# The Independent and Interactive Effects of Nitrogen Enrichment and Heat Stress in the Model Sea Anemone *Aiptasia*

JK Da-Anoy, Alexandra Geisser, Kian Thompson
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Department of Biology, Boston University, Boston, MA 02215, USA

**ABSTRACT:** Reef-building corals and their symbiotic algae face a multitude of unprecedented environmental stressors due to anthropogenic global change. However, how the coral host or the algal symbiont affects the holobiont's stress tolerance is unknown. This study aims to understand how multiple stressors, specifically elevated temperature and nitrogen enrichment, impact *Exaptasia pallida*, a model organism for studying reef building corals and algal symbiont dynamics. Aiptasia were exposed to independent stressors, high temperature (35°C), and nutrient enrichment (5μM nitrate). These two stressors were also combined to investigate whether any synergistic effects would result. Phenotypic changes and photosynthetic efficiency were measured to determine holobiont health. Results suggest that the dynamics between symbiont and host may allow the holobiont to be more resilient to short-term stressors than initially hypothesized.

KEYWORDS: *symbiosis*, *nitrate, thermal stress*, *cnidarian*

## INTRODUCTION

Coral reefs are home to some of the most biodiverse and productive ecosystems on the planet. These important habitats are being affected by anthropogenic global change and are declining in population. Corals are pillars of many marine ecosystems, so their deterioration would further detriment many tropical, coastal marine environments. Coral reefs face many stressors, but these stressors' independent and combined significance is still under-investigated (Muthukrishnan et al., 2014). More so, the effect of symbiotic algae on coral reef resilience or mortality to these stressors is even less explored. Two of the major stressors that corals face are eutrophication/ nutrient stress and ocean temperature increase, which may cause coral bleaching. Exploring the effects of algal symbionts is not feasible in most obligate symbiosis reef-building corals, so model organisms who exhibit facultative symbiosis are used.

Coral bleaching is a phenomenon that has become alarmingly frequent and more severe in recent years, mainly due to increased ocean temperatures. Due to increased temperatures, corals may expel their algal symbionts and become bleached. Increased ocean temperatures may also negatively impact coral size and range, and fecundity (Riegl et al. 2018).

Nutrient loading, or eutrophication, has also been shown to cause the degradation of corals in the form of bleaching and disease prevalence (Vega Thurber et al., 2013). Eutrophication can occur naturally, but anthropogenic activities have increased these events dramatically in frequency and magnitude (Rees et al., 2006). The most common nutrients to cause eutrophication events are nitrogen and phosphorus. Nitrogen is especially of interest in environmental studies because it is often the growth limiting nutrient in oceanic primary productivity

(Billen et al., 2013). Eutrophication events are projected to worsen alongside ocean warming due to climate change and anthropogenic activity (Sinha et al., 2017). The combined effects of increasing sea surface temperatures and nutrient enrichment (Halpern et al. 2008) are harming reef-building corals worldwide. Particularly, this experiment is interested in the dynamics between algal symbiont primary productivity and host, with nitrogen loading.

Here, we will use the coral model *Aiptasia* (sensu *Exaiptasia pallida,* Grajales and Rodríguez, 2014) to investigate the effects of nutrient enrichment and elevated temperature on cnidarian-microbial interactions and holobiont physiology. The advantages of using *Aiptasia* as a system are that (1) they can be easily cultured in the lab, unlike corals, (2) have a faster regeneration rate to produce clones, and (3) *Aiptasia* are facultatively symbiotic anemones that can survive with or without the presence of their photosynthetic dinoflagellate algae in the Family Symbiodiniaceae. The symbiont-host relationship dynamics play in the interactive role of nutrient enrichment with heat stress remains largely unexplored. It is hypothesized that the combination of heat and nutrient stressors will lower photosynthetic efficiency (Fv/fm) and physiological performance; nutrients alone will increase Fv/fm and physiological performance; the elevated temperature will decrease Fv/fm and physiological performance.

## MATERIALS AND METHODS

### *Aiptasia preparation and husbandry*

Clonal populations of CC7 anemones (Sunagawa et al., 2009) were reared in six-well cell culture plates filled with 10 mL seawater at 24 °C under white light on a 12:12 h light: dark cycle (daytime of 06.00–18.00). Symbiotic CC7 anemones were menthol-induced bleached (Matthews et al., 2016) to generate aposymbiotic individuals. After bleaching, aposymbiotic anemones were maintained in the dark to ensure there was no re-establishment of symbiosis. Anemones were fed with freshly hatched Artemia brine shrimps twice per week and were cleaned with a cotton Q-tip 2–3 times per week.

Anemones were then transferred to a standard petri dish containing artificial seawater (ASW) and dissected half longitudinally with a scalpel to generate clones. Animals were transferred to new Petri plates with ASW and were allowed to heal and acclimate for 15 days before the experiment. Anemone clones were tagged to keep track of their origin.

### *Thermal and nutrient stress mesocosm experiment*

Each *Aiptsia* (32 symbiotic, 32 aposymbiotic) were transferred to a scintillation vial filled with 10 mL artificial seawater (ASW) and were allowed to acclimate to the experimental tanks for 24h at ambient temperature (27C ± 1, mean ± SD) before initiating the temperature ramping. Each symbiotic or aposymbiotic aiptasia clone was assigned to either control or heat-treated tanks. Thermal stress experiments were conducted in 60 L tanks filled with deionized water. Two independent replicate tanks were used for each temperature treatment (27°C: control, 34 °C: heat-ramped), and the temperature in individual experimental tanks was adjusted using submersible thermostat heaters. 100 L h−1 pumps provided additional mixing within the tanks. All setups received illumination under low photosynthetic photon flux density of ∼40 μmol m−2 s−1 on a 12:12 light-dark

cycle (**daytime of 19.00–07.00**). The light levels are similar to those reported by other studies working with Aiptasia to support optimal growth of the animals' optimal growth and because all symbiont strains used here perform well under this irradiance (Cui et al., 2019). Light and temperature in each experimental tank were monitored using submersible loggers (Onset HOBO). The elevated-temperature treatment tank was set to increase by ~1°C every day for a total of 7 days until it reached 34°C. Nutrient enrichment was done on the fourth day by replacing the ASW with 5 µM $NH_4NO_3$. This resulted in 4 experimental treatments:

| | |
|---|---|
| control temperature + no enrichment | (CT+NE) |
| control temperature + enrichment | (CT+E) |
| elevated temperature + no enrichment | (ET+NE) |
| elevated temperature + nutrient enrichment | (ET+E) |

Experimental temperature treatments and nutrient concentrations were selected based on the conditions in the ocean−estuary interface in Narragansett Bay (Oviatt et al., 2017). After nine days, anemones were immediately flash-frozen in liquid nitrogen and stored at −80°C until further processing.

### *Photosynthetic efficiency of Aiptasia algal symbiont*

The dark-adapted photochemical efficiency (Fv/Fm) of aiptasias was performed daily using non-invasive Walz Junior pulse-amplitude modulated (PAM) fluorometry (Ralph et al., 2015). PAM measurements were taken from the oral disc (n=4). A negative control (seawater or enriched seawater) was used.

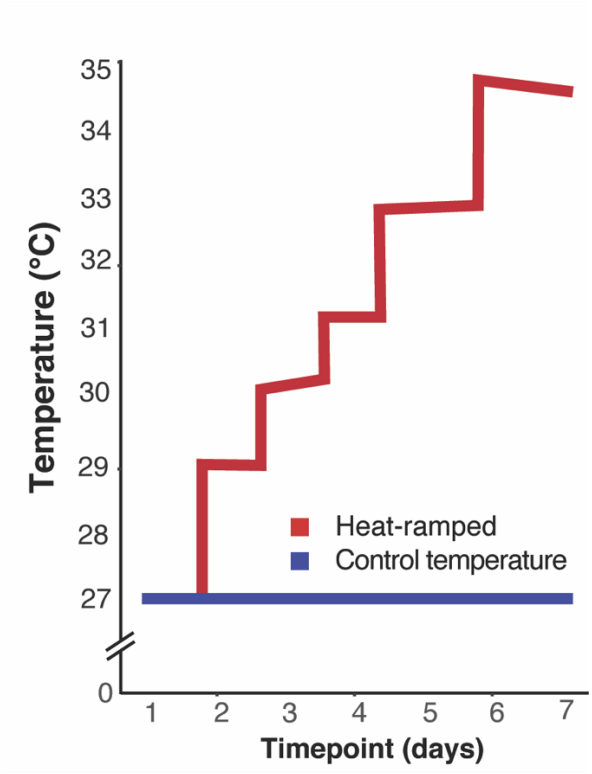



**Figure 1.** Temperature profiles of the experimental tanks. The temperature of each experimental tank was monitored using submersible loggers (Onset HOBO) logged every 30 mins for seven days. The Control tank (blue line) was maintained at 27°C, while the elevated-temperature treatment tank (red line) was set to increase by ~1-2°C/ day for a total of 7 days until it reached 34°C.

### *Feeding Behavior*

On experimental days 2, 4, and 6, Aiptasia were each fed about 20-30 freshly hatched Artemia brine shrimp. Observations were noted before and 30 minutes after feedings. The behavior of the Aiptasia's tentacles was indexed (henceforth ABI for Aiptasia

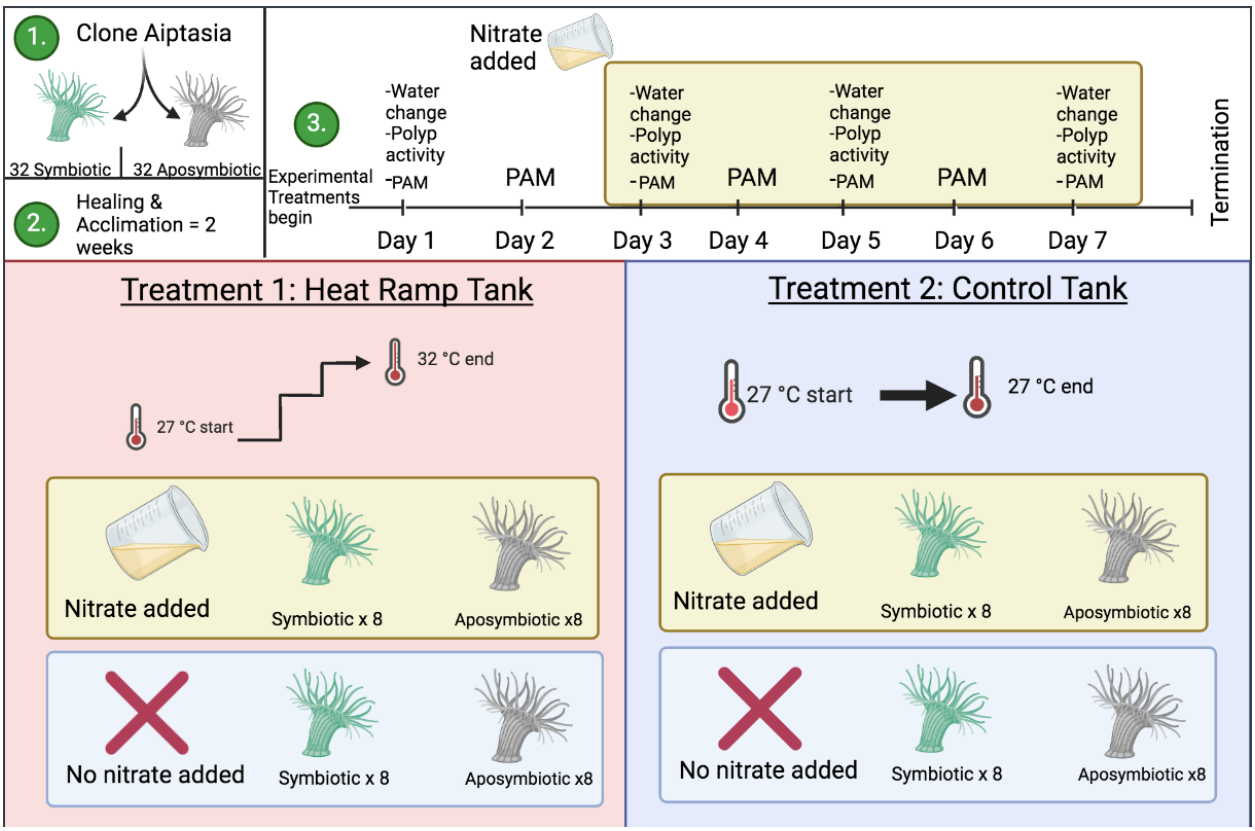



**Figure 1.** Experimental Design and Timeline. Overview of the rearing and nutrient and thermal stress treatments of Aiptasia for a 9 day experiment. Grey anemones represent aposymbiotic individuals, while green represents symbiotic individuals. A timeline of the experimental progress is pictured in the top right of the figure, indicating when the Aiptasia were fed, water changed, and observed for phenotypic change as well as photosynthetic efficiency. The red tank indicates the heat treatment tank which started at 27°C and ended at 34°C. The yellow boxes indicate groups that were treated with 5 µM NH4NO3.

behavioral index) with 0 (closed tentacles), 1 (half extended tentacles), and 2 (fully extended tentacles). The body ABI was denoted with 0 (very compressed body and/or closed tentacles), 1 (slightly compressed body with extended tentacles), 2 (rounded body with extended tentacles), and 3 (fully extended body with extended tentacles). This metric was inspired by the polyp extension score from Burmester et al. 2018. 5 hours following the feedings, the water in the vials would be replaced with the aforementioned nutrient enriched or non-enriched stock water based on treatment groups.

### *Statistical analysis*

Differences of Fv/Fm in treatments across time points were analyzed using repeated-measures ANOVA (analysis of variance) following Shapiro–Wilk normality tests for ANOVA assumptions. The fixed factors were clone/host genotype, treatment, and time, which were repeated measures. Symbiotic and aposymbiotic aiptasias' Fv/Fm were analyzed separately. Significant ANOVA results were further tested using a post-hoc Tukey's HSD test to compare

pairwise differences in Fv/Fm between treatments or time points. A Bonferroni correction was applied on P-values to account for increased statistical error in multiple comparisons (Zar, 1984). All statistical analyses were conducted in the R statistical environment version 4.1.1 (2021-08-10, https://www.r-project.org/).

The ABI were plotted as line graphs in RStudio and compared amongst treatment groups. ABI from experimental day 6 were statistically compared across treatment groups after conducting Welch's t-tests.

## RESULTS

### *Effect of ocean warming and nitrogen enrichment on photochemical efficiency*

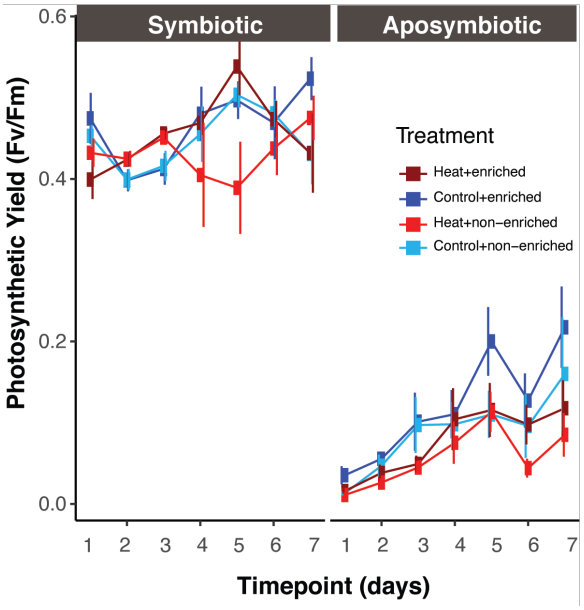
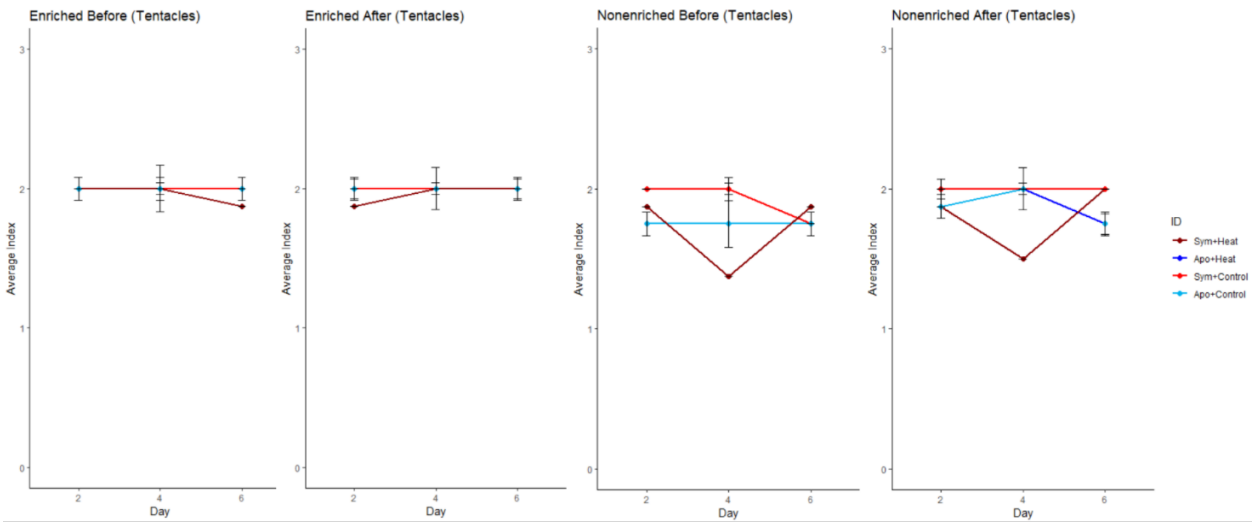


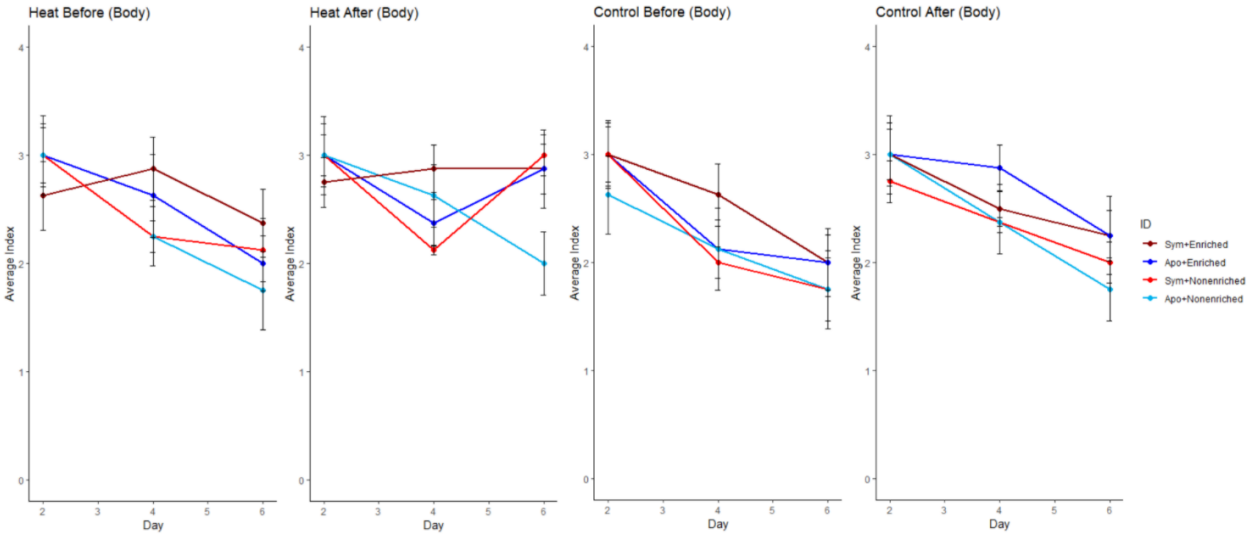

**Figure 2.** Photochemical efficiency in symbiotic and aposymbiotic aiptasia exposed to four treatments over seven days. Aiptasia clones were subjected to a treatment temperature of 34 °C (Heat+nonenriched, red-bordered squares), control temperature of 27°C (Control+nonenriched, light, blue-bordered squares), nitrate enrichment (Control+enriched, blue-bordered squares), and combined elevated temperature and nutrient enrichment (Heat+enriched, dark red-bordered squares) for 1, 2, 3, 4, 5, 6, 7 days. Data are presented as means ± standard deviation of N = 8 aiptasia examined each time for each symbiotic and aposymbiotic sample.

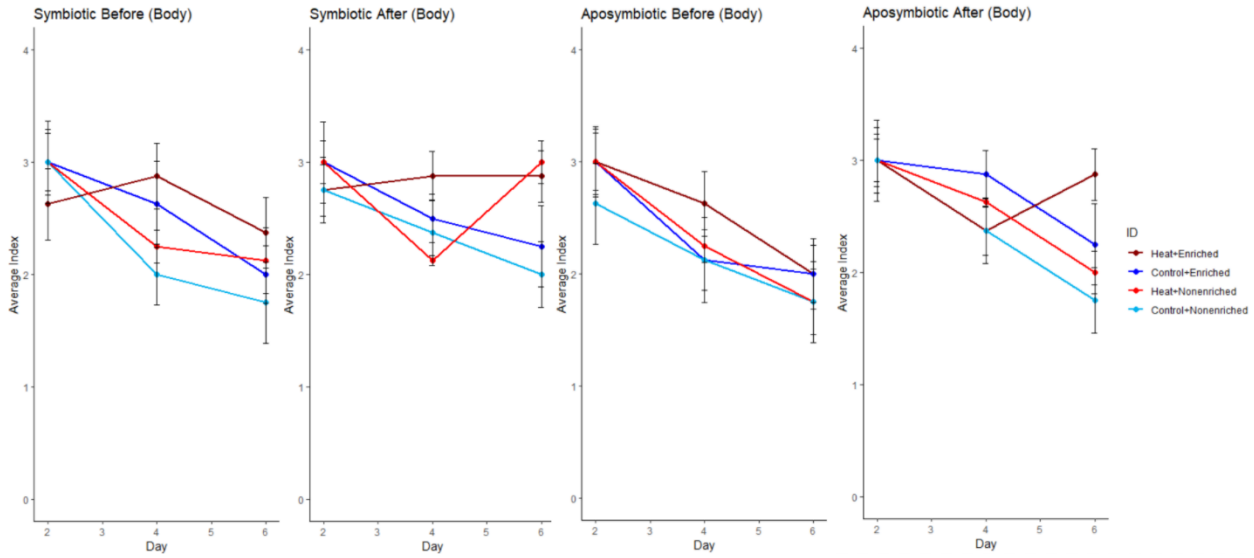
Symbiotic aiptasias maintained at the control temperature exhibited a dark-adapted photochemical efficiency (Fv/Fm) of 0.44 ± 0.036541561 (mean ± SD), while aposymbiotic aiptasias had 0.08 ± 0.05. None of the treatments resulted in a significant change in the dark-adapted quantum yield of photosystem II (Fv/Fm) of aiptasias over seven days for both symbiotic and aposymbiotic aiptasias (Figure 2, ANOVA, $p > 0.05$). Moreover, symbiotic aiptasia showed a reduction of Fv/Fm after 4 and 5 days, while aposymbiotic aiptasia showed an increase of Fv/Fm after 4 to 7 days. Still, these were insignificant relative to controls and appeared to recover by 6 and 7 days. It is also noteworthy that we observe pigmentation on aposymbiotic aiptasia after four days, indicating algal-symbiont starting to increase.

### *Feeding Behavior*

The mean ABI of each treatment group per day (separated by data from before and after feeding) was plotted in [Figure]. The bars on each point signify standard error. There is a general trend for tentacle ABI to stay rather stable from day 2 to day 6, although the SHN group(symbiotic, heat ramp, non-enriched) had a visible drop in ABI on day 4 before recovering on day 6. Body ABI can be seen


Enriched Before (Tentacles)
Enriched After (Tentacles)
Nonenriched Before (Tentacles)
Nonenriched After (Tentacles)
Average Index
Day
ID
Sym+Heat
Apo+Heat
Sym+Control
Apo+Control


Heat Before (Body)
Heat After (Body)
Control Before (Body)
Control After (Body)
Average Index
Day
ID
Sym+Enriched
Apo+Enriched
Sym+Nonenriched
Apo+Nonenriched


Symbiotic Before (Body)
Symbiotic After (Body)
Aposymbiotic Before (Body)
Aposymbiotic After (Body)
Average Index
Day
ID
Heat+Enriched
Control+Enriched
Heat+Nonenriched
Control+Nonenriched

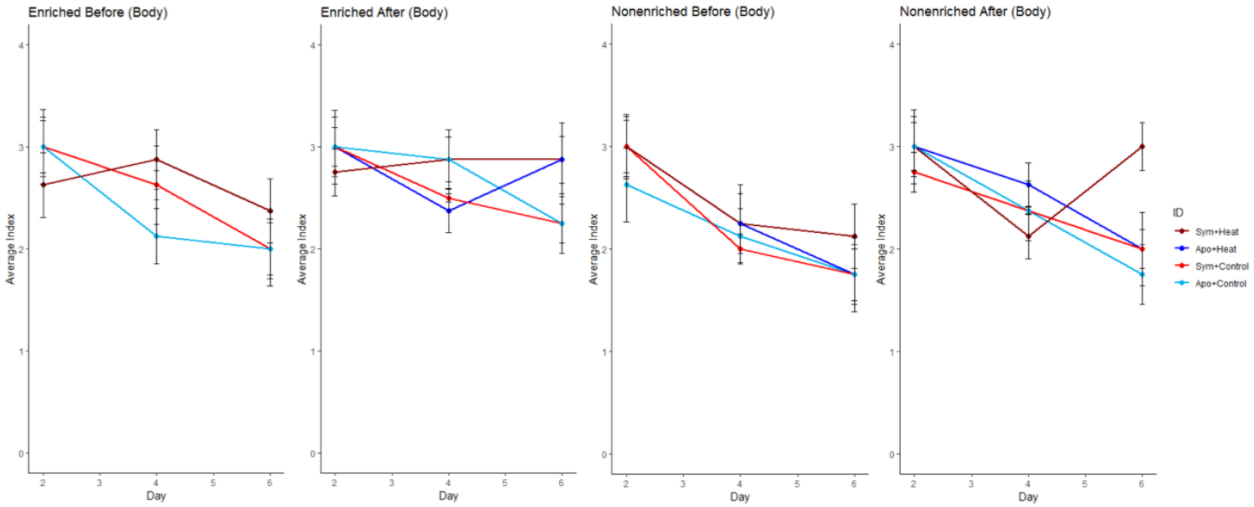



**Figure 3.** Line graphs showing average ABI of each treatment group over the days of feeding, showing both behaviors of tentacles and bodies before and after feeding. The bars on each point represent standard error.

**Table 1.** P-value results from Welch's t-test of mean ABI of tentacles and body for all treatment groups. This compares the behaviors of both combined and before/after feedings of *Aiptasia*. Significant values are bolded.

| *Tentacles* | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Treatment* | *SymState* | *Heat* | *Nutrient* | | | | |
| *p-value* | 0.3003 | 1 | **0.005703** | | | | |
| *p-value (Before)* | 1 | 1 | **0.005424** | | | | |
| *p-value (After)* | 0.1817 | 1 | 0.1817 | | | | |
| | | | | | | | |
| *Before vs After* | *Overall* | *Sym* | *Apo* | *Heat* | *Control* | *Enriched* | *Nonenriched* |
| *p-value* | 0.2983 | 0.09172 | 1 | 0.4691 | 0.5374 | 0.391 | 0.2978 |
| | | | | | | | |
| *Body* | | | | | | | |
| *Treatment* | *SymState* | *Heat* | *Nutrient* | | | | |
| *p-value* | 0.2419 | 0.05475 | 0.1376 | | | | |
| *p-value (Before)* | 0.2667 | 0.2667 | 0.1083 | | | | |
| *p-value (After)* | 0.3953 | 0.06708 | 0.3068 | | | | |
| | | | | | | | |
| *Before vs After* | *Overall* | *Sym* | *Apo* | *Heat* | *Control* | *Enriched* | *Nonenriched* |
| *p-value* | 0.05394 | 0.153 | 0.2529 | 0.06794 | 0.2382 | 0.075 | 0.3105 |

with a general downward trend on the plot,in some cases with the groups with heat ramp treatment having a higher ABI compared to their non-heat ramp counterparts.

P-values by Welch's t-test were calculated for the mean ABI of each group to compare between treatment groups, along with comparing data from before and after the feedings (Table 1). Significant p-values are bolded; there were only significant differences comparing the tentacle ABI of nutrient enriched and non-enriched groups, for combined before and after as well as exclusively calculating from ABI before feedings. The t-tests were conducted in a two-sided manner, although the sample estimates confirm that the nutrient-enriched groups had higher mean ABI; 1.984375 (enriched) and 1.828125 (non-enriched) for the combined p-value, and 1.96875 (enriched) and 1.78125 (non-enriched) for before feeding.

## DISCUSSION

Physiological responses represent the first line of defense of an organism in reducing the potentially harmful effects of unfavorable environmental conditions (Rosic et al., 2014). However, our understanding of the underlying mechanisms of the coral holobiont to multistressors, particularly ocean warming and nutrient enrichment, is still limited. Here, we subjected aptasia to ocean warming and nitrate enriched conditions to determine the photosynthetic fitness by measuring the dark-adapted yield of PSII (Fv/Fm). Previous studies have shown that Fv/Fm is a good indicator of chronic photoinhibition (Gorbunov et al. 2001), with bleached corals exhibiting reduced values for Fv/Fm (Warner et al. 1999). Our results showed that ocean warming and nutrient stress allowed aiptasia's algal symbiont to maintain healthy photosynthetic fitness based on stable or increased dark-adapted Fv/Fm. Similarly, reef-building corals such as *Acropora aspera*, *Montipora digitata*, and *Porites cylindrica* did not show significant changes in the PSII activity (Fisher et al., 2012). Short-term thermal shock exposure of *Pocillopora damicornis* and S. hystrix also showed insignificant differences in Fv/Fm after 12 h at 30, 28, and 21°C (Putnam et al., 2010).

Additionally, Rosic and colleagues (2014) have shown that although physiological performance was stable in *Acropora aspera* exposed to elevated temperatures and nutrient stress, the signs of oxidative stress were developing at the molecular level, as shown in the enrichment of genes involved in oxidative stress and cell death. The breakdown of coral host-algal symbiosis often starts with ROS production and oxidative stress (Lesser 2002). Studies in corals have shown that increased antioxidants that scavenge ROS during heat stress improve photosynthetic activity and decrease bleaching events (Lesser 1997).

Our results showed both symbiotic and aposymbiotic aptasia were able to withstand multistressors. The robust response of aiptasia host to warming and nutrient stress could be attributed to the heat ramping employed in the study. Studies in corals have shown that exposure to slow warming rates also leads to overall differences in the severity of bleaching responses exhibited during thermal stress events (Brown et al., 2002; Middlebrook et al., 2008; Bellantuono et al., 2011). This exposure may stimulate protective mechanisms, such as UV-protective compounds (Yakovleva et al., 2004; Baird et al., 2009), enzymatic antioxidants (Richier et al., 2005; Lesser, 2006), or expression of heat shock proteins (Rodriguez-Lanetty et al., 2009; DeSalvo et al., 2010; Traylor-Knowles et al., 2017), which help reduce decline in coral health. Therefore, it would be of interest to determine the gene expression dynamics that

accompany the robust physiological responses of aiptasia host to multistressors.

Our behavioral observation showed general trends in the behavior of tentacles where they stabilized and showed similar results by day 6 of the experiment. One treatment group to note is the tentacle behavior of the nutrient-enriched group mentioned, which exhibited a drop in ABI on day four before recovering by day 6. Because this was the only group for tentacle behavior to show this pattern, there is a curiosity for the effect of heat stress alone and possible adaptation to stress conditions after initial exposure. Black et al. 1995 discussed the presence and impact of heat shock proteins in Aiptasia; an analysis of the proteins in our Aiptasia specimens may show similar behavior. These heat shock proteins may have been produced starting after day 4 of the experiment, possibly explaining why the nutrient-enriched group recovered by day six after heat stress.

Unlike the behavior of the tentacles, the behavior of the bodies of Aiptasia are shown to have an overall declining trend over the experimental period. Some treatment groups are seen to drop in ABI on day four and recover by day 6, while others observe a spike in ABI on day four before falling back down by day 6. Because all plots seem to show an overall downward trend, including control groups, this could signify a flaw in the experimental setup where all groups were affected, possibly the environment in which Aiptasia was kept or other controlled factors. This downward trend in body behavior was observed in a spike in the frequency of an ABI of 2 in the specimens, where most of them would exhibit a rounded body but with extended tentacles. Previous research discussing this behavior in the bodies of Aiptasia was not found, so further experiments should be conducted to explain its causes.

Only the p-values for tentacle ABI of nutrient-enriched versus non-enriched groups (combined and exclusively before) showed significance. This result was expected and agrees with our hypothesis based on Rädecker et al. 2019, where Aiptasia with long-term exposure to higher nutrient levels induced reduced tentacle size, as they had conducted their experiments with a lack of water changes. This especially applies to the calculated p-value of before feeding, with lack of feeding for a certain period. The consistency with the results from Rädecker et al. 2019 suggests further research should be conducted, especially investigating the behavior of coral polyps to long-term nutrient exposure or lack of feeding. Our experiment could also be repeated, however, with a longer trial period and observing different behaviors during the growth of Aiptasia rather than changes in their mature forms.

None of the remaining calculated p-values showed significant differences in the means of the ABI data, although a few were close to the 0.05 threshold. The p-value for tentacle ABI of before versus after feedings of all symbiotic Aiptasia suggests a trend with them changing behavior after feeding. Other noteworthy p-values were the body ABI for heat treatments, suggesting that heat stress causes a trend in changing behavior of the body. Lastly, the overall heat and nutrient-enriched body ABI for before versus after feeding were similarly near the threshold, suggesting a trend in feeding causing different behaviors in the body of Aiptasia under these two stressors.

Overall, the only significant feeding behavioral differences were found in the tentacles of *Aiptasia* in the nutrient treatment groups. We had hypothesized this occurrence, although failing to meet our

hypothesis of the effect of both stressors; there is a possibility, however of an observance of moderation, rather than amplification, of observed results when the two stresses are combined, which has been seen in the behavior of marine fungal disease under ocean warming and acidification (Williams et al. 2014).

In summary, we have shown a robust physiological response of both symbiotic and aposymbiotic aiptasia to ocean warming and nutrient enrichment using photochemical efficiency and behaviorl observation. The findings highlight the role of the host in stress response. The exact mechanisms underlying the host response remain to be explored.

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