Investigating the physiological resilience of the northern star coral *Astrangia poculata* to nutrient enrichment under thermal stress

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I. Abstract

As a result of urbanization and anthropogenic activity, Earth's oceans are warming and receiving an influx of nutrients such as inorganic nitrogen. Marine organisms located adjacent to human development may be vulnerable to the interactive effects from inorganic nitrogen enrichment and increasing sea surface temperature. The impact of these two stressors have been investigated in obligate symbiotic tropical corals, but the specific effects of nitrate and thermal stress on the coral animal & symbiont remain unknown. One seemingly resilient coral is *Astrangia poculata*, a facultatively symbiotic temperate coral that exists with *and* without symbionts along the eastern United States coast, including highly urbanized areas such as the New York Harbor. This study aimed to examine the effect of nutrient enrichment, specifically elevated nitrate, on the physiology of symbiotic and aposymbiotic *A. poculata* fragments at constant *and* elevated sea surface temperatures. Four treatments were run with both aposymbiotic and symbiotic colonies of *A. poculata* experiencing either a ramping temperature from 27-33°C or a constant 25°C, and presence or absence of 5 µM of $Na^{15}NO_3$ nitrate over the course of a 7-day experiment. Coral color, photosynthetic efficiency, polyp behavior, and metabolic activity (measured via oxygen consumption on a respirometer) were used as a read-out to determine lethal & sublethal response to stressors. Overall, we found no effect of nitrate enrichment on coral physiology, but found significant decreases in photosynthetic efficiency (Fv/Fm), polyp behavior, and increased bleaching beginning at 31°C temperature stress. Interestingly, there were no observed differences between Fv/Fm or polyp extension score between symbiotic states under 31°C+ thermal stress. Taken together, our results suggest that *A. poculata* corals are more resilient to nitrate enrichment than thermal stress, and that symbiotic state cannot predict thermal and/or nutrient enrichment resilience. As anthropogenic sources continue to input nitrates into warming coastal waters, it is essential to understand the impacts on coastal marine ecosystems. Our study lays the groundwork for more studies focusing on the intersection of ecotoxicology and global change stressors in temperate coral communities.

II. Introduction

Marine organisms located downstream from human activity face both potentially negative interactive effects of anthropogenic pollutants (heavy metals, microplastics, pharmaceuticals, increased nutrients) and global change stressors (increasing sea temperature and acidity) (Raju et al. 2018, Todd et al. 2019). Wastewater effluents and industrial runoff that are discharged to urban harbors contain a suite of contaminants that are not properly removed (Magadini et al. 2020, Benotti & Brownawell, 2007). In addition, marine coastal communities are vulnerable to agricultural runoff of pesticides and fertilizers (Burkepile et al. 2019, Holcomb et al. 2010). Though the impacts of anthropogenic pollutants on marine organisms are well studied, it is unclear if these impacts will be amplified by the effects of global change, specifically, temperature (Raju et al. 2018, Rotjan et al. 2019, Holcomb et al. 2010, Martínez-Castillo et al. 2020, Hadjioannou et al. 2019, Burkepile et al. 2019). Some marine organisms appear to be more resilient than others in coping with these challenges, but these interacting stressors may affect an organism's physiology in different ways than each stressor independently.

One ubiquitous marine ecotoxicological stressor is inorganic nitrogen in the form of nitrate. While the gaseous form of nitrogen (N_2) comprises 78% of the atmosphere's air, most organisms cannot use this form due to the strong triple bond and it must first undergo nitrogen fixation (Stevens et al. 2019). Nitrogen fixation is a process by which N_2 is converted into different nitrogenous compounds, such as ammonia, nitrites, and nitrates. After the nitrogen has been fixed, the forms nitrate and ammonium become

usable to a greater diversity of organisms for use in protein and DNA building (Smil et al. 1997). With the onset of fertilizer and pesticide production using the Haber-Bosch nitrogen fixation process, environmental nitrogen levels have dramatically increased (Razon et al. 2014). In urban settings, nitrates and ammonium enter waterways as waste and stormwater run-off from industrial and household waste (Jani et al. 2020). However, rural waste accounts for a greater proportion of nitrogenous runoff, with synthetic fertilizers contributing 41% of anthropogenically sourced nitrates and ammonium. As a result, eutrophication, or an overloading of nutrients, occurs. These nutrients are taken up by algae, causing an oxygen deficiency via algal blooms, a process quite harmful to marine ecosystems (Sobota et al. 2013).

Marine invertebrates, specifically corals, that live downstream from these nitrate sources experience these elevated levels of nitrate in addition to rises in global sea surface temperature. Warming temperatures have been found to increase the frequency and scale of coral bleaching events in which corals experience a breakdown of their symbiosis with their endolithic algae, *Symbiodinium spp.*, which assists in food production and maintaining coral health (Middlebrook, 2008, Chimienti et al. 2021). Studies have suggested that reduced water quality due to nitrate enrichment can reduce tropical coral thermal tolerance, increase bleaching/mortality, and slow bleaching recovery times versus the readily taken up ammonium which benefits corals (Nordemar et al. 2003, Wiedenmann et al. 2013, Burkepile et al. 2019). Interestingly, Hadjioannou et al. 2019 found that the temperate coral *Cladocora caespitosa* from high nutrient zones were able to maintain high levels of photosynthesis and saw no change in protein levels while their low nutrient counterparts exhibited lower gross and net photosynthesis and a decline in proteins, suggesting that nitrate enrichment may be beneficial in the absence of thermal stress (Hadjioannou et al. 2019). However, unraveling the impacts of global sea surface temperature increases and nutrient inputs from anthropogenic sources can prove difficult due to the obligate symbiosis between tropical corals and *Symbiodinium*. In order to probe the contribution of the symbiont to coral health nutrient enrichment, we can leverage a model facultatively symbiotic coral, *Astrangia poculata* (Neff et al. 2020).

A. poculata is a temperate stony coral that lives along the eastern coast of the Americas, with a range spanning as far north as Cape Cod and as far south as the Gulf of Mexico and the northern edges of South America (Dimond et al. 2013). *A. poculata* in the North Atlantic, an oceanic province of enhanced warming (Saba et al. 2016), experience a large temperature range from -2 to 26 degrees C (Wuitchik et al. 2021). These corals demonstrate resilience in response to stressors, and live in close proximity to urban environments— making *A. poculata* a great model organism to study the influence of warming sea temperatures and urban stressors (Rotjan et al. 2019, Neff et al. 2020, Holcomb et al. 2010). Much like other coral species, *A. poculata* exhibits a symbiotic relationship with an endolithic species of algae called *Brevioulum psygmophilum*, providing it shelter in exchange for some of the outputs of photosynthesis and nitrogen assimilation (Chan et al. 2021). This symbiosis is facultative, however, enabling the corals to exist as either symbiotic, aposymbiotic, or mixed colonies in the wild (Sharp et al. 2017). This unique facultative relationship and its influence on the coral's response to urban stressors of nitrogen warrants additional study.

A previous study on *A. poculata*'s response to enriched nitrogen focused on the uptake of ammonium and nitrate by symbiotic and asymbiotic colonies (DiRoberts et al. 2021). The study quantified the level of nutrient absorption by the *Astrangia* colonies, and examined how the intake of this nutrient influenced photosynthetic efficiency. Ultimately, researchers found that nitrate was only assimilated by the algal symbiont, while ammonium was taken up by members of all three subject groups. After nitrate intake, corals experienced a decline in photosynthetic efficiency, while the opposite was observed in individuals who took up ammonium, indicating the potentially positive effects of ammonium enrichment on the coral (DiRoberts et al. 2021).

Although *A. poculata* individuals are relatively resilient to thermal stress (Aichelman et al. 2019, Wuitchik et al. 2021) they have been found to exhibit tissue loss, lower calcification rates, and shifts in their

microbiome community from thermal stress (Sharp et al. 2017, Wuitchik et al. 2021). Shifts in photosynthetic activity from temperature have been found to vary with the colony's symbiotic state (Aichelman et al. 2019). Temperate corals like *A. poculata* have also been found to display varying thermal performance depending on the region from which they originate. Temperate corals found in the northeastern US tend to have lower thermal performance curves than temperate corals from warmer regions. Regardless of origin, *A. poculata'*s symbionts are most photochemically efficient in 18°C, and symbiotic corals have greater photosynthetic efficiency than their aposymbiotic conspecifics (Aichelman et al. 2019). While studies have shown how *A. poculata* responds to nitrogen enrichment (DiRoberts et al. 2021) and temperature effects (Wuitchik et al. 2021), there is a gap in our understanding of how *A. poculata* health will respond to these combined effects.

Here, we aim to study the interactive effects of nitrate pollution and ocean warming on the physiology of *A. poculata* via photosynthetic efficiency, coral color (a proxy for coral bleaching), polyp activity, and respiration rate under elevated nitrate and temperature regimes. In accordance with DiRoberts et al. 2021, we hypothesize that *A. poculata* corals experiencing nitrogen stress will experience a decline in photosynthetic efficiency, a decrease in polyp extension, and a significantly different (either higher or lower) metabolic rate than control colonies. In addition, we hypothesized that these impacts will be more drastic in colonies undergoing both nitrate and heat stress. We also predicted that symbiotic colonies would perform better under these stressors when compared to aposymbiotic colonies due to the energetic buffering effect of symbionts (Burmester et al. 2017). This study is critical for understanding the marine stressors of nitrate enrichment and increased sea surface temperature to evaluate the physiological tolerance of temperate corals in the face of a changing ocean.

III. Methods

Coral Collection & Fragmentation

Ten symbiotic and ten aposymbiotic lab-acclimated colonies of *A. poculata* were selected for the experiment from corals originally collected from Fort Wetherill, Rhode Island. Using a dremel, each colony was divided into 4 fragments of varying sizes (one fragment assigned to each treatment), for a total of 80 fragments. Fragments were given 12 days of recovery time in a common garden aquarium under approximately 21˚C and 35ppt conditions before experimentation. The four experimental treatments were: no nitrate enrichment and held at 25˚C (25˚C Control), nitrate enrichment but held at 25˚C (25˚C + N), no enrichment but exposed to a heat ramp from 27-33˚C (RAMP Control), and nitrate enrichment and heat ramp (RAMP + N). One ramet of each genet was represented in each of four treatments (20 fragments per treatment).

Coral Sorting and Tank Setup

All materials used in this experiment were acid washed to remove residual nitrogen contamination according to methods applied in DiRoberts et al. 2021. Two tanks were set up for each temperature regime: one with water held at 25°C, and one in which the temperature was increased by 1°C at the same time each day, from 27°-33°C by the end of the 7-day experiment. Temperature was controlled by a TR115SN controller from Aqualogic, Inc., and two powerheads were placed inside the water bath to maintain even temperatures throughout the tank. Temperature and salinity (water quality) within jars at opposite sides of each tank were measured and recorded three times per day each day with a Hach© probe. All systems were exposed to light for 12 hours per day, from 7 pm to 7 am at an intensity of 35-44W. Each jar was submerged and provided with an individual airstone attached to an airflow pipe for constant oxygenation. Low rates of bubbling were maintained.

Fragments were sorted into color-coded, acid-washed glass jars, which were filled with 200 ml artificial sea water (ASW) at 35 ppt salinity ensuring that fragments were fully submerged. Each fragment was placed in an individual glass jar to avoid nutrient sharing between fragments and to properly assess the absorption of nitrates into *A. poculata* colonies. Fragments assigned the nutrient enrichment treatment were maintained at a nitrate concentration of 5 μM $Na^{15}NO_3$ (Sigma-Aldrich©) nitrate. To ensure randomization of treatments, we organized fragments

from the same colony in descending order according to size and then rotated the treatment receiving the largest ramet so that each treatment would have a roughly even distribution of sizes. A random number generator was used to select the position of each jar within each of the two experimental systems (one held at 25°C that included 25°C Control & 25°C + N treatments and one that was heat ramped that included RAMP Control & RAMP + N treatments).

Corals were fed a high food regime of actively swimming 24-hour old hatched brine shrimp at a concentration of 500 nauplii/L (Aichelman et al. 2016) every other day. Air stones were turned off during feeding to allow for polyp extension and catching of the nauplii prey. Water was changed every day to ensure stable salinity, and Saran Wrap was used to cover experimental jars overnight to prevent evaporation.

Pulse Amplitude Modulation (PAM)

A Walz Junior PAM was used to measure photosynthetic efficiency of photosystem II (Fv/Fm) every two days, on days 1, 3, 5, and 7 of the experiment. Corals were dark-acclimated for at least half an hour prior to measuring Fv/Fm. At least two polyps per fragment were measured until a replicate reading within 0.05 was produced, with values ideally ranging between 0.25 and 0.75 for symbiotic fragments and often below 0.25 for aposymbiotic fragments.

Polyp Extension Score

Polyp extension scores were measured on days 2, 4, and 6 of the experiment one hour post feeding with aeration off. Values scaled from 0 (all polyps completely retracted, no active polyps) to 6 (all polyps fully extended with outstretched tentacles) according to the scale created by Burmester et al. (2017). The same investigator scored all polyps on each day to reduce observer bias. Each coral fragment's jar was kept stationary in its bath during data recording to ensure that motion did not disrupt the observed coral behavior.

Baseline Net Respiration

Baseline net respiration (baseline metabolic rate) was calculated from changes in dissolved oxygen concentration in approximately 400mL Plexiglass chambers, measured by a fiber-optic oxygen sensor probe connected to a 10-channel Fiber Optic Oxygen Transmitter (OXY-10 mini, Pre-Sens Precision Sensing GmbH, Regensburg, Germany). Oxygen concentrations at 0.1 Hz were recorded by Pre-Sens software v3.03. A 10-chamber system with a submersible magnetic stir plate was placed in a recirculating 34ppt seawater bath, calibrated to 25˚C by Apex temperature controllers. Powerheads in the water bath and stirbars inside individual chambers ensured uniform water flow. Due to the small size of coral fragments, two fragments from the same treatment (25˚C Control, 25˚C + N, RAMP Control, RAMP + N) and symbiotic state (symbiotic or aposymbiotic) were glued to a single tile and placed in a single respiration chamber. Coral fragments selected for respiration runs were picked by a random number generator from healthy corals (as defined by active polyp extension). It should be noted that for two treatments (aposymbiotic RAMP + N, symbiotic 25˚C Control), only one fragment was healthy enough to undergo respirometry, but all data were corrected by total surface area within a chamber (see below). Eight experimental respiration chambers (one per symbiotic state at each of four treatments) were run in addition to two seawater blanks without coral fragments inside. One 20-minute dark respiration run and 20-minute light respiration run were completed for two sets of corals on a total of 29 coral fragments. Blank metabolic rate was subtracted by rates in the experimental chambers in order to account for microbes in the water or instrumental changes (Aichelman et al. 2019). Net respiration was calculated from recorded oxygen concentrations using linear regression techniques in the LoLinR package (Olito et al. 2017, Aichelman et al. 2019). Calculated net respiration rate was normalized to the sum of the surface area of the two coral fragments in a chamber (Aichelman et al. 2019) and respiration due to photosynthesis was subtracted for a final rate in nmol/min cm^2. Surface area of coral fragments was determined using the freehand tool in ImageJ software.

Photo Analysis

Photos of each coral fragment were taken using an Olympus TG-6 macro-lens camera before the experiment began and on the final day, recording

images of each fragment's polyps and its corresponding beetag identifier. The corals were set up on a CORAL WATCH Coral Health Chart constructed by the University of Queensland, Australia. These pictures were calibrated in photoshop to account for lighting differences, and then analyzed in Matlab (Version 4.0) to extract RGB values. To extract these values, 10 points were randomly selected from the total area of living coral tissue on each fragment. The Analyze Intensity Macro used in the MATLAB analysis was provided in an article by Winters et al. 2009. RGB values were not inverted, meaning a higher RGB value is indicative of a more bleached or aposymbiotic coral.

Statistical Analyses

To investigate the effect of treatment and symbiotic state on PAM value, a mixed random effects repeated measures linear model (R package lme4) was generated with coral identification tag as a random factor, repeated measures on day, and treatment as a fixed factor. Estimated Marginal Means (EMM) were calculated as a post-hoc pairwise comparison between treatments. Akaike information criterion (AIC) tests and residual plots were used to complete model selection. The effect of treatment on polyp extension score was tested using Welch's Test for non-normal heteroscedastic data. Each day was analyzed separately to deal with repeated measures, omitting the first day of the experiment due to complete inactivity of polyps. Changes in color of coral fragments pre and post experiment were analyzed using a paired t-test with Bonferroni correction. Color differences between treatments and assigned symbiotic states before and after the 7-day experiment were assessed using separate (pre and post experiment) linear models with treatment and symbiotic states as fixed factors and EMM post-hoc comparisons, with models selected according to AIC values. Baseline net respiration data corrected to surface area and converted to nmol/minute cm2 were non-normal and homoscedastic. The effect of treatment on baseline net respiration was analyzed using a Kruskal-Wallis test. The effect of symbiotic state on baseline net respiration was calculated using a Wilcoxon rank sum exact test for non-normal homoscedastic data with two levels. All statistical analyses were completed in RStudio computing software, version 4.0.

IV. Results

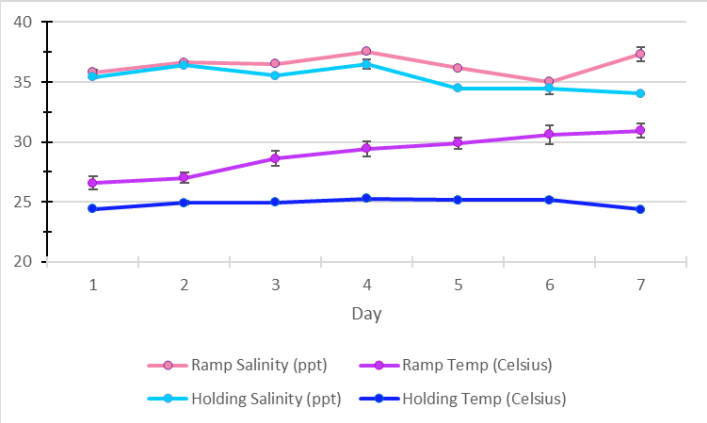

Figure 1. Average temperature and salinity in experimental jars across days in 25°C holding & 27-33°C ramped tanks. Temperature and salinity levels in experimental jars on opposite sides of each experimental tank (25°C holding & ramped) were taken with a Hach© probe three times per day and averaged. Ramped tanks were increased 1°C per day from 27-33°C. Error bars represent standard error of the mean.

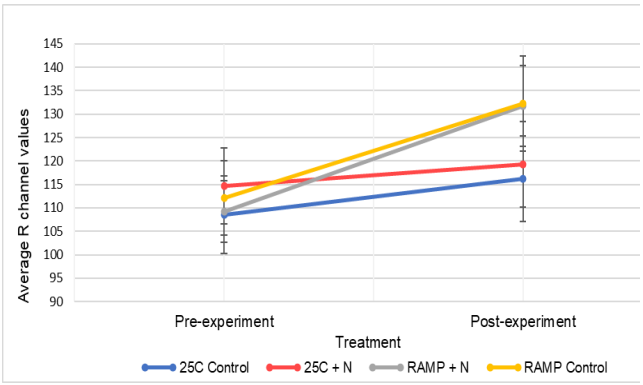

Figure 2. Pre vs. post-experiment red channel values of *Astrangia poculata* fragments. Photographs of *A. poculata* fragments in each experiment were analyzed for red channel color values on day 0 (pre-experiment) and on day 7 (post-experiment) for each of the four treatments (25°C Control, 25°C + N, RAMP Control, RAMP + N) using Adobe Photoshop and MATLAB computing software. Error bars represent standard error of the mean.

Water Quality & Tank Conditions

Water quality conditions in the 25°C holding tank ranged from 24.38°C (±0.0177, n=2) to 25.25°C

(±0.159, n=2) and from 34.05ppt (±0.035, n=2) to 36.43ppt (±0.412, n=2) in salinity, while the RAMP tank ranged in temperature from 26.58°C (±0.530, n=2) to 30.95°C (±0.566, n=2) and from 35.04ppt (±0.097, n=2) to 37.53ppt (±0.141, n=2) ppt in salinity (Figure 1), climbing approximately 1°C per day.

Pre and post-experimental red channel coloration of Fragments

Initial R channel values from photographs of the fragments did not differ between treatments (P=0.958, Figure 2), but did differ between symbiotic states (P=0.0097), with aposymbiotic colonies exhibiting higher R values. R channel values for fragments compared with a Bonferroni-adjusted paired t-test between initial (experiment day 0) and final (experiment day 7) were overall significantly different (Figure 2, P=0.023), and demonstrated an overall increase in R values (color bleaching) throughout the 7-day experiment in all treatments. There was no significant difference in R channel values between treatments post-experimentation (P=0.48, Figure 2), but there was a significant effect of symbiotic state (P=0.017), with aposymbiotic colonies maintaining higher R values relative to symbiotic colonies. Overall, corals in the 25°C control treatment moved from an average R channel value of 108.5 (±8.24, n=20) to 116.27(±9.16, n=20), 25°C + N treatment moved from 114.66(±8.08, n=20) to 119.275(±9.20, n=20), RAMP control treatment which increased from 112.11 (±7.96, n=20) to 132.205 (±10.14, n=20, Figure 2), and RAMP + N treatment moved from an average R channel value of 109.22 (±6.57, n=20) to 131.735 (±8.61, n=20).

Photosynthetic efficiency

Initial mixed linear models included symbiotic state and average fragment R channel color as fixed factors, but AIC analysis revealed that treatment alone as a fixed factor generated the most predictive model for Fv/Fm trends. The linear model produced revealed that there was no significant effect of treatment (P=0.725) PAM values. There was, however, an overall significant effect of day (P=<0.0001) on Fv/Fm under RAMP Control (P=0.0024) and RAMP + N treatments (P=0.017). Both RAMP Control and RAMP + N exhibited decreased Fv/Fm as temperature progressed throughout the days (Figure 3a-b). Specific analyses performed on the final day of the experiment (day 7) demonstrated that there was a significant effect of treatment on Fv/Fm (P=0.0006), with corals experiencing the RAMP temperature treatment either with (Tukey's HSD P=0.045) or without (Tukey's HSD P= 0.005) nitrate enrichment experiencing decreased Fv/Fm versus their 25°C holding counterparts (Figure 3a). There was no significant effect of symbiotic state (Figure 4, P=0.545) on Fv/Fm on day 7.

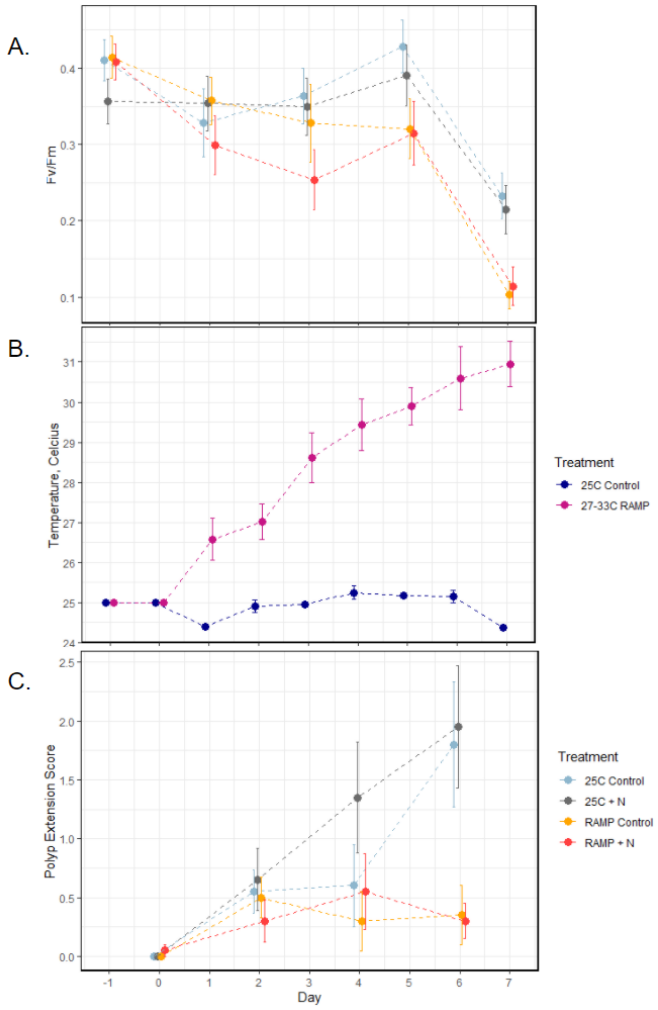

Figure 3. (A) Photosynthetic efficiency across nutrient and temperature treatments. Pulse Amplitude Modulation (PAM) photosynthetic efficiency data measured in Fv/Fm was recorded in *Astrangia poculata* using a JuniorPAM following a 30-minute dark acclimation 2 days prior to experimental start, and then on days 1, 3, 5, and 7 of the experiment. Fv/Fm data were plotted across days and treatments (25°C Control, 25°C + N, RAMP Control, RAMP + N). Error bars represent standard error of the mean. **(B) Average water bath temperatures over the**

course of the 7-day experiment. Temperature data in experimental jars on opposite sides of each experimental tank (25°C holding & ramped) were taken with a Hach© probe three times per day and averaged. Error bars represent standard error of the mean **(C) Polyp behavior across nutrient and temperature treatments.** Polyp extension score data were recorded one hour post-feeding according to the scale provided in Burmester et al. 2017. Data was recorded one day before the experiment began and then on days 2, 4, and 6 of the experiment. Score data were plotted across days and treatments (25°C Control, 25°C + N, RAMP Control, RAMP + N). Error bars represent standard error of the mean.

Polyp Extension Score

Welch's t-tests conducted on polyp extension scores on each day of data collection (day 2, 4, and 6) found a significant effect of treatment only on day 6 (the last day of scoring) (Table 1, P=<0.0001), with fragments in RAMP Control and RAMP + N treatments exhibiting lower polyp extension scores than those in the 25°C holding tank (Figure 3c). There was no statistically significant effect of symbiotic state on day 6 (P=0.131, Figure 4). However, there was a statistically significant effect of symbiotic state on polyp extension score on days 2 (Table 1, P=0.003) and 4 (Table 1, P=<0.0001), with symbiotic fragments exhibiting greater polyp activity (higher polyp extension scores) on these days.

Day	Effect of Treatment	Effect of Sym State
0	No polyp activity	No polyp activity
2	P=0.678	**P=0.003**
4	P=0.303	**P=<0.0001**
6	**P=<0.0001**	P=0.131

Table 1. Welch's t-test statistical summary table for polyp extension behavior. Separate Welch's t-tests for non-normal heteroscedastic data were performed on the effect of treatment (25°C Control, 25°C + N, RAMP Control, RAMP + N) and symbiotic state (aposymbiotic and symbiotic) on the polyp extension scores of *Astrangia poculata* on days 0, 2, 4, and 6 of the experiment. Significant p values greater than 0.05 are bolded.

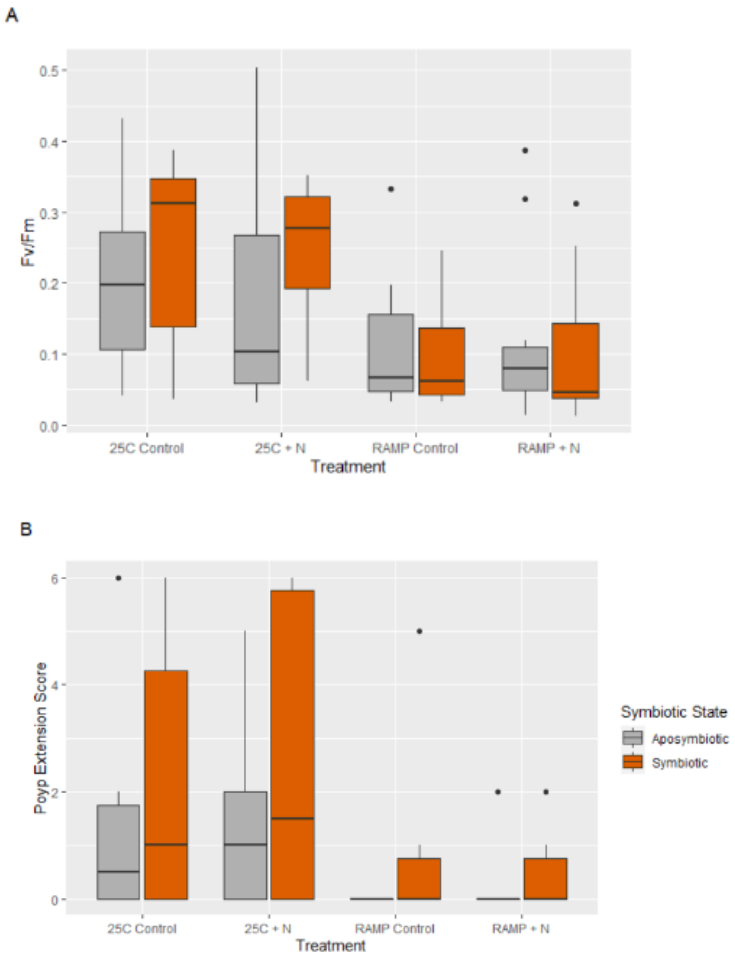

Figure 4. (a) End point photosynthetic efficiency across nutrient and temperature treatments by symbiotic state. Pulse Amplitude Modulation (PAM) photosynthetic efficiency data measured in Fv/Fm for *Astrangia poculata* were recorded using a JuniorPAM following a 30-minute dark acclimation. Fv/Fm data from the final experimental day (day 7) were plotted for each symbiotic state (symbiotic & aposymbiotic) at each nutrient and/or temperature treatment (25°C Control, 25°C + N, RAMP Control, RAMP + N). Lines within each boxplot represent the median, with whiskers from the upper or lower quantile to the upper or lower extreme. **(b) End point polyp behavior across nutrient and temperature treatments by symbiotic state.** Polyp extension score data were recorded one hour post-feeding according to the scale provided in Burmester et al. 2017. Scores from each symbiotic state (symbiotic & aposymbiotic) and treatment (25°C Control, 25°C + N, RAMP Control, RAMP + N) at the final day of polyp extension recording (experiment day 6) were included in the box plot. Lines within each boxplot represent the median, with whiskers from the upper or lower quantile to the upper

or lower extreme, and black points represent outliers. Ramped treatments experienced a 1°C increase every day, from 27℃-33℃ over the course of the 7-day experiment.

Respiration

Respiration data generally lacked a clear pattern (Figure 5), with particularly high standard error bars in aposymbiotic fragments at the RAMP + N treatment and lower respiration observed in Control 25°C symbiotic colonies, although these colonies exhibited the greatest Fv/Fm values on day 7 and polyp extension scores at day 6 (Figure 4). A Kruskal Wallis test revealed that there were no significant differences of baseline net respiration between treatments (P=0.9535), and a Wilcoxon rank test indicated no significant differences between symbiotic states (P=0.1143).

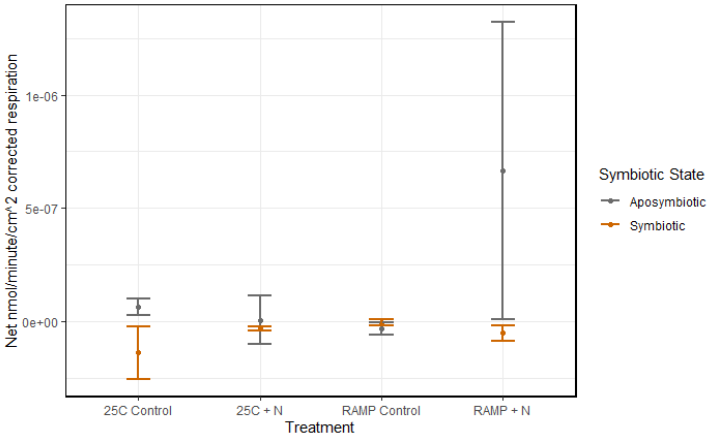

Figure 5. Net baseline respiration across treatments and symbiotic states. Baseline net respiration of *Astrangia poculata* fragments at 25°C in nmol/minute after 20 minutes of dark respiration and 20 minutes of light respiration were recorded by PreSens software with fragments from each of the four experimental treatments (25°C Control, 25°C+ N, RAMP Control, RAMP + N) at the two symbiotic states (aposymbiotic & symbiotic) and corrected for fragment surface area. Error bars represent standard error.

V. Discussion

Understanding the relationship between temperature, and nitrate enrichment, and symbiotic state in corals is critical for understanding how global and local anthropogenic activities affect marine species. Previous studies have shown that bleaching events are exacerbated by both nitrate inputs and heat stress, with an additive effect of their combination (Blanckaert et al. 2021, Wiedenmann et al. 2013, Burkepile et al. 2019). However, as many of these studies investigated tropical corals with obligate symbioses, and there is a lack of research on nitrate enrichment and heat stress on the coral animal or the symbiont alone. The decoupling of symbiont and host that can be achieved with our facultative temperate coral species of interest, *A. poculata*, allows us to ask these pointed questions. The overarching aim of our study was to analyze how nitrate enrichment and temperature stress influenced a coral's physiology at various symbiotic states. We hypothesized that the temperate coral *A. poculata* would demonstrate a lower photosynthetic efficiency, reduced polyp extension, an increase in R channel color values (i.e. bleaching), and metabolic stress (either depression or overactivity) under nitrate enrichment. We also predicted that corals undergoing heat stress would experience negative physiological effects proportional to increases in temperature they experienced over the 7-day heat ramp from 27°C-33°C, with individuals that underwent heat stress in addition to nitrate stress being more severely affected. Finally, we hypothesized that symbiotic corals would exhibit less severe physiological effects of each stressor (or a combination thereof) compared to aposymbiotic corals due to the energetic buffering effect of symboints (Burmester et al. 2017).

Throughout the 7-day experiment, the 25°C holding tank ranged in temperature from 24.38°C (±0.0177, n=2) to 25.25°C (±0.159, n=2) and from 34.05 (±0.035, n=2) to 36.43ppt (±0.412, n=2) in salinity, while the RAMP tank ranged in temperature from 26.58°C(±0.530, n=2) to 30.95°C (±0.566, n=2) and from 35.04ppt (±0.097, n=2) to 37.53ppt (±0.141, n=2) in salinity (Figure 1). This demonstrates that while salinity was relatively similar across days in both the 25°C holding tank and the RAMP tank, temperature steadily increased in the RAMP tank, while the 25°C remained relatively constant.

R channel color data was used as a proxy for bleaching, with higher R channel values indicative of more bleached fragments. Results show that the pre-experiment color values did not exhibit significant differences between treatments (P=0.958), indicating that there was an even distribution of symbiotic and aposymbiotic colonies across treatments. Consistent

with previous *A. poculata* research, there was found to be a difference based on symbiotic states (P=0.0097), with aposymbiotic corals possessing higher R values than symbiotic counterparts (Dimond & Carrington 2008). We predicted that all corals experiencing nitrate, temperature stress, or a combination of the two would experience bleaching when coloration was compared pre and post experiment. Interestingly, there was an overall difference in R channel values when comparing pre vs. post experiment fragments, with all treatments experiencing bleaching over the course of the experiment. (P=0.023). The two treatments that experienced the greatest change to their color values were the RAMP + N treatment, which moved from an average R channel value of 109.22 (±6.57, n=20) to 131.735 (±8.61, n=20), and the RAMP Control treatment which increased from 112.11 (±7.96, n=20) to 132.205 (±10.14, n=20, Figure 2). The change in R color intensity was similar for RAMP and RAMP + N treatments, indicating no effect of nitrate enrichment on bleaching. However, the colonies in the RAMP tank did experience greater bleaching than those in the 25°C tank, which aligns with previous studies that have demonstrated the bleaching effect of increased sea temperatures (Middlebrook et al. 2008). Corals in the Control 25°C treatment moved from an average R channel value of 108.5 (±8.24, n=20) to 116.27 (±9.16, n=20) and the 25°C + N treatment moved from 114.66 (±8.08, n=20) to 119.275 (±9.20, n=20). These results suggest that control 25°C corals experienced more bleaching than those under the 25°C + N treatment, reinforcing our conclusion that there was no effect of nitrate enrichment on bleaching. It is likely that all fragments in the 25°C tank exhibited bleaching over the course of the experiment due to the pre-experiment 36 hour 42 ppt stress event. Therefore, we can conclude that the observed increases in R channel values indicate dysbiosis or a shift in the coral's microbiome community, with a larger effect seen in corals experiencing the RAMP (27°C-33°C) temperature regime (Sharp et al. 2017).

We predicted that the presence of nitrate would cause significant declines in the Fv/Fm of *A. poculata*. However, our results displayed that there was no overall significant effect of either treatment (P=0.66) or symbiotic state (P=0.8898) on photosynthetic efficiency. Despite this, the results showcased in Figure 3A do note that the steepest declines in Fv/Fm during the experiment's duration took place in the treatments of RAMP Control and Ramp + N, indicating that temperature may have played a stronger role in altering photosynthetic efficiency than nitrate presence (Figure 3A). When we investigated the effect of treatment and symbiotic state on the final day (7) of the experiment, we found that there was a significant effect of treatment on Fv/Fm (P=0.0006), and that corals in the RAMP treatments exhibited lower photosynthetic efficiency Fv/Fm values. This is likely due to the increased bleaching we saw in these colonies–with decreased levels and/or disfunctional photosystem II machinery in their *Breviolum psygmophilium* symbiont, *A. poculata* corals in the RAMP treatment had decreased photosynthetic efficiency. Surprisingly, there was no significant effect of symbiotic state on photosynthetic efficiency on day 7. PAM measures the efficiency of all photosynthetic organisms, including the endolithic algae that was observed encrusting the skeleton of aposymbiotic *A. poculata*. Therefore, we hypothesize that Fv/Fm values were similar across symbiotic states due to the effect of endolithic non-*Breviolum* algae, rather than the efficiency of *B. psygmophilium* photosystem II. A potential next-step to clarify the effect of treatment on symbiont health would be to measure chlorophyll A concentrations in symbiotic and aposymbiotic colonies across treatments, with the expectation that there would be lower chlorophyll A presence in aposymbiotic colonies exposed to thermal stress. In addition, repeating this experiment with cultured *B. psygmophilium* would give us a more clear picture of the effect of nitrate and thermal stress on symbiont physiology.

Polyp extension, in which *A. poculata* polyps emerged from their corallites in response to a food cue was used as a determinant of overall colony health, as healthy corals are more likely to extend their tentacles to feed (Burmester et al. 2017). On days 2 and 4, there was a significant effect of symbiotic state on the corals' polyp extension ($P = 0.003$ and $P < 0.0001$, respectively), with symbiotic corals exhibiting much greater polyp activity than their aposymbiotic counterparts. This greater polyp activity in symbiotic

corals on day 2 and 4 is likely a response to the 36-hour 42ppt stress event that occurred pre-experiment, which is likely to have negatively affected the health of the aposymbiotic corals more than symbiotic, as they symbiotic corals have energetic buffering symbionts that make them more resilient to acute wounding and stress (Burmester et al. 2017). The trend of increased polyp extension in symbiotic colonies does not hold on the last day of recording, however, perhaps due to the aposymbiotic corals having fully recovered from the salinity stress event.

On day 6, the final day for recording polyp extension, there a significant effect of treatment on polyp extension ($P < 0.0001$), with fragments in the RAMP Control and RAMP + N treatments exhibiting significantly lower polyp activity than those within the 25° C holding tank (Figure 3C, and 4B). This suggests that the temperature in the RAMP tank on day 6, 30.6°C (±0.78) may be a critical temperature for *A. poculata* corals that results in a decrease of health and food capture ability. These results are similar to a recent study of *A. poculata* thermal stress, which also observed decreased polyp extension at 31°C (Wuitchik et al. 2021).

Baseline net respiration analysis (corrected to coral surface area) was performed at the end of the experiment (day 7) in order to assess coral health, with the expectation that corals that had been exposed to temperature or nitrate stress would exhibit baseline net respiration that was significantly different (either metabolic depression or overactivity) than the 25°C control corals. Interestingly, there was no observed significant effect of either treatment or symbiotic state ($P = 0.9535$ and $P = 0.1143$) on baseline net respiration rate, and no general trends could be observed. Notably, the highest respiration value occurred in the aposymbiotic corals within the RAMP + N treatment, but this was also accompanied by the largest amount of error (Figure 5). Limitations of this method & analysis include the low respirometry experiment duration (40 minutes) and low sample count (29 corals), therefore more robust and length respirometry experiments should be conducted before we can confidently assume there is no relationship between thermal/nitrate stress and baseline net respiration.

There were various limitations encountered in this study. The corals were originally collected in Ft. Wetherill, not originally intended for the purpose of fragmentation, and therefore varied in size and ability to be fragmented. In addition, the fragmentation of colonies into four genetically identical ramets induced a significant wounding stress. Although the corals were able to recover for 12 days, it is possible that their integrity was compromised. Unfortunately, the corals experienced a 36-hour 42 ppt salinity stress before the start of the experiment, potentially compromising their health and fragmentation recovery process. We did implement a survivorship status for every day of our experiment following the salinity stress, but due to the retraction of *A. poculata* polyps into their skeleton this metric was not reliable and therefore the experiment proceeded with all fragments (*pers. obs.)*. The salinity issue was corrected and corals were allowed 1 day without nitrate stress to recover, but the corals may have sustained irrevocable damage. Previous studies have demonstrated that salinity stress over 40 ppt is not always lethal to corals, but it is high enough to start the process of coral bleaching and damage tissues (Chavanich et al. 2009). Also, we found the PAM to be rather variable in its Fv/Fm readouts, and although we decided to keep the two values closest to each other, the values did vary quite drastically in some cases. Finally, there were various different investigators that took PAM data over the course of the days, which may have introduced bias in the polyps selected for PAM analysis.

Taken together, these results demonstrate a strong influence of temperature on the health of *A. poculata*. Importantly, we found the negative effects of thermal stress began to occur at approximately 31°C, with consistent impacts of color bleaching, reduced photosynthetic activity, and lowered polyp extension observed at this temperature. This suggests that 31°C is a critical threshold for *Astrangia poculata* health (Figure 3). Overall, nitrate presence was not found to have a significant impact on any of the surveyed variables. This suggests that *A. poculata* corals are relatively resilient to nitrate enrichment stress as compared to thermal stress.

This work lays the foundation for future research on the physiological effects of various

nutrients (including nitrates, nitrites, ammonium, and phosphates) on facultatively symbiotic corals, as each may pose different challenges. In fact, ammonium and phosphates tend to benefit corals, so research into conservation strategies concerning ammonium inputs may be worth investigating (DiRoberts et al. 2021). Capitalizing on the facultative nature of the *A. poculata* model organism to investigate the effects of nutrient pollution, thermal stress, and a combination of the two will allow the scientific community to gain insights into how the coral animal, symbiont, and holobiont will survive (& perhaps thrive) in the decades to come.

VI. Acknowledgements & Works Cited

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