL of artificial seawater and 5 mL of algal culture was placed into the algal wells by pipette delivery. Anemones were collected using a razor blade and added to wells via transfer pipette, resulting in 10 mL ± 1 mL ASW in each of the anemone wells. At the time of transfer, the salt water used in the first rendition of the experiment was 32 ppt.

Each anemone in the experiment was given a specific code to provide all necessary information about its experiment, treatment, genet, plate, and symbiont state. It is important to note that while anemones were described as symbiotic or aposymbiotic in the anemone code, aposymbiotic anemones were not fully without symbionts. Therefore, in this paper they will be described as symbiont depleted.

The three experiment codes were RS (ramp/shock), RO (ramp only), and SO (shock only) to describe the experimental conditions for each plate. In the RS experiment, the temperature of the stress treatments was gradually raised or lowered, followed by a period of this stress temperature being held constant. After a recovery period, RS anemones were then exposed to a sudden temperature change and held constant at this stress temperature. RO anemones were

only exposed to the gradual temperature change and temperature hold portion of the experiment. In the shock treatment, which was applied to both RS and SO anemones, the temperature in the hot incubator was brought immediately up to 32℃ and the temperature in the cold incubator was brought immediately down to 16℃ (shocked to 32℃ and shocked to 16℃) without any acclimatization to in-between temperatures between 24℃ and the shock temperatures. Shock temperatures were maintained until experiment breakdown.

Treatment codes were H (hot), C (cold), and N (control/neutral). The genet codes were C (CC7), H (H2), V (VWB), and B (Bulk/CBS). The symbiont state was described as S for symbiotic and A for symbiont depleted.

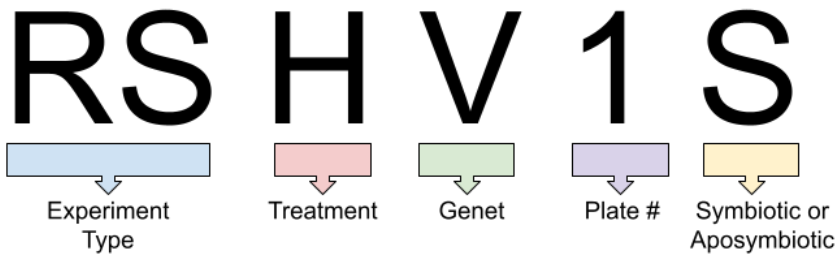

Figure 2: Anemone code breakdown detailing the explanation for each letter and number in the six digit code assigned to each individual anemone.

In the first rendition of the experiment, six plates were placed in the cold treatment, six in the control treatment, and three in the heat treatment because it was hypothesized that the heat treatment anemones would not survive the heat stress. Therefore, there were no anemones in the RS experiment in the first rendition. Three additional plates were placed in each treatment later for the shock treatment. In the second iteration, however, six plates were placed in each treatment, allowing for three plates per treatment per experiment. Three additional plates were later placed in each treatment for the shock experiment.

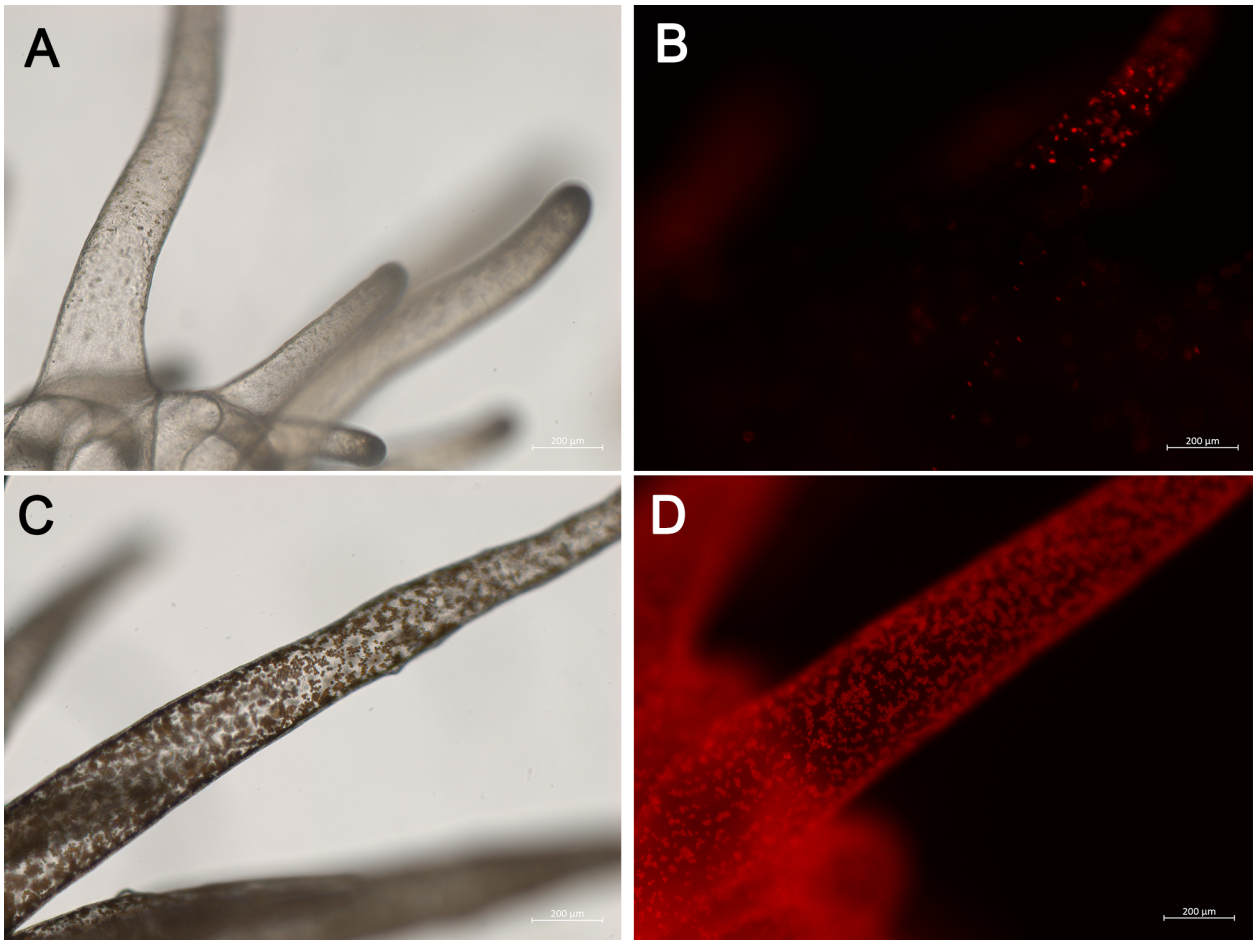

Figure 3: Microscope images of anemones of genet CC7 acquired with the Zeiss Axio Imager M2 Light Microscope and Zeiss Zen Image Acquisition and Processing software. A) Symbiont-depleted CC7 anemone under brightfield. B) Symbiont-depleted CC7 anemone under fluorescent light. C) Symbiotic CC7 anemone under brightfield. D) Symbiotic CC7 anemone under fluorescent light.

For the RO and RS experiment, hot and cold treatments were placed in their respective incubators and acclimated to 24°C for at least 24 hrs. Each day after the acclimation period, the temperature was increased 1°C for the hot treatment and decreased 1°C for the cold treatment until the cold treatment reached 16°C and the hot treatment reached 32°C. In the first rendition of the experiment, the temperature was raised or lowered in the incubators at 12 pm, and the lights were put on a 12 pm - 12 am lights on and 12 am - 12 pm lights off schedule. The lights in the incubator were set to 15% white light on the top and bottom shelf for an intensity of ~12 PAR. In the second rendition of the experiment, the temperature was raised or lowered in the incubators at 10 am everyday, and anemones were put on a 10 am - 10 pm lights on and 10 pm - 10 am lights off light cycle. The top shelf of the incubator was set to 15% white light and the bottom shelf was set to 17% white light for a light intensity of ~12 PAR.

Control anemones were held at 24-25°C in a water bath with a heater set for 24-25°C. Lighting was provided by LED strips to match the incubator light settings at ~12 PAR. A HOBO sensor was placed in the water bath to collect temperature readings every minute, and a thermometer was placed in the control tank for manual temperature readings. A powerhead was placed in the control tank to mix the water and maintain a constant temperature throughout the tank. Anemones were placed on a rack above the water, with the anemone plates just slightly submerged in the water so their temperature was controlled by the water temperature in the control tank. The lights were attached to the lid of the tank and were set to the same lights on/lights off light cycle as the incubators.

Pictures of all plates were taken with a light box and a color card to normalize samples on day 0, and photos were taken of each plate after mortality occurred. For the second experiment in iteration two, photos were also taken of all RS and RO plates on the last day of the ramp experiment, June 10, prior to breakdown and freezing of RO anemone samples. Images of each plate were also taken on the final day of the RS experiment recovery period and on the day of the RS/SO experiment breakdown.

In the first rendition of the experiment, anemone checks were performed three times daily at 10 am, 3 pm, and 9 pm to record temperature and mortality data for all treatments. Due to a minimal amount of change in anemone health between the 3 pm and the 9 pm checks, in the second rendition of the experiment anemones were checked twice daily. In the second iteration, anemone checks were conducted at 9:30 am and 5 pm daily to determine whether mortality had occurred and to record observations on the temperature of the treatments and condition of the anemones.

In the first rendition, RS anemones were held at their final temperature for 10 days and then the temperature was lowered to 24°C and maintained at this temperature for eight days. RO anemones were then removed from their respective incubators or the control tank and flash frozen and stored according to the experiment breakdown procedure listed below. Before the beginning of the shock treatment, anemones for the RS and SO experiments were kept at 25°C for two hours before beginning acclimatization to 24°C for 24 hours. Anemones for the shock treatment in the first rendition were fed *Artemia* the same day they were transferred to their respective incubators or the control tank.

In the second rendition of the experiment, RS anemones were held at their final temperatures for five days and then brought back to a recovery temperature of 24°C. After a recovery period of three days at 24°C, the anemones in the hot incubator were shocked to 32°C and the anemones in the cold incubator were shocked to 16°C. Because all but one anemone in the heat treatment of the first rendition died by day five of the temperature hold at 32°C, in iteration two the temperature hold was maintained for only five days.

In the second iteration, due to a lot of mortality on plates RSH1 and RSC1, those plates were replaced with ROH3 and ROC3 respectively for the recovery and shock portions of the experiment. These anemones were discounted from the mortality data because all anemones

died in consecutive wells, which may indicate a plate malfunction. Therefore, there was a reduced number of anemones in the RO experiment in the second iteration. Because it was suspected that evaporation may also have been a reason for this mortality, a conical tube filled with DI water was placed in each incubator and the control tank for use with topping off wells if there was evaporation in further portions of the experiment. Some plates in the first iteration were also switched, but these changes did not impact the number of anemones in each experiment or treatment.

With the second rendition of the RO experiment, anemones were removed from the incubators and control tank after a recovery period of 1.5 hours at 24°C. These anemones were flash frozen and stored according to the experiment breakdown procedure listed below. For the SO experiment in the second rendition, anemones were acclimated to 24°C for three days, being placed in the incubators while the RS experiment anemones were in their recovery phase. Then, after three days at 24℃, anemones in the hot incubator were shocked to 32℃ and anemones in the cold incubator were shocked to 16℃.

For the second iteration, salinity of the artificial salt water was 33 ppt at time of transfer to RS and RO plates. RS and RO anemones were fed *Artemia* the day before transfer to the well plates. SO anemones were fed around 11:30 am on June 9 and added to their respective plates on June 9 around 4:00 pm. These plates were added to the incubators to acclimate to 24°C on June 10 around 1:10 pm and shock treatment began at 10:00 am on June 13. The salinity of the water was 36.4 ppt when SO anemones were added to the plates.

For both iterations, experiment breakdowns occurred after the final day of the shock treatment for the RS and SO anemones and after a recovery period for the RO anemones. Anemones were removed from their respective wells one at a time and transferred to a small piece of aluminum foil with a label listing the anemone's experimental code. Excess water was removed from the aluminum foil using a cotton swab, and then anemones were flash frozen by placing the aluminum foil on dry ice. Samples were stored in a -80°C freezer. When anemones were removed from the -80°C freezer, they were combined with other anemone samples of like genet and treatment and then weighed. Anemone samples were then transferred to conical tubes labeled by genet and treatment.

Tissue extractions were performed with pooled anemone samples to separate host tissue from symbionts. A 500 µL aliquot of extraction buffer (100 mM Tris, 100 mM NaCl, 10 mM EDTA) was pipetted into each conical tube, and samples were then homogenized with a mortar and pestle. The samples were then centrifuged at 8000 RCF for two minutes at 23°C to condense the algal fraction into a pellet at the bottom of the tube. Supernatant was transferred to a separate conical tube labeled for the host sample and stored in a -20°C freezer. The algal sample was then washed with 200 µL of buffer and vortexed at 3000 speed to resuspend the algal pellet. The sample was again centrifuged at 8000 rcf for two minutes at 23°C. Supernatant was removed and the washing procedure was repeated. After dumping supernatant a second time, the algal pellet was resuspended in 200 µL of buffer and resuspended with vortex at 3000 speed and stored in a -20°C freezer.

Peroxide measurements

Algal cells produce peroxide when they are stressed (Weis 2008), so measurements of peroxide in the wells was used as a marker to measure that stress response. Peroxide measurements were made using a plate reader (Molecular Devices Spectramax iD5) and a 3% w/w peroxide standard two-fold dilution series. Sample absorbance was read at 240 nm. In the first iteration of the experiment, peroxide measurements were made approximately five hours after the temperature change in the incubators, so anemones and algae had five hours to acclimate to the new temperature before the water was tested for peroxide concentration. In the second iteration of the experiment, water from the 6-well plates was tested approximately four hours after the temperature change occurred in the incubators. Peroxide levels were tested once per week. However, the use of peroxide measurements was discontinued in the second iteration of the experiment after week two because there were no non-zero measurements recorded between the first and second weeks of the experiment.

Peroxide measurements were made from 125 µL triplicates of seawater removed from each well in the 6-well plate and pipetted into assigned wells on the 96-well plate. Seawater was back-pipetted in each well of the 6-well plate prior to the removal of water samples to ensure accurate concentration readings. This volume of seawater was replaced using ASW at the same temperature as the water currently in the 6-well plate to avoid shocking anemones by adding water of a different temperature.

For algal samples or water samples with a lot of debris, 500 µL of water was removed from the well in the 6-well plate after back-pipetting and dispensed into a microcentrifuge tube. This tube was centrifuged at 16000 RCF for five minutes to make algal cells or debris into a pellet at the base of the tube, allowing for 125 µL triplicates of seawater to be aliquoted without algae or debris. When this method was used for the algal samples, water was not replaced with ASW to avoid diluting the algal cultures.

Carbohydrate measurements

Adapted from the protocol by (Dubois et al 1956), carbohydrate measurements were made using a plate reader (Molecular Devices Spectramax iD5) and a 2 mg/ml D-glucose standard two-fold dilution series. 50 µl of each host and algal sample was pipetted in triplicate into the 96-well plate according to the sample map created. Host and algal cells were separated previously via tissue extraction. A 5% phenol solution and concentrated sulfuric acid were added to each well of the 96-well plate. The plate was incubated at 90°C for five minutes and left to cool for an additional five minutes at room temperature before being loaded into the plate reader. Because the symbionts produce carbohydrates, some of which is in turn consumed by the host, this procedure can be used to determine whether symbionts or hosts are taking in more carbohydrates under stress treatments than when the symbiotic relationship is maintained at ambient temperatures.

Algal quantification

Algal counts were performed with a hemocytometer under a microscope. Algal samples were prepared using tissue extraction to separate host and algal cells, except for the wells with just algae in them for which this was not necessary. When algal samples were very densely packed with cells, dilutions were performed to make counting easier, and these dilution factors were recorded so that all samples could be normalized.

Statistical analysis

All statistical analyses were performed in R version 4.1.1 (2021-08-10).

RESULTS

Peroxide concentrations in algal cultures increased under heat stress

We reliably quantified differences in peroxide concentrations in culture media for the algal cultures. However, we did not observe non-zero peroxide measurements in the well water from wells containing anemones. Peroxide levels in algal cultures were significantly higher in the heat treatment than control ($p = 0.004$) (Figure 4). In addition, peroxide levels were significantly higher on 3/18 (16 days of stress exposure) than on 3/11 in the algal cultures ($p = 0.046$) (Figure 4).

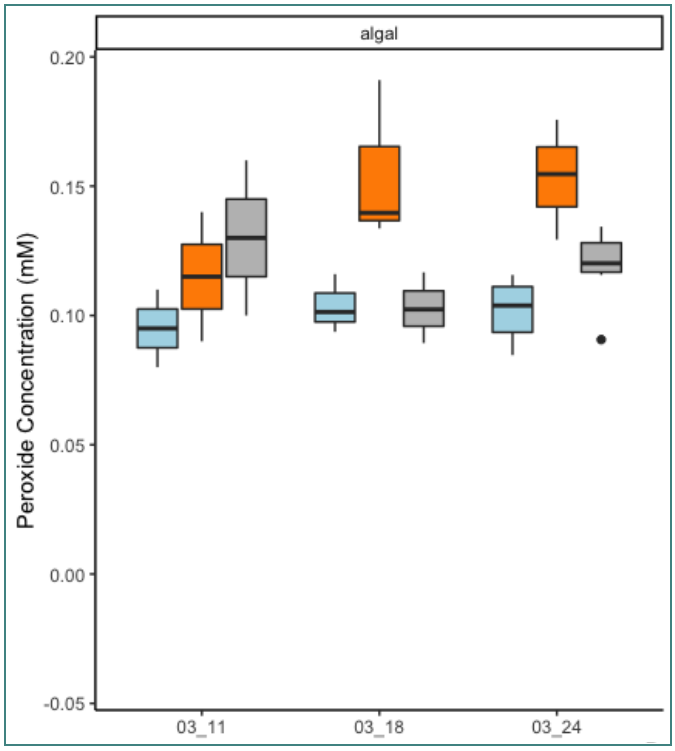

Figure 4: Peroxide concentration (mM) in the well water for algal samples recorded for three time periods: the 11th, 18th, and 24th of March 2022, which represent days 9, 16, and 22 of the experiment respectively.

Symbiont densities were lower for anemones under cold stress

Cold treatment was associated with significantly lower *in hospite* algal density relative to controls across all three experiment types (ramp only, ramp then shock, shock only, $p < 0.001$). Extensive mortality under heat stress precludes our ability to measure symbiont densities in the hot treatment.

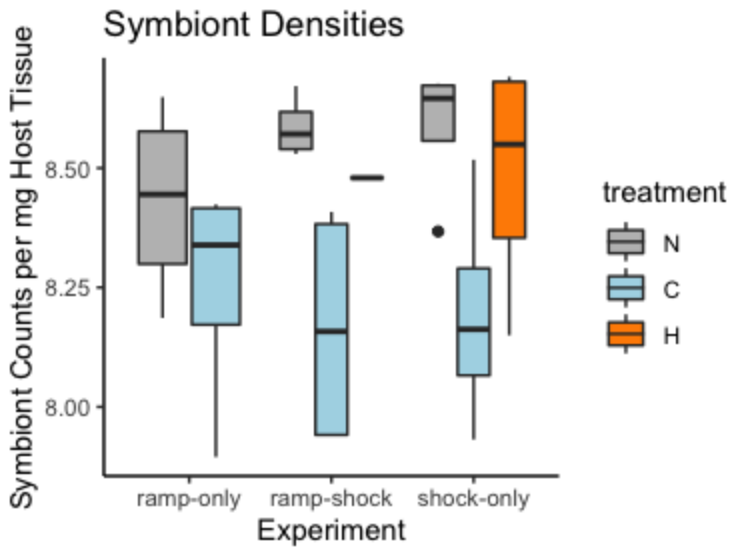

Figure 5: Symbiont densities normalized to the weight of anemone host samples to determine differences in symbiont densities between experiments and treatments ($p < 0.001$ for cold treatment)

Total carbohydrate content was lower under cold stress

Carbohydrate content was lower for dinoflagellates that were isolated from anemones in the cold treatments. SO hot treatment dinoflagellate samples have similar carbohydrate content to control samples for the same experiment.

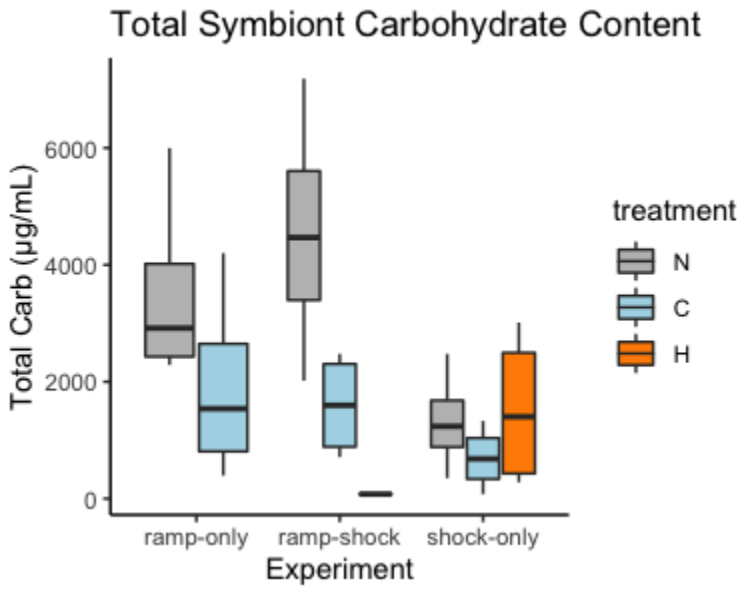

Figure 6: Total carbohydrate concentration for symbiont samples ($p = 0.010$ for cold treatment, $p = 0.142$ for heat treatment).

When normalized to the number of algal cells, carbohydrate content appears to be higher in cold treatments than in control treatments because symbionts were beginning to bleach due to stress. The SO hot treatment symbionts also have a higher carbohydrate content.

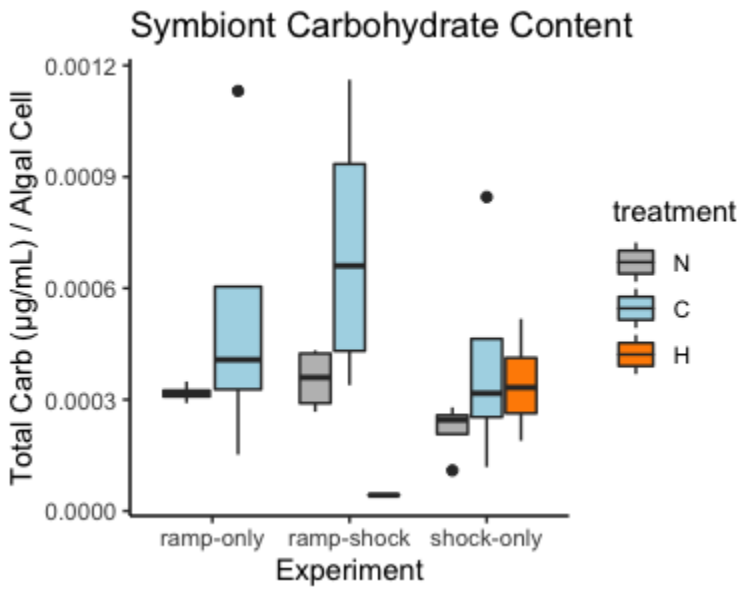

Figure 7: Total carbohydrate concentration for symbiont samples, normalized to the number of symbionts in each sample ($p = 0.018$ for cold treatment, $p = 0.910$ for heat treatment).

When normalized to the weight of anemone samples, host carbohydrate content is lower in the SO cold treatment compared to the control samples of the same experiment, but host carbohydrate content is higher than the control values in the cold treatments for both the RO and RS experiments. Host carbohydrate content in SO cold treatment anemones therefore follows the pattern exhibited in data for symbiont densities normalized to host weight (Figure 5). Carbohydrate content is higher in the hot treatment for the SO experiment compared to the control anemones.

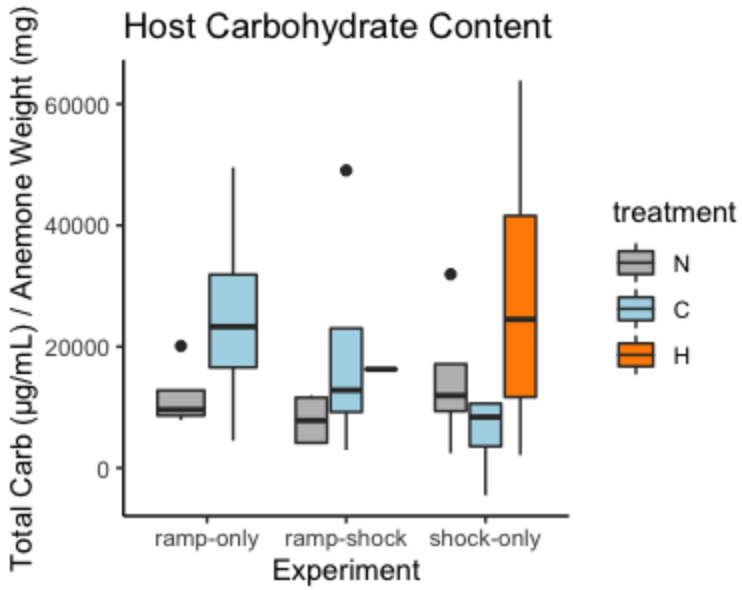

Figure 8: The total amount of carbohydrate content in the host samples standardized by the weight of the anemone sample (p = 0.402 for cold treatment, p = 0.066 for heat treatment).

Because carbohydrate content was tested only for the anemone samples from the first iteration of the experiment, there is no data to demonstrate whether there is a difference in the carbohydrate content of anemones with symbionts compared to anemones that have a depleted number of symbionts. However, the limited number of deaths of symbiont depleted anemones may suggest that a reduced number of symbionts may be beneficial for anemone survival during exposure to stress.

Limited anemone mortality occurred in stress treatments

In the first rendition of the three experiments, there was a large difference between the mortality data from the RO experiment and the SO experiment with the hot treatment. In this first iteration, only one anemone in the RO hot treatment survived, while all the anemones in the SO hot treatment survived, which was different than what was expected. There were no RO or SO cold treatment deaths, but there were two RS cold treatment deaths, which may demonstrate that repeated exposure to stress conditions may have an impact on anemone mortality. Although there were no RS hot treatment anemones in this iteration of the experiment, the one surviving anemone from the RO treatment was carried into the shock treatment, which is why it appears as an RS anemone in carbohydrate data.

Table 1: Mortality data for the first iteration of the hot and cold experiment organized by treatment type. NA is listed for the RS hot treatment because there were no anemones in the hot treatment exposed to the ramp/shock experiment in the first iteration.

	RO			RS			SO		
Treatment	Alive	Dead	Total	Alive	Dead	Total	Alive	Dead	Total
C	12	0	12	10	2	12	12	0	12
H	1	11	12	NA	NA	NA	12	0	12
N	12	0	12	12	0	12	12	0	12

In this first rendition of the three experiments, one VWB anemone survived the RO hot treatment, and both anemone mortalities in the RS cold treatment were of the genet CC7. This may demonstrate that VWB has more heat tolerance and CC7 has less cold tolerance than other genets, but due to the small sample size this cannot be declared from this data alone. More repetitions of the experiment are necessary to confirm whether this will be a trend.

In the second rendition of the three experiments, there was one death declared in the RO cold treatment and none declared for the heat treatment. However, one plate of heat treatment anemones and half a plate of cold treatment anemones in the RO experiment were removed due to suspected plate failure, as all deaths occurred on the same plate in wells that were adjacent to each other. It is possible there was an unknown crack in the plate that led to evaporation in that plate or that proximity to a certain part of the incubator led to greater exposure to the heat or cold, as both plates were in the top left of the incubator. There was one less anemone in the SO control due to a death as a result of a low water level in one well.

In the second iteration, there were more deaths observed in the heat treatment than there were in the cold treatment, with 5 RS heat treatment deaths and 3 SO heat treatment deaths, compared to 3 RS cold treatment deaths and no SO cold treatment deaths. However, it is suspected that some of the heat treatment deaths may have been the result of evaporation rather than heat stress, as hot treatment plates' wells tended to have more variable water level, even though it was topped off when it was observed that the wells appeared to be lower.

Table 2: Mortality data for the second iteration of the hot and cold experiment organized by treatment type. Lines with less than 18 anemones in the total are the result of anemone samples being removed due to plate or well malfunctions.

	RO			RS			SO		
Treatment	Alive	Dead	Total	Alive	Dead	Total	Alive	Dead	Total
C	14	1	15	15	3	18	18	0	18
H	12	0	12	13	5	18	15	3	18
N	18	0	18	18	0	18	17	0	17

The one cold death observed in the RO treatment was of the genet H2. In the RS cold treatment, the three observed deaths were of the genets CC7, H2, and VWB, and the cold deaths observed were only seen in symbiotic anemones. Symbiont depleted anemones had no observed cold mortality. RS hot treatment deaths were seen in CC7, both symbiotic and symbiont-depleted, and in VWB, with three VWB deaths and one each for CC7 symbiotic and

symbiont-depleted. This is in contrast to the first iteration of the experiment when the only RO anemone to survive was VWB. In the SO heat treatment, there were three anemone deaths, one each from each of the symbiotic anemones Bulk/CBS, CC7, and H2. There were no observed SO cold deaths.

Overall, there was very little anemone death in the second rendition of the three experiments for both hot and cold treatments, a difference from the first rendition when the majority of anemones died in the RO heat treatment. In addition, there were SO heat treatment deaths observed in the second rendition of the experiments, which did not occur in the first rendition.

There were three RS cold treatment deaths in the second rendition compared to two RS cold treatment anemone deaths in the first rendition of the experiments, which may demonstrate a greater potential for cold treatment deaths with multiple exposures to stress, though this will need to be tested further. There were no SO cold treatment deaths in either rendition of the experiments, but there was one RO cold treatment death in the second rendition of the experiments.

DISCUSSION

Anemone temperature stress response

The physiological responses tell us that cold and hot stress can impact both the anemone host and the symbionts, though the two organisms may demonstrate stress in different ways. In addition, gradual and sudden temperature changes for both temperature treatments cause stress responses. From these two renditions of the experiment, it is difficult to determine whether gradual or sudden temperature changes are more stressful, though it was hypothesized that sudden temperature changes would result in more severe stress responses. If more renditions of the experiment were conducted, it is possible that this hypothesis would be supported, but the use of live organisms as test subjects creates some degree of uncertainty.

While the sample size in this experiment was too small to make determinations regarding differences between genets, it is possible that different genets of *Exaiptasia diaphana* would have different adaptations depending on where the organisms are found regionally and what temperature conditions they may typically be exposed to. Therefore, future experiments with larger sample sizes by genet may see differences in the stress responses among the genets.

The sample size in this experiment was also too small to make concrete determinations regarding differences between symbiotic states. However, there is a possibility that having symbionts results in a cost to the organism, and therefore the expulsion of symbionts or living in a symbiont-depleted state allows the host organism to focus more energy on its own survival.

Future considerations

During the course of the experiment, it was noted that the water level of wells in the hot treatment did tend to fluctuate, despite best efforts to maintain a consistent water level and salinity. In future versions of this experiment, consistent water level and salinity monitoring, especially of anemones in the hot treatment, would be beneficial for preventing anemone mortality as a result of salinity changes rather than temperature stress.

The importance of repeated experiments

When working with live subjects, there is potential for variation in the responses of the organisms studied. Repeated experiments are necessary to ensure that results are accurate and not an isolated occurrence.

An improved metric for anemone mortality

This experiment used complete bodily disintegration as a metric for marking anemone death, but an earlier metric for declaring anemone death would be beneficial for narrowing down time of death for anemones. The use of a microscope to observe anemones helped with declaring anemone death when there was uncertainty. In one instance, it was observed that the anemone still retained some anemone shape but that symbionts had been expelled from the tissue into the surrounding water and that anemone cnidocytes had been expelled as well. There was also no anemone movement documented for this sample, further confirming that the organism was dead. In another instance, an anemone that looked to not have any shape was put under a microscope and movement was observed despite the anemone appearing to no longer have anemone features to the naked eye. Therefore, it is believed that complete bodily disintegration may provide inaccurate accounts of anemone mortality, and that the use of a microscope would provide a clearer definition of anemone death.

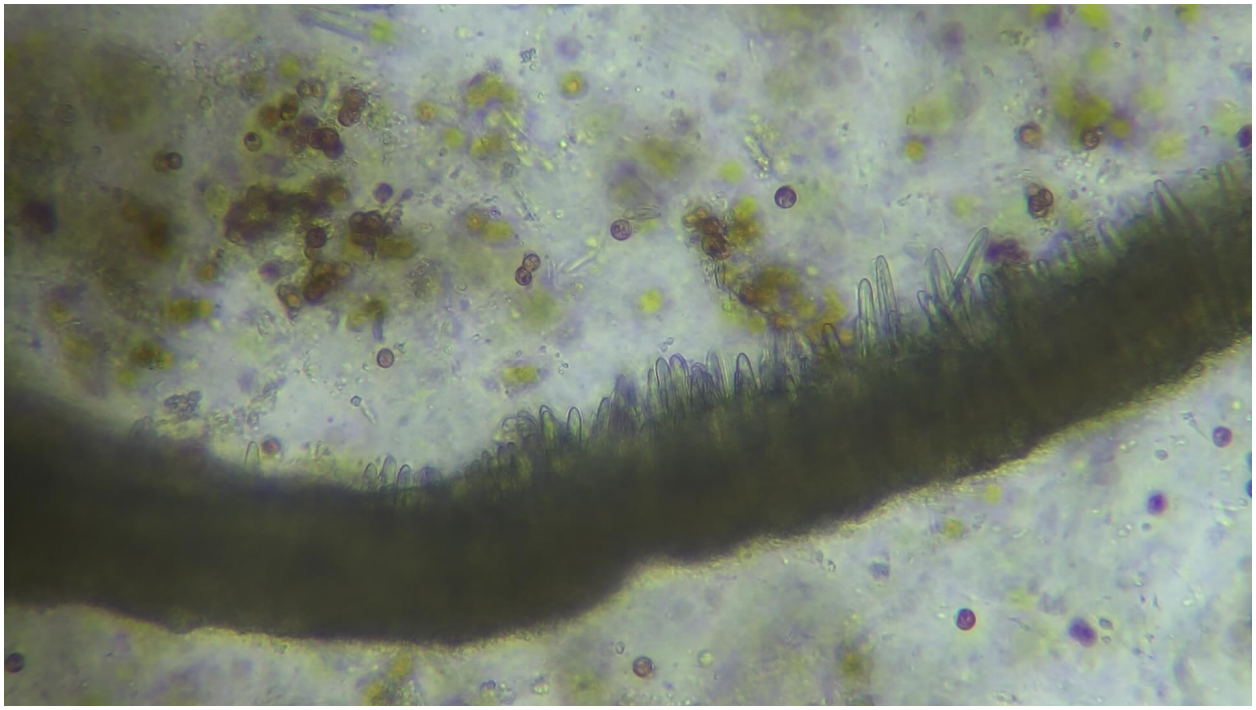

Figure 9: Image of anemone tentacle with expelled cnidocytes and symbionts.

CONCLUSION

From this study, it seems that the cold and heat stress temperatures used may not be as stressful as we thought for these organisms, which may be a good thing in the context of the changing climate, as it means these anemones may be better adapted to face changing temperatures. Further research should be done to determine if this tolerance will apply to other cnidarian species, such as coral. In addition, this study emphasizes the importance of repeating experiments in order to fully understand the impact of temperature conditions on cnidarian species, as different organisms even of the same clonal line may respond differently to temperature stress.

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