Cold stress inhibits photosynthesis in the coral model *Exaiptasia pallida*

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Abstract

Extreme changes in sea surface temperatures driven by climate change are the result of continuous anthropogenic activities increasing carbon dioxide concentrations in the atmosphere. Varying temperatures lead to coral bleaching, a process by which cnidarian hosts expel their endosymbiotic algae, rendering the host susceptible to disease or death. In response to bleaching, cnidarians must rely more heavily on their ability to capture food, rather than photosynthates from their symbionts. Vice versa, during food scarcity, cnidarians rely on their symbiont's photosynthetic ability to provide food. While the effects of temperature stress on cnidarians are well studied, the combined effects of thermal and food stress are not well known. Here, we consider the effects of both heat and cold thermal stress along with different feeding regimes on the host and symbiont of the anemone *Exaiptasia pallida,* a common model organism. *E. pallida* were exposed to three temperature treatments: cold (18^{o}-1^{o}C/day), hot (18^{o}+1^{o}C/day), and ambient (18^{o}) for 14 days. Within each temperature treatment, individuals were divided into two feeding regimes: fed and starved. Symbiont photosynthetic efficiency (Fv/Fm) was measured using pulse amplitude modulation (PAM), while chlorophyll concentration was determined using photograph-derived intensity in the red channel (IRC). Host physiology was assessed using pedal disk diameter measurements and mortality observations. Cold stress reduced symbiont photosynthetic efficiency, pedal diameter, and intensity in the red channel. However, feeding regimes had no influence on the form or function of the holobiont. As climate change continues to progress and temperatures shift beyond cnidarians' typical range, understanding how they respond to both cold and hot thermal stress is imperative to assess future survivability.

Introduction

The symbiotic relationship between cnidarians and their algal symbionts (*Symbiodiniaceae*) is essential for the maintenance of tropical coral reef ecosystems (Davy and Cook, 2001). The coral host provides structure and inorganic materials required for the algal symbiont to photosynthesize, while the symbiont provides photosynthetically-derived carbon sugars to the host (Rosenberg et al., 2007). Photosynthetic efficiency of symbionts can be decreased by photoinhibition in photosystem II, which is often caused by environmental stressors including temperature (Takahashi et al., 2009).

Elevated sea surface temperatures, which are increasing in frequency with climate change, cause a disruption between the endosymbiosis of cnidarians and their *Symbiodiniaceae* (Wild et al., 2011). Coral bleaching is the common response to these changes in temperature, as it is a process by which the coral or other cnidarian hosts

expel their zooxanthellae symbionts as a survival mechanism (Hoegh-Guldberg, 1999). This results in the loss of coral coverage and the overall destruction of reef ecosystems (Hoegh-Guldberg et al., 1989). Increased thermal stress affects the organism's ability to control and regulate incoming bacteria from their tissues resulting in higher symbiont densities (Ahmed et al., 2019). In turn, symbiont densities have been implicated in susceptibility of bleaching, which is likely a result of higher reactive oxygen species production past their antioxidant threshold (Cunning and Baker, 2013). Additionally, heat stress is also known to reduce overall photosynthetic efficiency but may not always lead to bleaching events (Gegner et al., 2017). This reduction in photosynthetic efficiency from decreased symbiont density may lead to an increased dependency on external food sources.

Starvation or infrequent feeding has been found to hinder both individual and overall population growth of cnidarians (Clayton et al., 1985). When faced with infrequent food sources or periods of prey scarcity, starvation is known to significantly reduce gross photosynthesis and algal density within the host due to nutrient deprivation, but does not reduce the photosynthetic capability of *Symbiodiniaceae* (Clayton et al., 1984). In these nutrient-poor environments, symbionts can revert to dark carbon fixation through NH4+ enhancement, continuing to provide essential energy to the host (Cook et al., 1992). In nutrient-rich environments, symbiont density may increase for some cnidarians due to the mitigating effects of heterotrophy (Aichelman et al., 2016).

Studying coral bleaching in tropical climates is challenging for many reasons: different coral species respond differently to the same stressors (Bove et al., 2019), it can be hard to determine which stressors are eliciting specific phenotypic responses, and these stressors are often not well defined (Brown, 1997). However, *E. pallida* are an attractive model species to understand coral bleaching and climate change resilience because they are closely related to corals taxonomically (Lehnert et al., 2012) and have been shown to similarly respond to stressors (Grajales, 2014). Understanding how *E. pallida* responds to food scarcity and thermal stress is crucial in determining its overall resilience to environmental changes. Additionally, many molecular and genetic tools are available to study *E. pallida* to better understand *cnidarian-Symbiodinium* symbiosis, marine toxins, and interactions between heterotrophy and autotrophy (Leal et al., 2013).

The direct influence of thermal stress and food availability on *E. pallida* has not previously been considered. In addition, little research has been done to assess the effects of both hot and cold temperature extremes on marine organisms. Climate change is making temperature extremes more prominent within the ocean (Riegl et al., 2011). Therefore, to better understand the resilience of marine organisms at extreme temperatures, we investigate thermotolerance and the impact of feeding regimes on photosynthetic efficiency of *E. pallida* in both increasing and decreasing oceanic temperatures. We hypothesize that

the combined effects of temperature extremes and food scarcity will inhibit photosynthetic efficiency and reduce chlorophyll density through symbiont bleaching. Examining the interaction between these stressors will be useful in determining how cnidarians respond to anticipated temperature shifts beyond their ecological range as a result of climate change. This study will therefore provide insight on future reef ecosystem health and recovery.

Methods

Experimental Design

Exaiptasia pallida (n=78) were obtained from Carolina Biological Supply Company. Individuals were gently removed from their original tank and randomly allocated to one of the three temperature groups such that each treatment had 26 individuals. Temperature treatments included a hot treatment, cold treatment, and ambient treatment (control). Within each treatment, the 26 individuals were split evenly between two feeding regimens: starved (n=13) and fed (n=13). All tanks started at 18˚C and were warmed or chilled by 1˚C each morning at 10 am for the hot and cold treatment respectively (Figure 1). Each bin within the larger tanks was equipped with water flow control and circulation. Aquaria water was made with DI water and Instant Ocean ® and was monitored twice daily for salinity to maintain a target concentration of 35 ppt. Cleaning and partial water changes occurred post-feeding every 3 days. Extra saltwater was prepared and kept at a constant salinity to be available for water changes. For detailed experimental design please refer to Figure S1**.**

Anemone Husbandry

Exaiptasia pallida were fed every three days, for a total of five feeding sessions throughout the two-week experiment duration**.** Prior to each feeding, sump flow was removed from feeding bins to isolate anemones from the larger tank and prevent food spillover into the system. Fed treatment groups were target fed Reef Chili brand coral food (2.64 cc / 135 mL seawater), while starved groups were given 135 mL plain seawater to mimic target feeding disturbance. All treatments were left for 1 hour. After feeding, water was removed from all isolated tubs to remove leftover or regurgitated food from the system, and replaced with freshly prepared saltwater. Flows were then replaced in each bin to reconnect water circulation through the tank.

Symbiont Physiology

To quantify photosynthetic efficiency, pulse amplitude modulation (PAM) measurements were taken from 8 am to 10 am on day 1, day 7, and day 14 of the experiment using Junior PAM and the PAM software WinControl-3. *Exaiptasia pallida* were dark-adapted for 12 hours before data collection. PAM parameters were set to the following specifications: saturation pulse width 0.6, saturation light intensity 12, electronic signal damping 2, electronic signal gain 4, and measuring light intensity 2 (Aichelman, 2017). Three replicate PAM measurements were taken for each individual and then averaged to obtain a

single value per individual. Values above 0.700 were not accepted as they were likely not real as noted by Thornhill et al., (2008). Chlorophyll density was determined using intensity in red channel (IRC) of photographs. By using IRC as a proxy of chlorophyll density, the integrity of the individuals could be maintained throughout the experiment. Images of *E. pallida* were taken after partial water changes on each feeding day using Duyoi Otoscope Wireless Endoscope accompanied by Y camera smartphone app. Two images per individual anemone were taken; one of the oral disk and one of an individual tentacle to ensure accurate representation of the symbiont distribution (Figure 2)**.**

Host Morphology

Pedal disk diameter (PDD) was measured on the last day to the nearest millimeter using standard laboratory calipers as described in Leal et al., (2013). Mortality was monitored to track organismal death and potential reproduction throughout the duration of the experiment (Figure S2). Organisms were removed from the aquaria and fixed on the final day in 200 proof EtOH and stored at -80˚C for future genomic analyses.

Statistical Analysis: Symbiont physiology and host morphology

Pulse amplitude modulation and PDD data were not normally distributed, but fell within the acceptable theoretical quantile regression parameters. Therefore, data was treated as parametric. To test the effect of temperature and feeding regime on photosynthetic efficiency and host morphology, one-way analysis of variance (ANOVA) statistical tests were performed, and significance was confirmed with the Tukey Honest Significant Difference (HSD) post-hoc test. The effect was considered significant if the reported p-value was < 0.05. All statistical analyses were performed with R software version 1.3.1073 using tidyverse and the base mosaic packages.

Statistical Analysis: Photocolor analysis/RGB

Exaiptasia pallida photographs were color corrected in Adobe Photoshop (2021) to find true white points for color comparison. To quantify color intensity, color corrected photos were analyzed in MATLAB following the AnalyzeIntensity protocol by Winters et al., (2009). Ten points were randomly selected within the photographs and were analyzed for their respective intensity in red channel (IRC) values. The values were then averaged to obtain a single IRC value per organism on days 1 and 14. To test the effect of temperature and feeding regime on *E. pallida,* an ANOVA test was run to analyze their respected IRC values as a proxy for chlorophyll density. Significance was confirmed with the Tukey Honest Significant Difference (HSD) test. The effect was considered significant if the reported p-value was < 0.05. Statistical analyses were performed with R software version 1.3.1073 using tidyverse and the base mosaic packages.

Results

Photosynthetic efficiency of Symbiont

The photosynthetic efficiency of *Exaiptasia pallida* photosystem II (Fv/Fm) symbionts was significantly affected by temperature treatments (Figure 3. ANOVA, $p = 3.7e\text{-}4$). For cold treated anemones, Fv/Fm values were significantly lower than photosynthetic efficiency of hot treated individuals (Figure 3. TukeyHSD, $p = 3.2e\text{-}4$). Over the course of the 14-day experiment, time did not significantly affect Fv/Fm for any treatment (Figure S3. ANOVA, $p > 0.05$). However, the photosynthetic efficiency of cold-exposed *E. pallida* decreased over the duration of the experiment. Individuals in differential feeding treatments were not significantly affected by the presence or absence of food, independent of temperature treatment (Figure 4. TukeyHSD, $p = 0.47$).

Chlorophyll concentration of Symbiont

Chlorophyll density of *E. pallida*, determined by intensity in red channel, was significantly affected by temperature treatments when compared to the ambient treatment (Figure 5. ANOVA, $p = 0.0408$). Intensity in red channel for cold-treated anemones was significantly lower than hot treated *E. pallida* (Figure 5. TukeyHSD, $p < 0.05$). Feeding regime did not significantly affect the intensity in red channel for *E. pallida* across any temperature treatments for all days of experimentation (Figure 6. TukeyHSD, $p = 0.135$). However, chlorophyll density of *E. pallida* on day 14 was significantly impacted by temperature treatments (Figure 6. ANOVA, $p = 3.89e\text{-}09$).

Host morphology

Pedal disk diameter (PDD) of *E. pallida* was measured on day 14 of the experiment. Temperature treatment significantly affected average anemone size at the end of this experiment (Figure 5. ANOVA, $p= 1.6e\text{-}13$). Anemones exposed to the cold treatment were significantly smaller compared to individuals in both ambient and hot treatments (Figure 7. TukeyHSD, $p < 0.05$). Again, the feeding regime did not affect average PDD across any treatment groups (Figure S4. Tukey HSD, $p = 0.8$).

Discussion

The present study aims to understand how feeding regime and thermal stress affect *Exaiptasia pallida* to better understand the response of cnidarians to climate change. We hypothesized that exposure to extreme hot and cold temperatures, in addition to starvation, would lead to an overall decrease in photosynthetic activity (Fv/Fm) and chlorophyll density due to symbiont bleaching. Contrary to our hypothesis, cold stress more negatively impacted both physiology and morphology of *E. pallida* resulting in a decrease in the holobiont photosynthetic activity, intensity in red channel, and pedal disk diameter (PDD) than heat stress or feeding regime.

Response to thermal extremes

Overall, exposure to cold stress had more of a negative effect on *E. pallida* across all variables measured compared to heat stress. These negative effects were consistent in photosynthetic efficiency,

chlorophyll density and morphology. Surprisingly, elevated temperatures had minimal effect on host-symbiont physiology compared to the control ambient temperature. However, given their natural tropical/subtropical range, *E. pallida's* resilience to higher temperatures should not be overlooked (Trenfeld et al., 2017). In contrast, exposure to extreme cold temperatures negatively affected them more intensely.

Photosynthetic efficiency was significantly reduced in cold treated *E. pallida* compared to hot treated individuals. Lower efficiency therefore indicates that cold treated individuals had reduced photosynthetic activity (Howe et al, 2017). Typical temperature treatment experiments on cnidarians focus on elevated temperatures, however few have documented the effects of cold stress on photosynthetic efficiency. Bellis and Denver (2017) noted that long-term exposure to cold stress (4^{o}C) consistently resulted in the death of *E. pallida* colonies. After two consecutive cold-shock exposures, all *exaiptasia* anemones moderately bleached their symbionts regardless of host-strain specificity (Bellis and Denver, 2017). Cold stress has also been shown to inhibit photosynthetic efficiency in the coral species *Montipora digitata*, and elicits a response similar to that observed in heat-stressed individuals (Saxby et al., 2003). This photosynthetic inhibition response is consistent with our findings across the decreasing temperature gradient for *E. pallida*.

Not only did symbiotic efficiency decrease in response to cold temperatures, but symbiont density was also negatively affected. Cold treated *E. pallida* had reduced chlorophyll density (IRC) compared to hot treated *E. pallida.* Lower chlorophyll densities are attributed to coral bleaching, as the host expels the photosynthetic symbionts within their tissues and decreases their internal algal concentration (Hoegh-Guldberg, 1999). Pulse cold-shock techniques have been shown to induce substantial expulsion of *E. pallida* symbionts (Muscatine et al.,1991). The loss of *symbiodinium* due to short term cold stress is concerning in respect to the increasing frequency of cold-water upwelling events and decreasing seasonal low temperatures in tropical reef habitats.

Pedal disk diameter was measured on day 14 of the experiment in order to provide a morphological comparison between temperature exposures. Cold stressed *E. pallida* was significantly smaller in size compared to both ambient and hot treatments. Anemone size determination has been associated with assessment of overall biomass, and suggests that cold-treated *E. pallida* have reduced biomass compared to ambient and heat stressed individuals (Leal et al., 2013). Distinct morphological changes have been observed in cold-treated *E. pallida* that are not seen in heat-treated individuals. Cold treated *E. pallida* display shorter and stubbier tentacles, possibly from degradation or tentacle tissue contraction (Bellis and Denver, 2017). Anecdotally, although not directly measured in this study, substantial tentacle retraction was observed in cold-treated anemones which resulted in the inability to collect tentacle intensity in red channel on day 14 of the experiment.

For each measured variable, we saw substantial deleterious effects of cold exposure on *E. pallida,* suggesting their heightened vulnerability to decreasing oceanic temperatures.

Response to feeding regime

Conversely, feeding regime had no significant effects on photosynthetic efficiency, chlorophyll density, and morphology of *E. pallida*. We expected to see a decrease in photosynthetic efficiency of symbionts in the absence of food due to the decreased availability of inorganic compounds from the host species. In the presence of thermal stress, it was anticipated that the chlorophyll density and host morphology would not decrease in the absence of food because of the host coral's increased dependence on symbionts as a food source. Our results indicated a potential decrease in chlorophyll density on day 14 for hot-starved and cold-starved individuals compared to their respective fed individuals. This could be indicative of the anemone's inability to provide sufficient nutrients, such as ammonium, to their symbionts through heterotrophy (Aichelman et al., 2016).

Experimental Limitations

While our study brings light to the effect of cold stress on cnidarians, there were limitations that resulted in the need for more expansive methods. The extent of the experimental design was limited by time, resources, and scope. Due to the short duration of the experiment, our results were not representative of long-term effects of thermal stress and food availability. Some studies have assessed the physiological response of thermal stress in *exaiptasia* over the duration of multiple years (Ahmed et al., 2019).

Specifically, the short time series could explain the lack of starvation induced response in *E. pallida*. An extended experimental duration, in addition to a wider range in temperature variation might have intensified the difference in photosynthetic efficiencies and chlorophyll concentrations across temperature treatments and feeding regimes. The heat stress maximum did not exceed the ideal aquaria temperature of 26^{o}C for *E. pallida* species (Leal et al., 2013). Future related studies should increase the time series and expose them to higher temperatures, such as in Sunagawa et al.'s 2008 study where *E. pallida* were exposed to temperatures of 33^{o}C as a heat stressor. In addition to limitations in quantifying photosynthetic efficiency and chlorophyll concentrations over time, proper assessment of mortality was hindered by time and metrics. Future studies should track individuals over time in order to better quantify individual organism's response to the stressors examined.

Cold treated *E. pallida's* lower chlorophyll density, smaller biomass, and slower photosynthetic efficiency demonstrate that cold stress was a greater stressor on *E. pallida* than heat stress and starvation, hinting at their trouble surviving in cold waters. Overall, this study highlights the need for expanded research on lower limit thermal tolerance as climate change shifts toward both warmer and colder thermal extremes.

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Figures

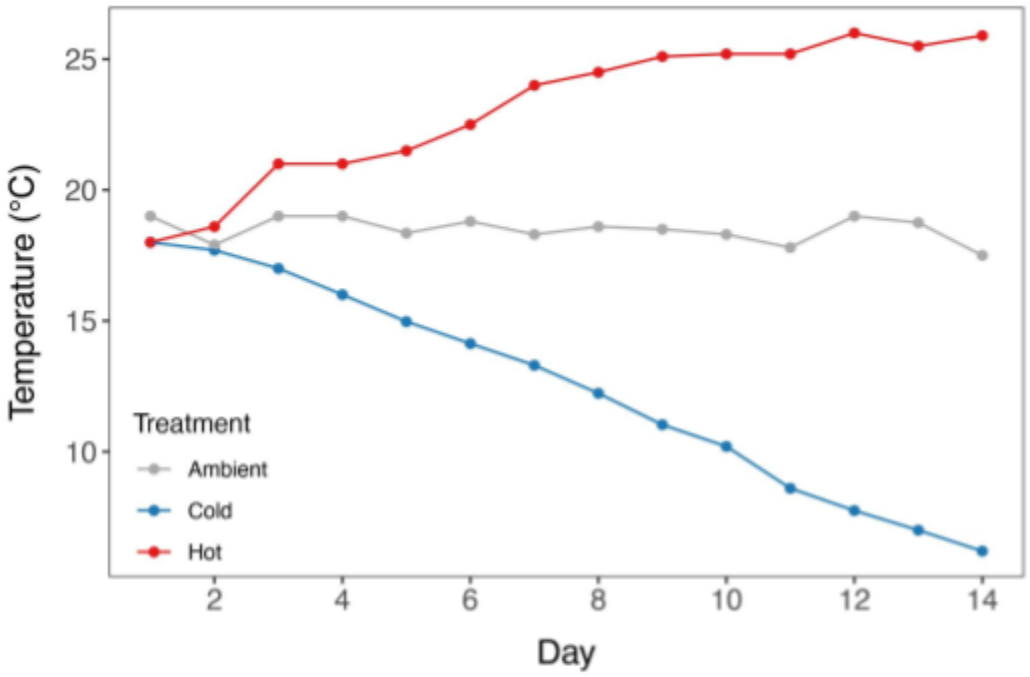

Figure 1. Experimental Water Temperatures. Temperature was monitored at least once daily in all treatments for the duration of the 14-day experiment. All treatments began at 18°C and increased, decreased, and remained consistent by 1°C per day for hot, cold, and control treatments respectively. The hot-treatment tanks reached a maximum temperature of 26.5°C, while the cold-treatment tanks reached a minimum temperature of 4°C.

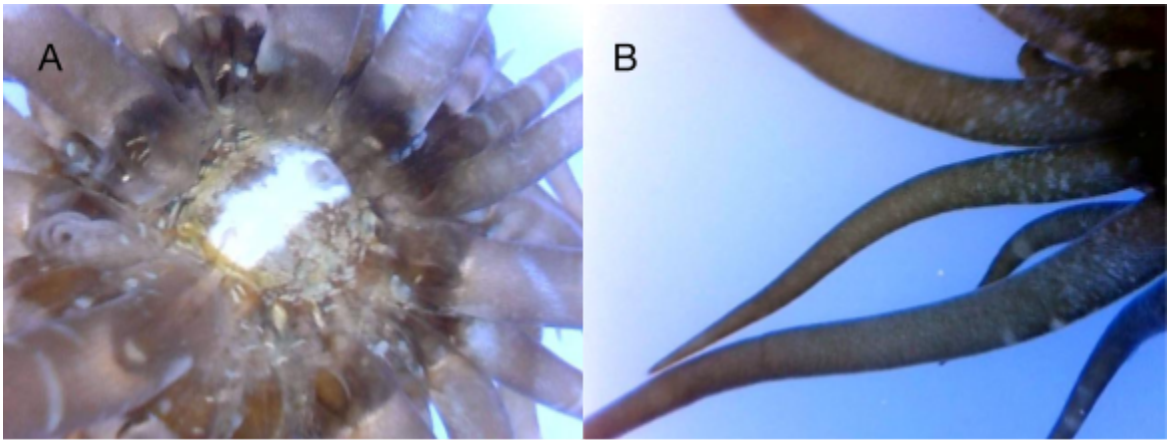

Figure 2. Images of *Exaiptasia pallida* for color analysis. Color corrected images of (A) an oral disk and (B) a tentacle of *Exaiptasia pallida*. Images were taken on day 1 of the experiment using Duyoi Otoscope Wireless Endoscope accompanied by Y camera smartphone app. Images were manipulated to find their average intensity in red channel as a proxy for chlorophyll density.

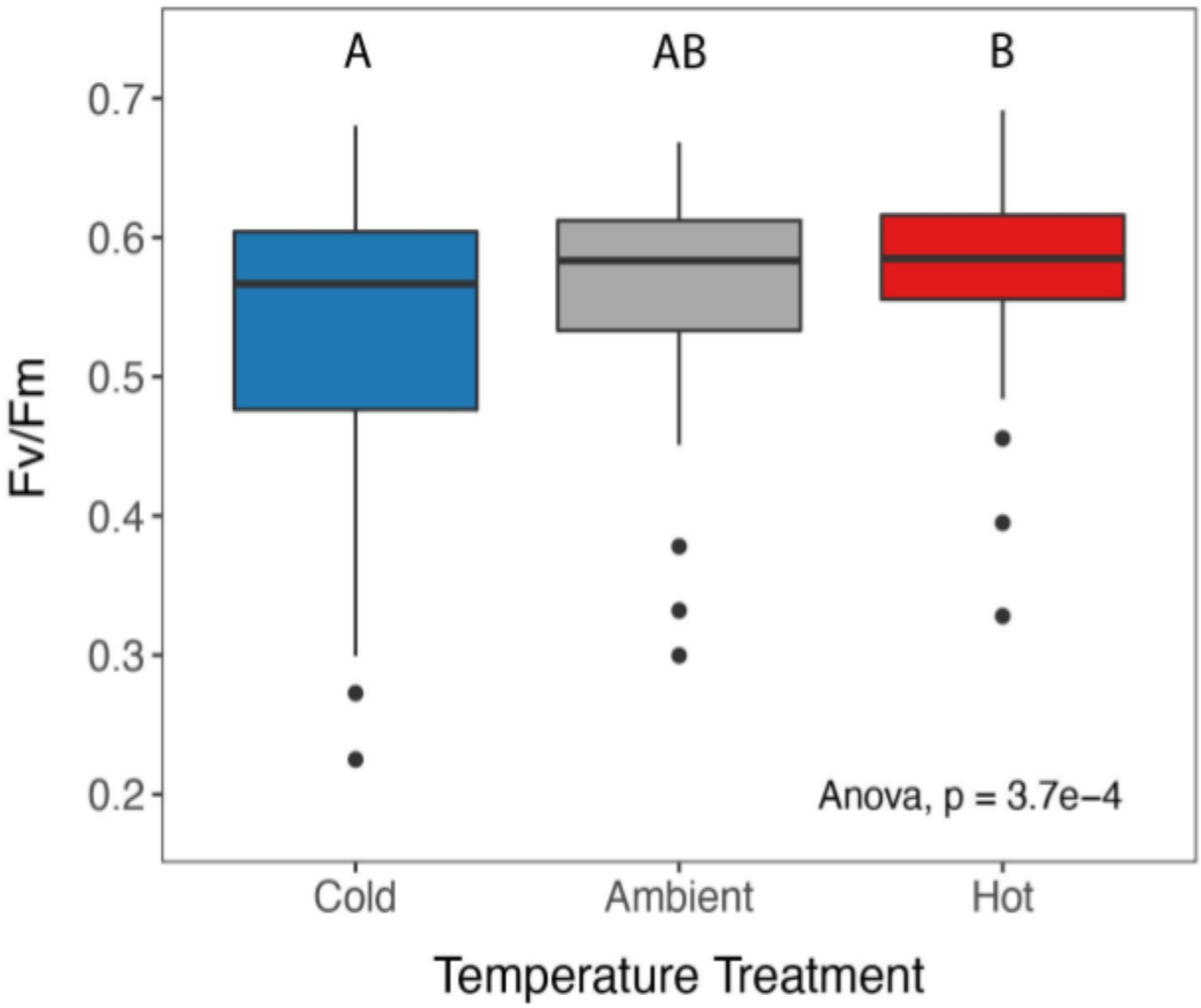

Figure 3. Average symbiont dark adapted photosynthetic ability of photosystem II (Fv/Fm) across all temperature treatments. Fv/Fm was measured and then averaged on days 1, 7 and 14 of the experiment using Junior PAM and PAM software WinControl-3. Fv/Fm was significantly affected by temperature (ANOVA, p = 3.7e-4). TukeyHSD post-hoc analysis indicated a statistically significant difference between cold and hot treatment groups, indicated by differential letter annotations (TukeyHSD, p = 3.2e-4). However, statistical analysis showed no significant effect of feeding regime on photosynthetic efficiency in any temperature group (TukeyHSD, p = 0.47).

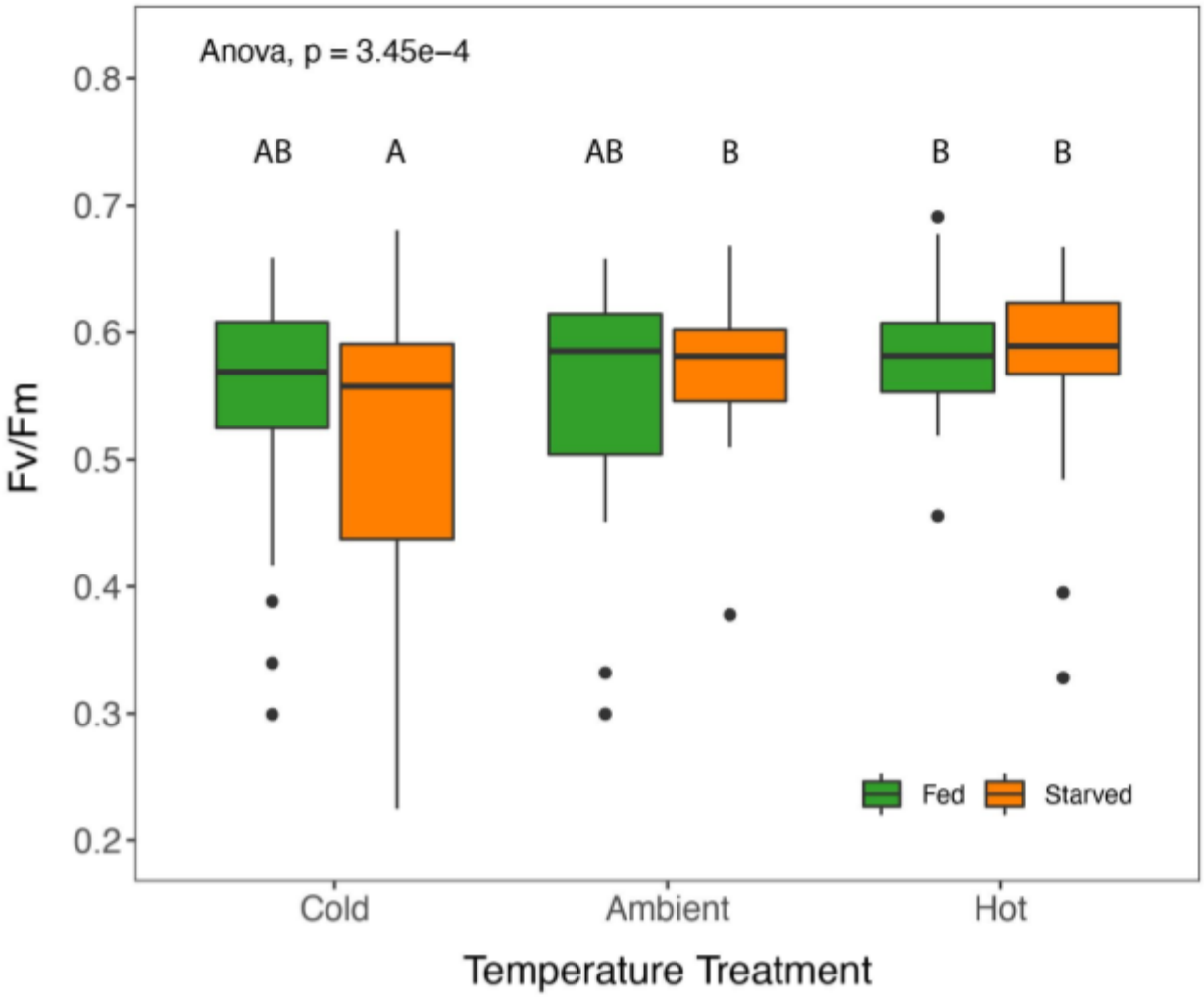

Figure 4. Average symbiont dark adapted photosynthetic ability of photosystem II (Fv/Fm) for fed and starved *Exaiptasia pallida.* Fv/Fm was measured on days 1, 7 and 14 of the experiment and then averaged using Junior PAM and PAM software WinControl-3. In contrast to temperature, feeding regime did not significantly affect Fv/Fm (ANOVA, $p = 3.45e\text{-}4$, $p = 0.47$ respectively). However, TukeyHSD showed that cold-starved anemones were significantly different from ambient-starved, and both hot-fed and hot-starved treatments (TukeyHSD, $p < 0.05$).

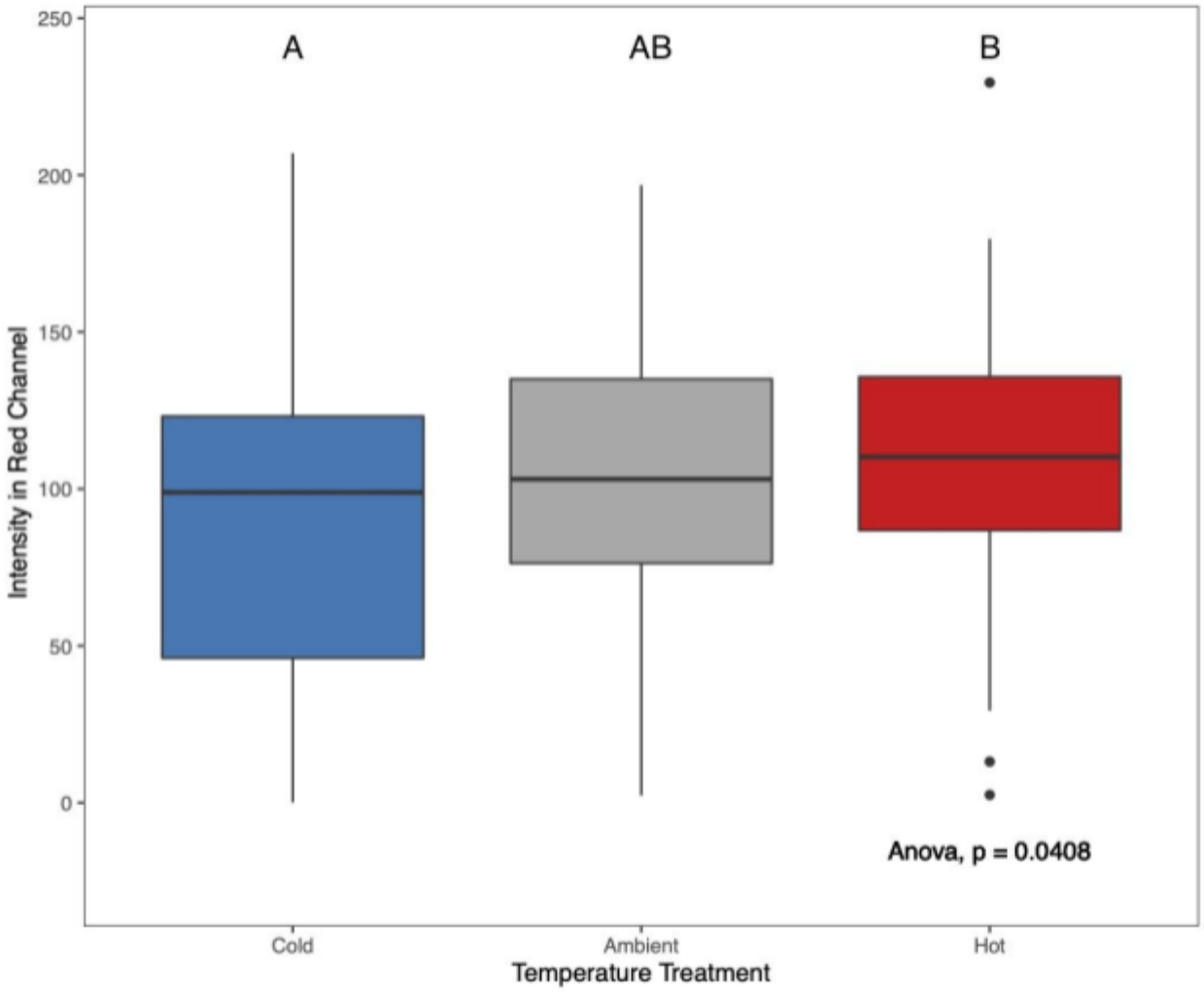

Figure 5. Average intensity in red channel for *E. pallida* across temperature treatments. Red channel intensity was measured using MATLAB following the AnalyzeIntensity protocol by Winters et al., (2009). Red channel intensity differed significantly across temperature treatments (ANOVA, $p = 0.0408$), where cold and hot treatments were significantly different (TukeyHSD, $p < 0.05$). Feeding regime had no significant effect on red channel intensity (ANOVA, $p = 0.135$).

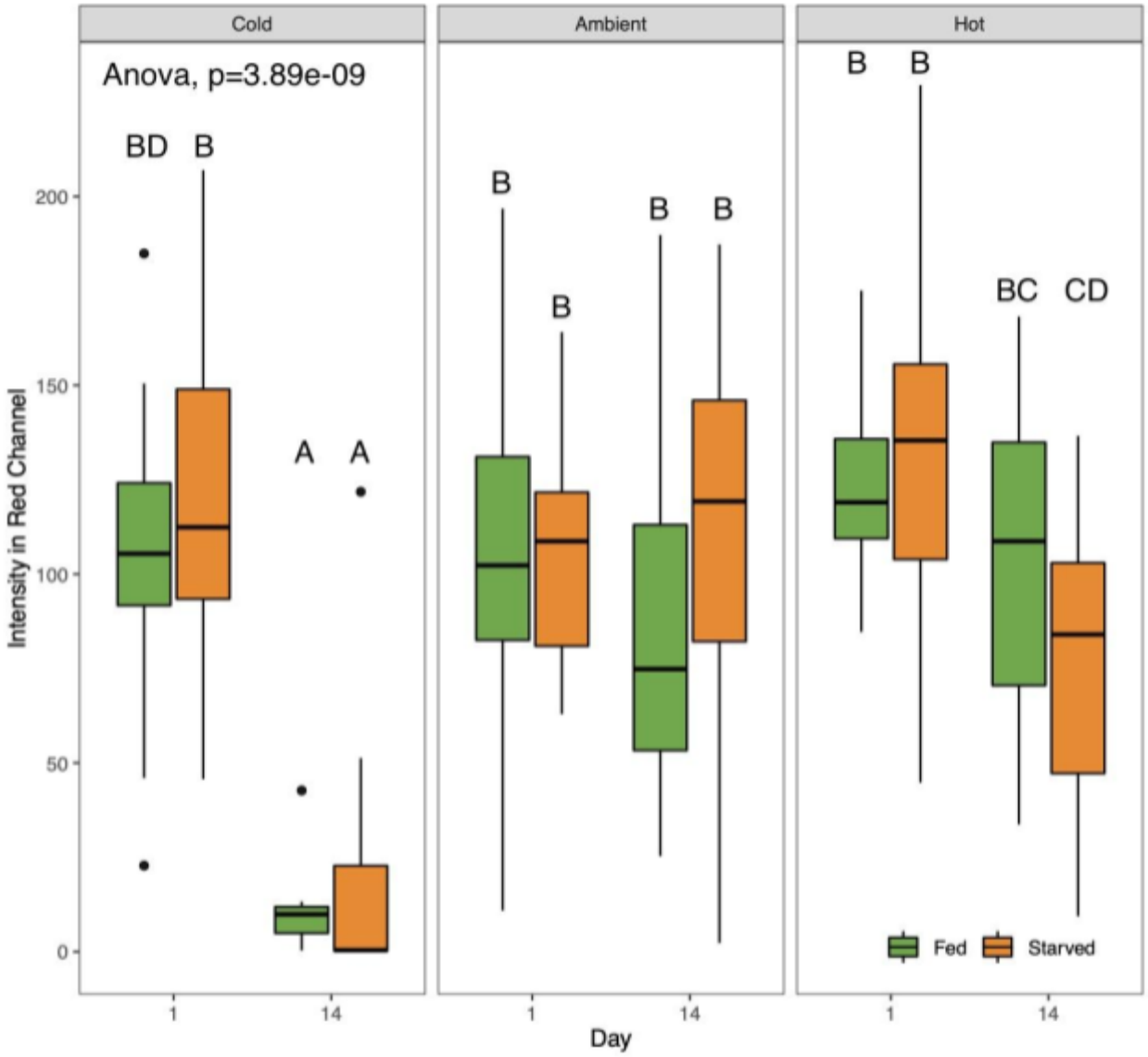

Figure 6. Change in average intensity in red channel from days 1 and 14 of the experiment. Red channel intensity was measured using MATLAB following the AnalyzeIntensity protocol by Winters et al., (2009). Red channel intensity differed significantly across treatments (ANOVA, $p = 3.89e\text{-}09$). Significant differences are indicated by different letters (TukeyHSD, $p < 0.05$).

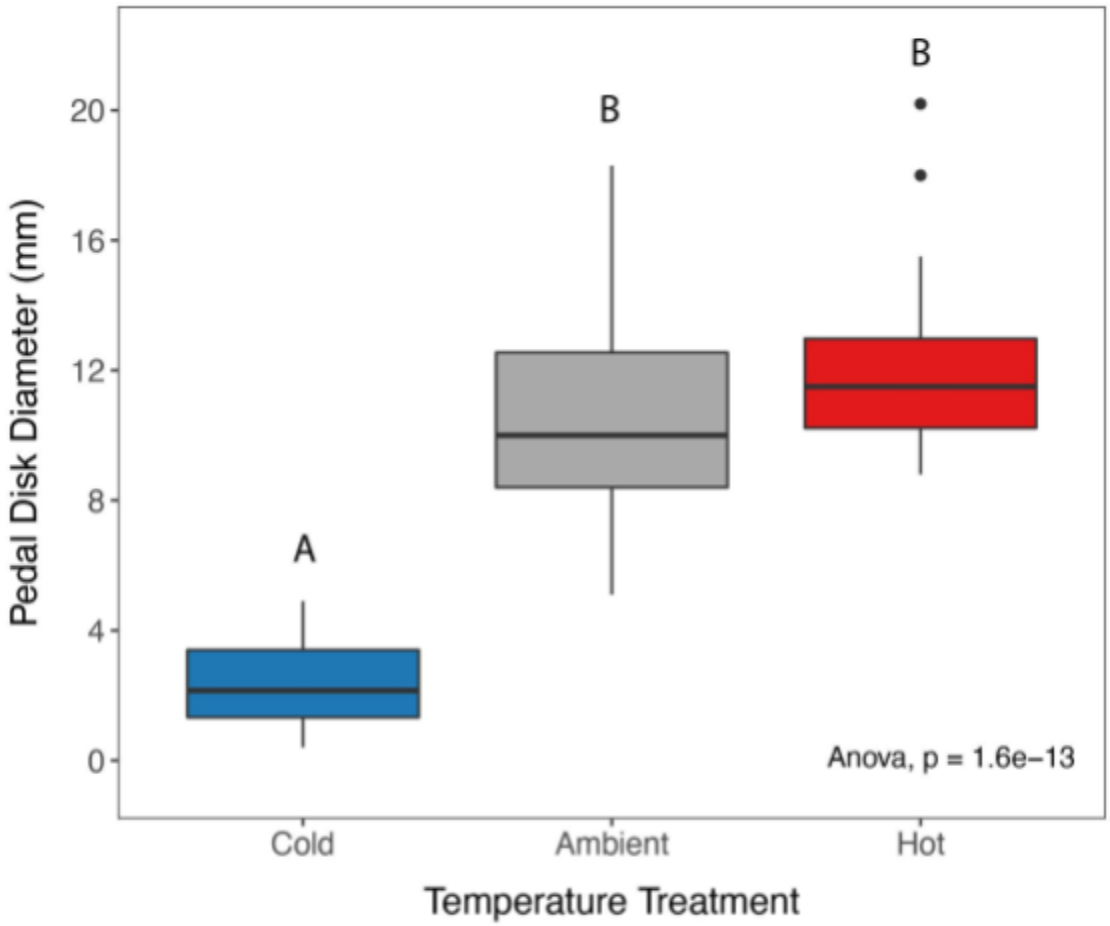

Figure 7. Average *Exaiptasia pallida* pedal disk diameter (mm) between temperature treatments. Host morphological differences were examined using pedal disk diameter (PDD) to the nearest mm as a proxy for anemone size and therefore biomass. Temperature had a significant effect on average PDD, (ANOVA, $p = 1.6e\text{-}13$), where cold-treated anemones differed significantly from both ambient and hot treatment groups (TukeyHSD, $p < 0.05$).

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Supplementary Materials

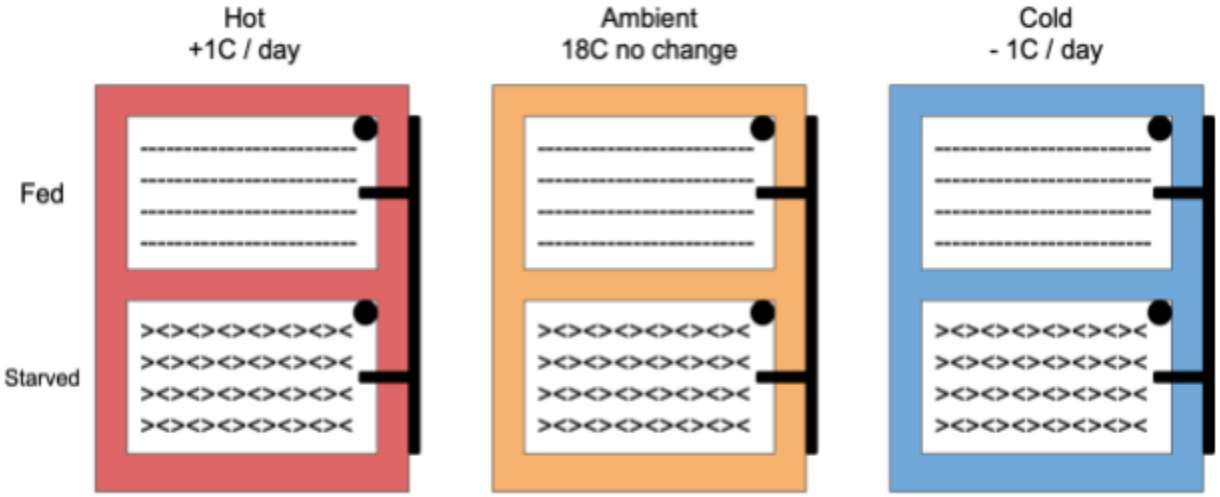

Figure S1. Experimental Design Scheme. Overview of the heat and food stress experimental design treatments of *Exaiptasia pallida* for a 14-day common garden experiment. Color represents the respective treatment temperatures, red: hot, beginning at 18°C and increasing by 1°C/day until maximum temperature was reached (26°C), yellow: ambient (18°C), and blue: cold, beginning at 18°C and decreased by 1°C/day until temperature reached 4°C. Dashed (-) and diamond areas (<>) represent fed (n=13) and starved (n=13) treatments. Black circles represent the water circulation pumps, and black bars represent water flow for the entirety of the system.

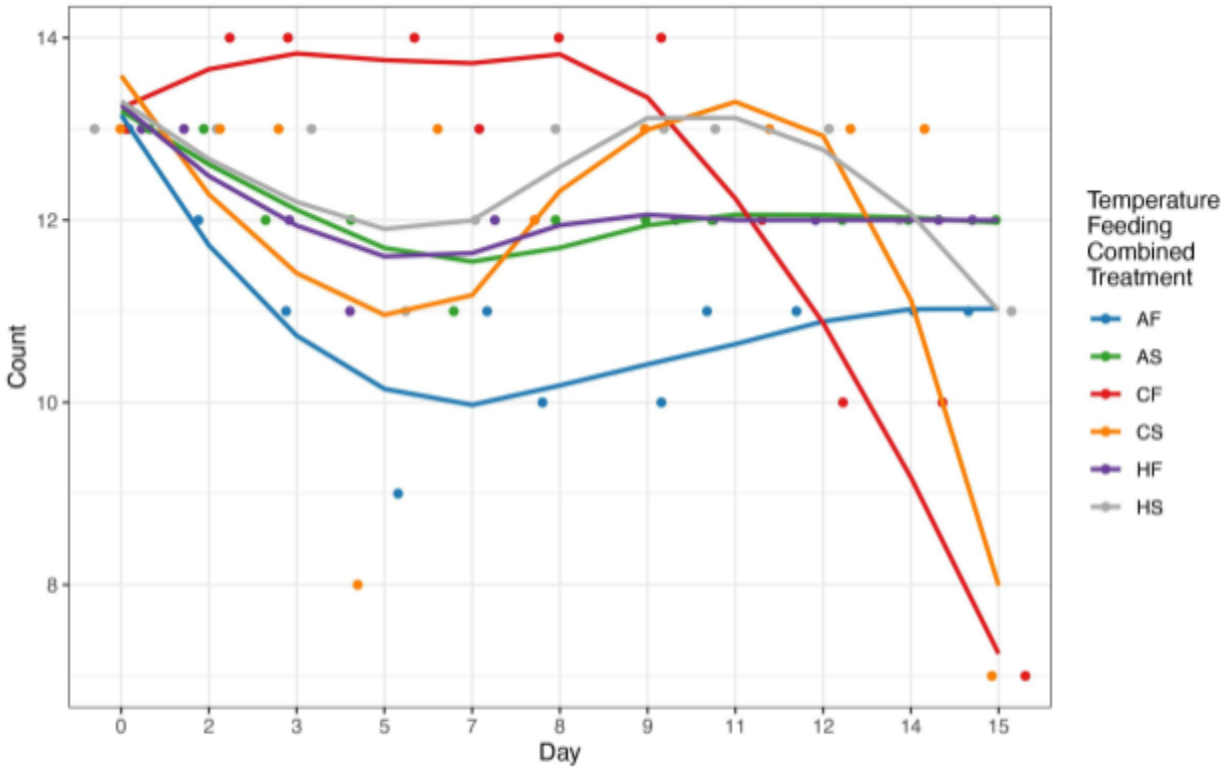

Figure S2. Count of *E. pallida* individuals by day over the 14-day experiment. Individuals were observed daily and total counts were recorded.

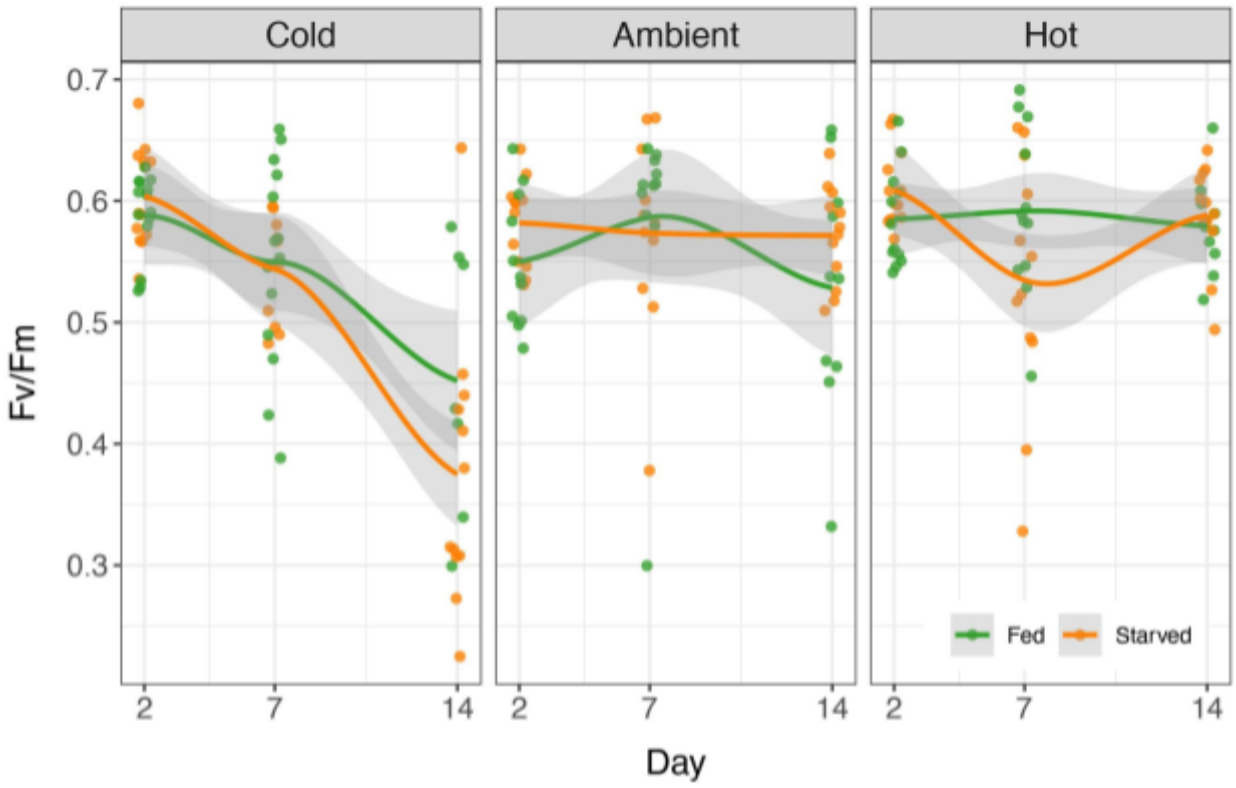

Figure S3. Change in *Exaiptasia pallida* symbiont dark adapted photosynthetic ability of photosystem II (Fv/Fm) over 14 day experimental period. Fv/Fm was measured on days 2, 7 and 14 of the experiment and then averaged using Junior PAM and PAM software WinControl-3. Time did not significantly affect Fv/Fm (ANOVA, $p > 0.05$). On day 14, Fv/Fm did not differ significantly between cold-fed and cold-starved groups (ANOVA, $p = 0.16$) despite the apparent drop in photosynthetic efficiency.

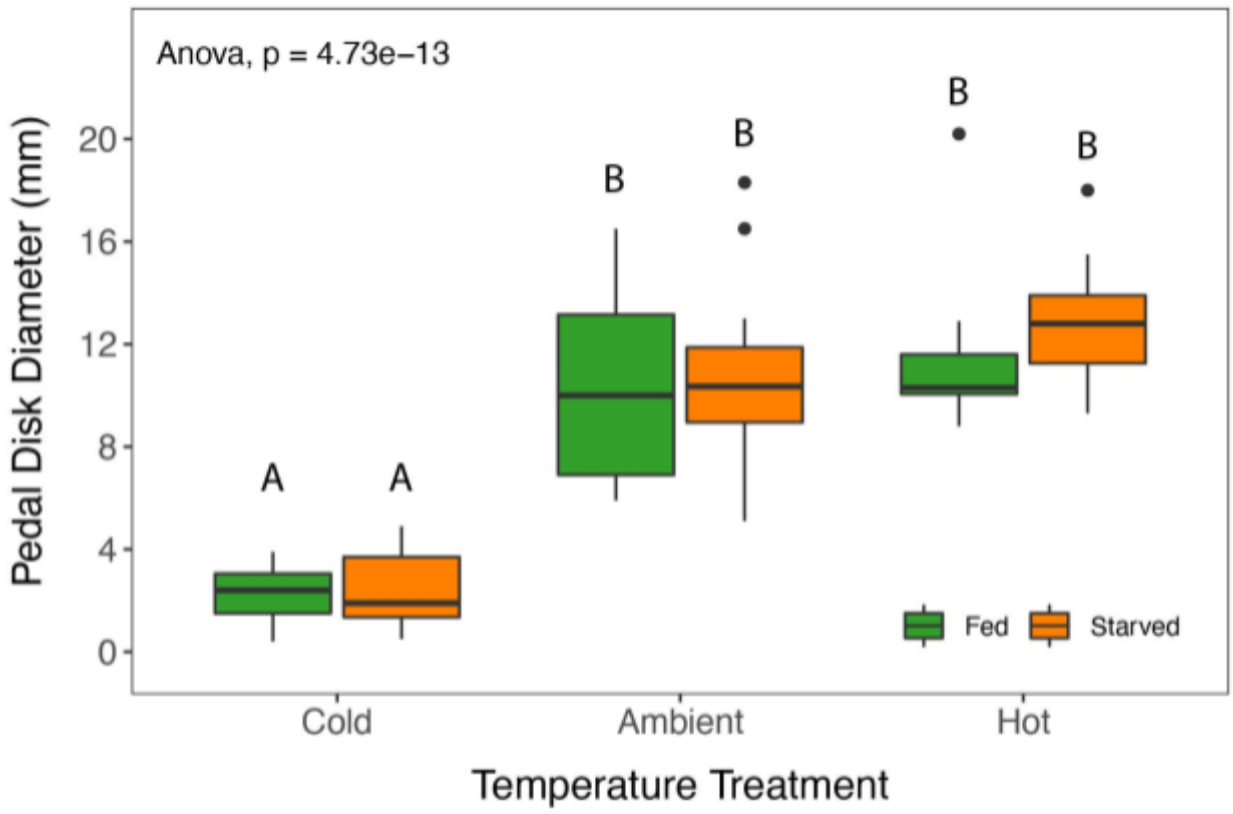

Figure S4. *Exaiptasia pallida* pedal disk diameter (mm) on day 14 of the experiment. Host morphological differences were examined using pedal disk diameter (PDD) to the nearest mm as a proxy for anemone size. Feeding regime had no effect on PDD (ANOVA, p = 0.8), while temperature accounted for the significant difference between treatment groups (ANOVA, p = 4.73e-13).