Thermal Stress Response Variation in *Exaiptasia pallida* is Dependent on Location and Exposure Time

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ABSTRACT

Increasing sea surface temperature (SST) reduces coral growth rates and disrupts the relationship between corals and their symbiotic dinoflagellates, thus affecting coral productivity. *Exaiptasia pallida* are small sea anemones shown to have a high resistance to temperature variations and are often used to study the effects of diurnal thermal variation (DTV) on bleaching and gene expression. Little is known about phenotypic response and gene expression across aposymbiotic and symbiotic strains and how this relates to increased resistance to heat stress. This two-week experiment measured and compared the productivity, bleaching, reproductivity, and phenotype of three strains of *E. pallida* in response to thermal stress. Additionally, we observed how their thermal thresholds and resilience changed depending on pre-exposure to DTV. Our results show a significant difference in response to temperature variation across strain types, with DTV samples demonstrating a lower overall resilience to heat stress than replicates not pre-exposed to DTV. Symbiotic samples of *E. pallida* experienced an overall reduction in productivity and phenotype, however some DTV symbiotic samples retained high productivity levels and phenotypic health. Aposymbiotic anemones experienced similar results, with a more linear decline as they have no thermal threshold upheld by symbiotic algae. However, some aposymbiotic samples regained their algae as a method of thermoregulation against DTV. Our findings indicate the need for understanding strain-specific responses to thermal variation in *E. pallida* for use as an indicator species on the effects of increasing SST induced by anthropogenic forces.

INTRODUCTION

With expected warming of 1.5°C over the next 20 years, marine organisms of all trophic levels face unprecedented challenges in mitigating the adverse effects of climate change (IPCC, 2022). The consistent rise of ocean temperature caused by pollution during the last several decades disrupts the biodiversity and productivity of coral reefs, primarily through significant bleaching events that are increasing in frequency. In many coral species, responses to heat stress result in the expulsion of its symbiote (if present) when exposed to a prolonged increase in water temperature. Coral reefs are essential in protecting land masses from large bodies of open ocean and act as a line of first defense from large waves. Additionally, it is a natural habitat for thousands of marine creatures, driving high biodiversity and production in healthy reefs (Milius, 2019). However, coral reefs are highly susceptible to changes in the ocean; they depend on interlocking environmental cues, including daylight and DTV. These changes to the natural environment are explored further in

this experiment on *Exaiptasia pallida* anemones, measuring bleaching, mortality, reproductivity, and overall health.

Exaiptasia pallida are anemones that have emerged as models for coral symbiosis because they are in the phylum cnidaria. The species has a habitable range and distribution along the western Atlantic coast, from Maine to Florida, extending to the Caribbean and the Gulf of Mexico (Glon et al. 2020). Also known as the Glass Anemone, *E. pallida* occur in tropical and subtropical shallow waters, with an extensive record of withstanding broad temperature and salinity ranges (Gegner et al. 2017). They are an endosymbiotic species, hosting many dinoflagellate algae of *Symbiodinium sp.* (Thornhill et al. 2013). When stressed, it is common for *E. pallida* to retract its body and expel clumps of symbiotic algae and stem cells, known as pedalacerates (Lam et al. 2017). As a result of its high tolerance for environmental variability, *E. pallida* has been labeled as an invasive species and is known to replace communities of local corals, reducing biodiversity in many areas it may spread to occupy (Okey et al. 2003). However, *E. pallida* are well known as a model organism for researchers to study in place of coral, as their growth rate and survivability tend to be higher than most coral species (Hartman et al. 2019). The organism is regenerative in practically any state, either from one single cell or from being horizontally or vertically bisected (Hartman et al. 2019). Additionally, phenotypic changes in *E. pallida* act as easily quantifiable indicators of agitation. These factors make it an ideal specimen for studying how temperature and salinity affect the host anemone and its symbiotic algae.

Our experiment aims to study the effects of heat stress on *E. pallida* by measuring the effects of heat stress across different symbiotic and aposymbiotic *E. pallida* strains, with some samples having been pre-exposed to high Diurnal Thermal Variation (DTV). By manipulating the host for photosynthetic efficiency via host buffering, the study aims to better understand how replicates of *E. pallida* acclimate to thermal stressors and how their response applies to mitigating the effects of increasingly high thermal variation observed globally across susceptible coral species. Previous studies on similar *Exaiptasia* species (*E. diaphana*) investigate the effects of thermal stressors on their endosymbiotic relationships. One study grew one group of *Exaiptasia* samples at 25˚C and maintained another group at 32˚C for over two years. They found that the 32C treatments remained stable and had higher bacterial diversity. However, no signs of relative heat stress suggested that both the anemones and the bacteria had adjusted or acclimatized to temperature elevation (Hartman et al. 2019). Knowledge of *Exaiptasia's* bacterial microbiome remains relatively underexplored, giving *E. pallida* the potential to provide essential information on the effect of heat stress on the relationship between the host anemone and its symbiotic algae.

MATERIALS AND METHODS

Experimental Design

To monitor the effects of heat challenge on *Exaiptasia pallida*, we tested three strains of the anemone: one strain from Virginia West Beach (VWB), one from Hawaii (H2), and one collected off the coast of New England (CC7). A total of 68 anemones were used in this experiment, 33 of which experienced DTV pre-conditioning and 35 maintained in stable 26˚C conditions. DTV-conditioning was conducted 10/14/2022- 11/04/2022, where daily temperatures varied from 24°-34°C. Of the total 68 anemones, 33 were in an aposymbiotic state, and 35 were in a symbiotic state.

Control anemones were maintained at 26˚C for the duration of the experiment. These experiments were conducted in 12 trays, each containing 6-well plates containing replicates in ~10 mL of salt water.

Initially, the trays containing *E. pallida* replicates were placed in two incubators, with set temperatures of 24˚C for the control incubator and temperature ramping from 24˚C for the heated incubator. However, on the first day of data collection, both incubators experienced malfunctions where the temperature inside each incubator was elevated to 30˚C overnight. As a result, our design changed to have the trays set to float on top of a water-cooling system. The water temperature in the cooling system was

Strain		CC7		H2		VWB		
State		Apo	Sym	Apo	Sym	Apo	Sym	
								Total
DTV	Control	3	3	3	2	1	3	15
	Heat	3	3	3	3	3	3	18
NO DTV	Control	3	3	3	3	2	3	17
	Heat	3	3	3	3	3	3	18
Total		12	12	12	11	9	12	68

Table 1. Experimental distribution of *E. pallida* replicates across 12 trays, having six wells per tray containing *E. pallida* anemones. Each tray contains up to three symbiotic replicates and three aposymbiotic replicates of the same strain, treatment status, and exposure status (DTV or non-DTV exposure). Some trays contain a reduced number of replicates due to an initial lack of replicates distributed across trays, resulting in 68 replicates.

In order to test if DTV can prime anemones for resistance to heat change, we compared the performance of DTV-primed anemones relative to anemones that were pre-conditioned at a constant 24 °C. Half of the DTV-primed anemones from each strain experienced a heat change treatment that increased temperatures by 1°C per day every day for seven days until the treatment reached 34˚C, after which temperatures were maintained at 34˚C for four days (Figure 1).

timed constantly at 9:30 AM and 2:00 PM. Temperature data were collected using the Onset HOBO Bluetooth TidbiT 5000 Temperature Data Logger and the app HOBOconnect, with one device placed in the sumo of each system. All tanks had a timed LED light system maintained at 120-130 photosynthetically active radiation units (PAR) 12:12 hour light: dark cycle. The salinity of all wells was maintained between

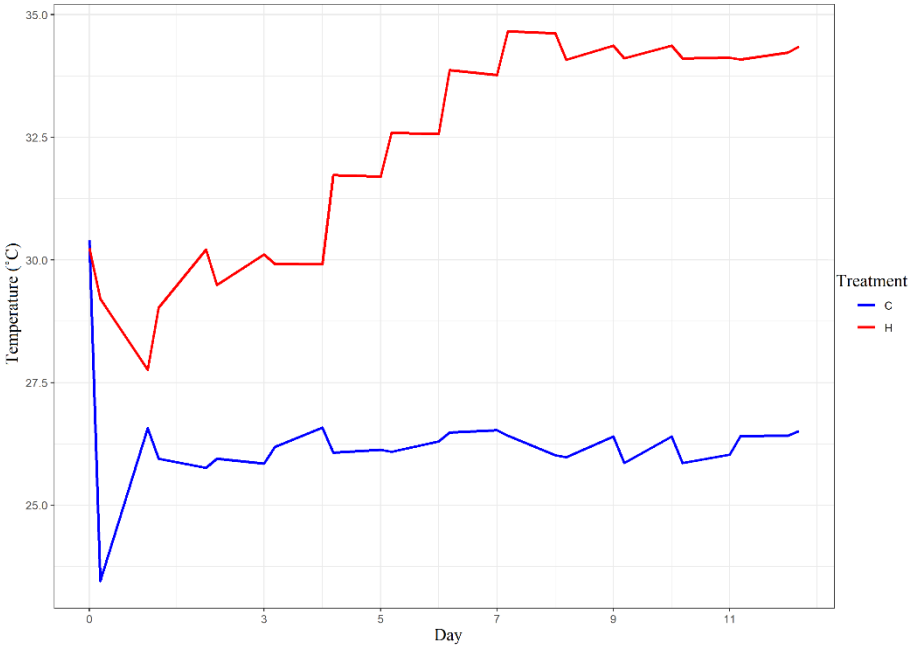


Figure 1. Linear progression of temperature readings taken twice daily. Contains temperature data for treatments at constant temperature (blue) and elevated temperature (red) over time.

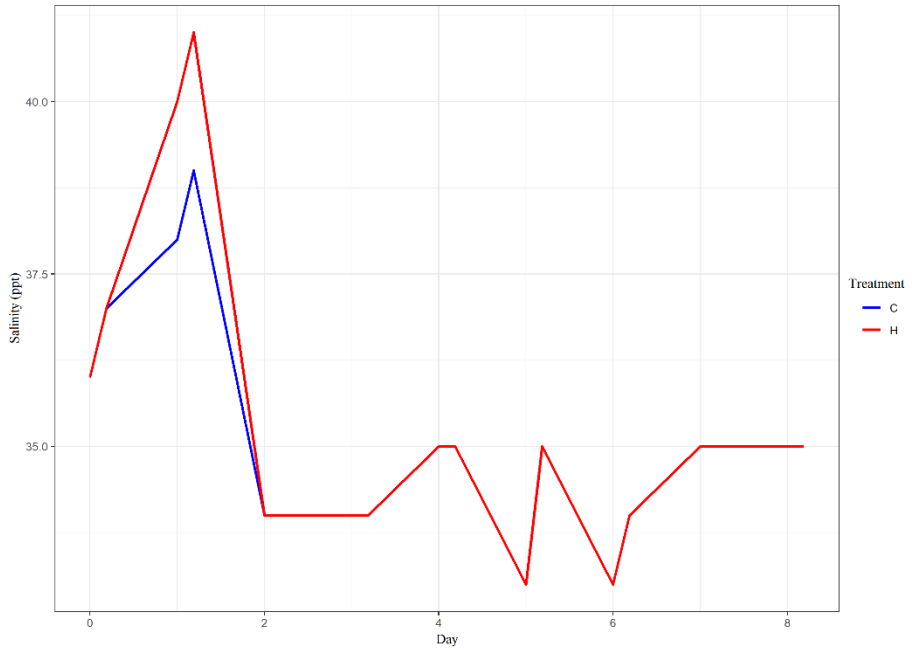


Figure 2. Daily salinity of seawater inside experimental trays. Shows salinity readings in ppt for the control group (blue) and experimental group (red) over eight days following initiation.

32-34 ppt. Salinity data was collected using a Dual Scale Sea Water Salinity Refractometer, recorded in tandem with temperature data. The seawater inside the trays was taken from the same source, and no pumps were used, causing salinity recordings to have little to no effect on experimental data excluding days affected by equipment malfunction. Following day 8 of data collection no further salinity measurements were performed.

Throughout the experiment, maintenance of the anemones was necessary to remove the possibility of death via starvation or polluted wells. Feeding of the anemones was conducted approximately every two to four days. This process consisted of feeding brine shrimp to each

individual via a pipette. After waiting 1 hour to allow for digestion, half of the water was replaced with seawater from the system, and each well was thoroughly cleaned with a Q-tip to remove algae and waste.

Mortality and Anemone Health

Observational counts on overall anemone health were paired with morning temperature checks. A qualitative health score on a scale of 0-4 was assigned to each individual (Table 2), with four being the healthiest, one being the least healthy, and 0 meaning the replicate had died. Health was determined by the phenotypic appearance of the anemone, with the healthiest anemones showing full tentacle and stalk extension, while the least healthy anemones showed little to no tentacles and partial retraction of the stalk. Mortality was also tracked throughout the experiment as the temperature gradually increased. For reference of the health score taken, refer to table 1. Note that 0 is not present, as in stage 1 they still had some chance of rebounding.

Photosynthetic Efficiency

Every two days, pulse-amplitude modulation (PAM) readings were taken from each individual after 1.5 hours of dark acclimation to measure the photosynthetic efficiency of photosystem II (Fv/Fm). PAM data were collected using the JUNIOR-PAM fluorometer paired with the software WinControl-3 to collect Fv/Fm readings.

Table 2. Qualitative Health Score of E. pallida anemones using phenotype, designed by JP Daanoy. Scale ranges from 1-4 for alive replicates, with 1 being the least healthy (full retraction, no visible tentacles) and 4 being the most healthy (full extension, fully visible tentacles). For organism death, replicate is assigned a score of 0.

When using the probe attached to the fluorometer, the data collected was 1 mm off the beak of each symbiote. Three PAM readings were conducted on each individual to calculate the average Fv/Fm for each replicate as time progressed. An additional fourth reading was taken if one of the readings seemed outside expected levels and was used as a replacement for other data points to reduce measurement error.

Anemone color

Photos of each anemone were taken immediately after Fv/Fm data were collected. Aposymbiotic *E. pallida* were placed on a black background, and symbiotic replicates were placed on a black background for easier visualization. Adobe Photoshop version 24.0 was then used to white-balance each image. The MATLAB package macro "AnalyzeIntensity" from Winters et al. was used to collect the average RGB values from 10 randomly selected points on the anemone from the white-balanced images. Half of the points were obtained from the anemone's body, and the other half from the tentacles. The RGB values measured pigmentation and color present in each photo. Red resolution was used for data analysis due to symbiotic algae causing the anemone to develop darker pigmentation when they are producing effectively. Resolution values are a measurement compatible with bleaching rate and anemone health, with a high resolution indicating a bleached organism. High initial resolution values are expected for aposymbiotic anemones, as they are not pigmented by symbiotic algae.

Statistics

Following data collection, coding and analysis were performed in R 4.2.2 using ggplot for PAM data, RBG resolution, and phenotypic observations. Cox's proportional hazards regression model was used to create mortality curves. Significance tests were conducted using Analysis of Variance (aov) tests, and p-values were displayed using asterisks. Boxplots were added to RGB resolution and phenotypic observation data, while linear regression lines were used for PAM data in R. This adjustment is due to the PAM data being averaged daily, making boxplots incompatible as they were incapable of averaging data by day. All data were differentiated via treatment, DTV exposure, and aposymbiotic and symbiotic state.

RESULTS

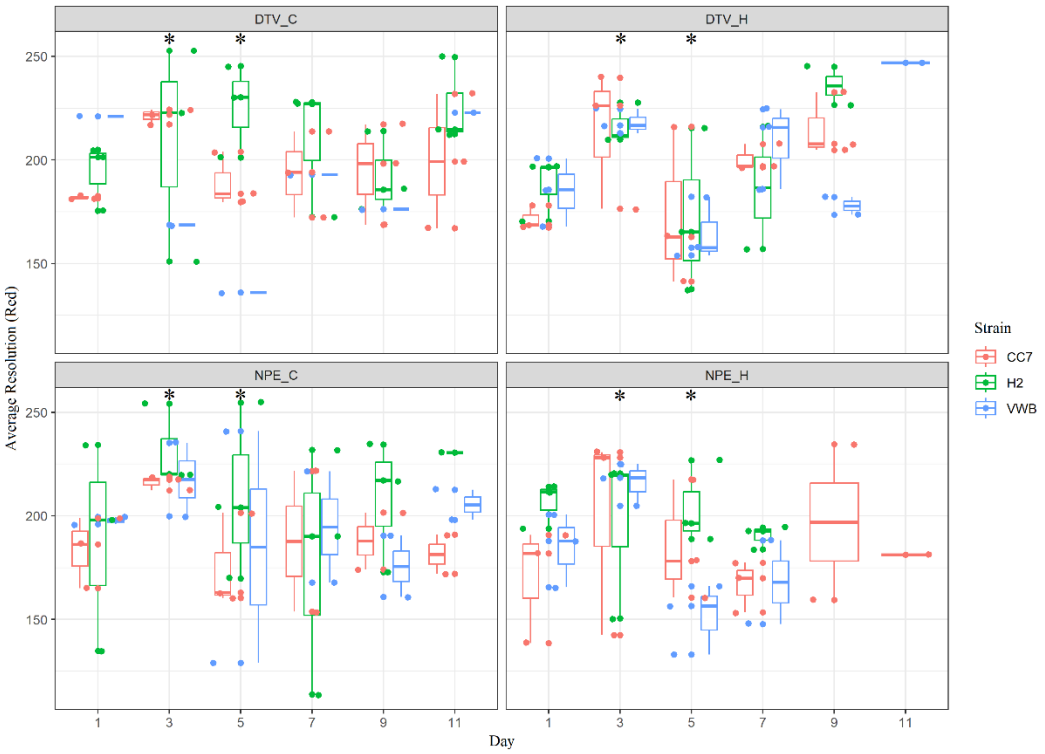
Water Quality and Conditions

The temperature in the control tank ranged from 25.86°C - 26.58°C for the duration of the experiment. Temperature in the heat tank ranged from 27.76°C to 34.66°C with an increase of 1°C per day and a holding temperature of 34°C for four days once reached (Figure 1). The salinity in both tanks stayed in the range of 33ppt - 35ppt for the duration of the experiment (Figure 2).

Photo Color Analysis

Photo color analysis data presented significant differences across strain and treatment data for days 3 and 5. H2 replicates consistently had the highest average red resolution among the aposymbiotic individuals and were visibly clearer. VWB had the highest variability, showing a reduced average resolution from day 5 onward ($pSTRAIN<0.05$) (Figure 3A). In symbiotic individuals, CC7 and VWB maintained low-resolution averages, while H2 developed higher average resolutions across treatments over time ($pSTRAIN<0.05$) (Figure 3B). Heated treatments showed significant changes in average resolution compared with control,

3A



3B

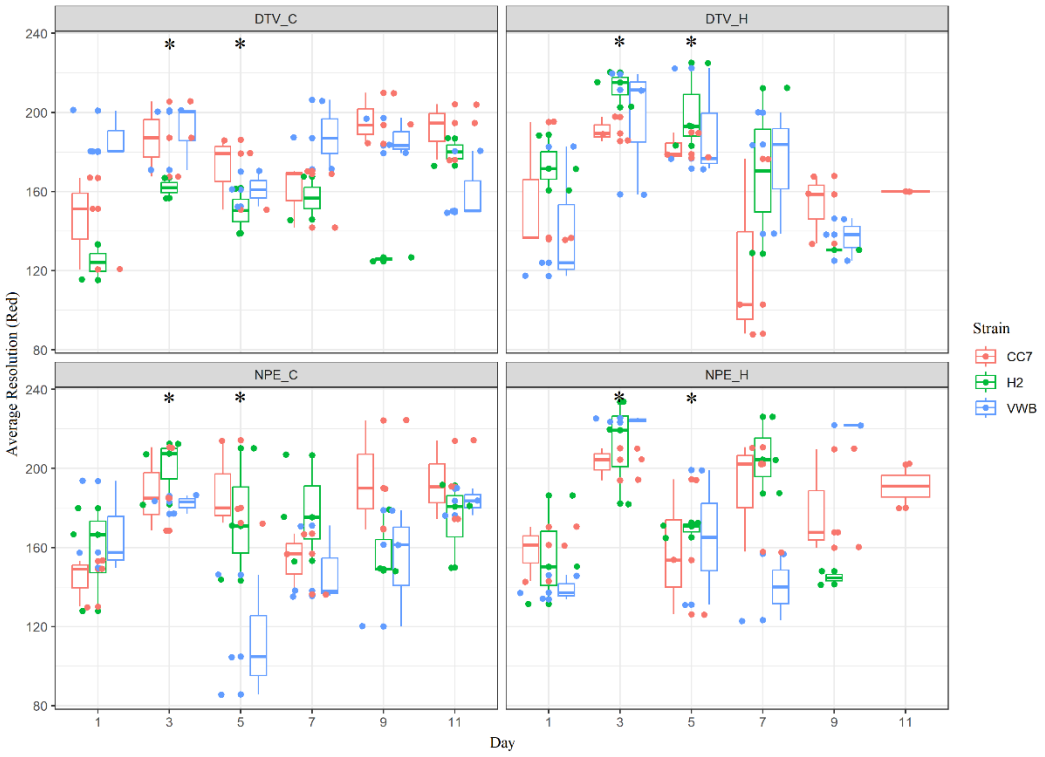


Figure 3. Average RGB red resolution of aposymbiotic (3A) and symbiotic (3B) groups of *E. pallida* collected via the use of white-balanced images in Adobe Photoshop and resolution data from Matlab "AnalyzeIntensity." Data is further separated into four treatments based on temperature (H = heated, C = control) and preexposure to Diurnal Thermal Variation (DTV = pre-exposed, NPE = non-pre-exposed). Boxplots are displayed per strain per day of data collection, and asterisks are indicative of a p-value of less than 0.05 using an Analysis of Variance (aov) test.

4A

4B

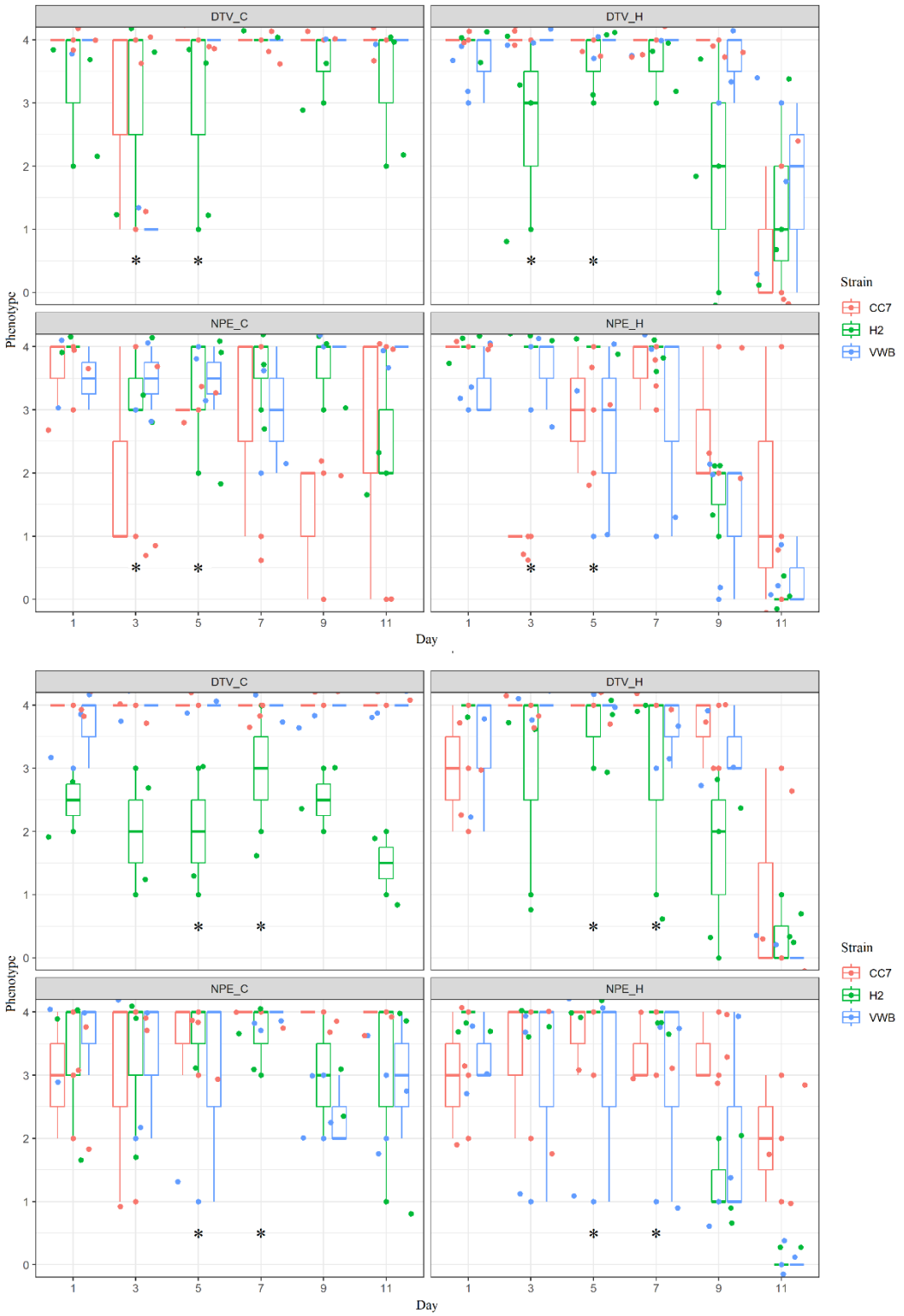


Figure 4. Qualitative Health Score (0-4) of *E. pallida* replicates per day, separated by aposymbiotic (4A) and symbiotic (4B) state. Data is further separated into four treatments based on temperature (H = heated, C = control) and preexposure to Diurnal Thermal Variation (DTV = pre-exposed, NPE = non-pre-exposed). Boxplots are displayed per strain per day of data collection, and asterisks are indicative of a p-value of less than 0.05 using an Analysis of Variance (aov) test.

especially in the symbiotic samples. Notably, some aposymbiotic samples decreased in resolution, gaining symbiotic algae as thermal stress increased. DTV pre-exposure seemed to have reduced the average resolution slightly for aposymbiotic individuals and increased the average resolution for symbiotic individuals ($p_{TREATMENT}<0.05$) (Figure 3).

Health Decline

Qualitative health score was a prominent indicator for overall health, resistance, and mortality in our experiment. For aposymbiotic individuals, day 3 saw significant reductions in health between strains, with H2 and CC7 performing the worst ($p_{STRAIN}<0.05$). Day 5 saw significant reductions between treatments, with H2 showing resilience in DTV, bouncing back from a low score of 1 to a 3 and doing the same in the control treatments ($p_{TREATMENT}<0.05$) (Figure 4A). Symbiotic data for day 5 showed a significant reduction in health for DTV and heat treatments, with DTV treatments having the highest overall reduction in health score ($p_{TREATMENT}<0.05$). Day 7 showed a reduction in health across strains, with H2 performing the worst in DTV_H treatment ($p_{STRAIN}<0.05$) (Figure 4B). Overall data showed reductions in health for both heated treatments as the experiment continued, showing variation between strain and treatment.

Photosynthetic Efficiency

When comparing the heated and control treatments, there were significant differences in Fv/Fm between strain and DTV treatment. Specifically, at day 3 different strains began to diverge between aposymbiotic individuals at 30°C, with H2 and CC7 having the highest Fv/Fm readings for Non-DTV and DTV treatments, respectively ($p_{STRAIN}<0.05$). Day 5 aposymbiotic showed significant declines in Fv/Fm in relation to treatment, with both DTV and control treatments declining ($p_{TREATMENT}<0.05$). Interestingly the relative performance of strains was flipped between DTV and Non-DTV treatments (Figure 5A). Symbiotic DTV had reductions in Fv/Fm along with heated treatments except for H2 in NPE_H, having notably higher levels on day 5 ($p_{TREATMENT}<0.05$). Day 7 symbiotic data revealed strain differences at 33.7°C, where H2 did much worse in both DTV_H and NPE_H treatments, having a much lower overall Fv/Fm reading, especially in NPE_H. VWB in NPE_H showed resilience with fluctuating Fv/Fm readings and eventually became the best performer in NPE_H treatment ($p_{STRAIN}<0.05$) (Figure 5B).

Mortality

Aposymbiotic individuals showed significant differences across strain and treatment for days 3 and 5. VWB had the highest mortality for both aposymbiotic and symbiotic individuals ($p_{STRAIN}<0.05$). Across treatments, non-DTV outperformed DTV, heated treatments had higher overall mortality than organisms in the controlled treatment, and all treatments experienced organism death as the experiment progressed ($p_{TREATMENT}<0.05$) (Figure 6).

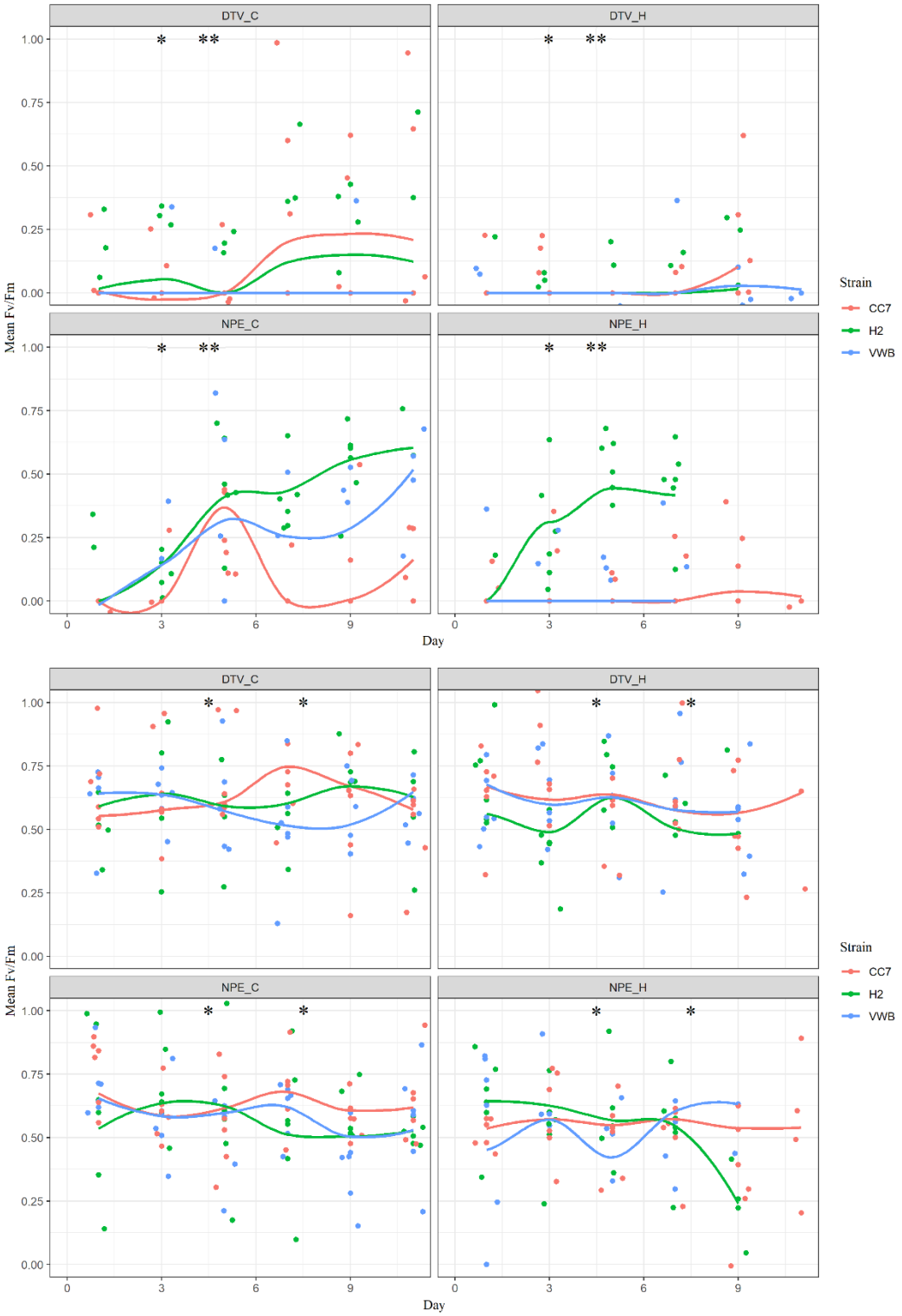


Figure 5. Average efficiency of photosystem II (Fv/Fm) of *E. pallida* replicates, separated by aposymbiotic (5A) and symbiotic (5B) state. Data is further separated into four treatments based on temperature (H = heated, C = control) and preexposure to Diurnal Thermal Variation (DTV = pre-exposed, NPE = non-pre-exposed). Linear regression lines for each strain are shown, and asterisks indicate p-values of less than 0.05 collected via Analysis of Variance (aov) tests.

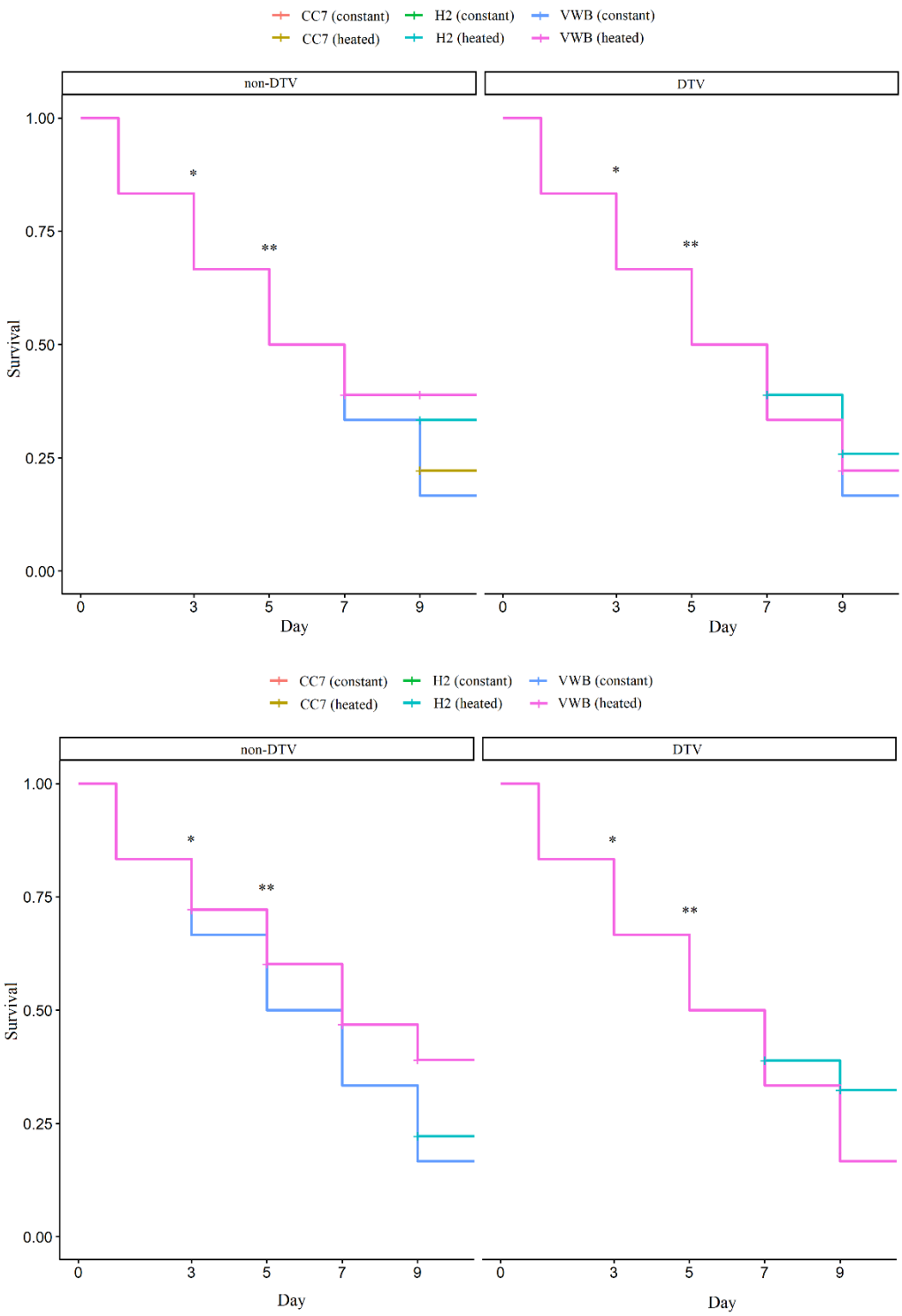


Figure 6. Survival plot of *E. pallida* replicates using Cox's proportional hazards regression model separated by aposymbiotic (6A) and symbiotic (6B) state. Data is further separated by preexposure status (DTV or non-DTV exposure), strain, and treatment. Asterisks are indicative of p-values less than 0.05 using an Analysis of Variance (aov) test.

DISCUSSION

The primary goal of this study was to examine the thermal stress response of *Exaiptasia pallida* in various ways. These included looking at response differences between strains, DTV versus Non-DTV, and aposymbiotic versus symbiotic individuals. Understanding this information is essential to the use of *E. pallida* as a future indicator species for climate change. Since *E. pallida* are extensively used in studies, we believe data gathered from this experiment, although partially inconclusive, can give way to future research on how anthropogenic forces are affecting our environment. We anticipated in this study that DTV samples would have a higher resilience to temperature change and that symbiotic individuals would perform better than their aposymbiotic counterparts in health, photosynthetic efficiency, and mortality. The observed patterns in the study varied by strain but were overall consistent, indicating that further research might validate our initial hypothesis. The data collected from our work indicates a more significant difference in thermal stress response between strains than simply DTV versus Non-DTV treatment.

Responses to Thermal Stress

As the experiment progressed and the temperature threshold was reached, replicates declined in overall health, with mass death recorded in the heated treatments on days 9 and 11. VWB experienced the most deaths, but H2 experienced the earliest deaths, likely due to differences in genetic structure between their microbiomes (Hartman et al. 2019). While not examined in this study, genetic variation by strain is associated with natural variation in temperature ranges by location. As stated earlier, the strain H2 was gathered from the tropical waters of Hawaii, while VWB and CC7 were from subtropical zones along the Eastern United States, which likely had a significant effect on the performance of each strain in the experiment. Despite being of the same genus, the variation in anthropogenic forces across *E. pallida's* wide habitable range likely resulted in the development of highly specialized population genomics within the host anemone and their symbiotic algae (Glon et al. 2020).

Within the heated treatment, each strain experienced significant declines in health, photosynthetic efficiency, and survivability. However, there was large variation in stress response across strains, notably in aposymbiotic strains when PAM analysis indicated replicates regaining their respective symbiotic algae. This behavior occurred most frequently with H2, to the extent that PAM readings from symbiotic and aposymbiotic replicates became virtually indistinguishable. Within days of the experiment beginning, H2 aposymbiotic individuals within the heated treatment had nearly fully regained their algae. This response is likely due to the tropical location of the strain driving the organism to regulate endosymbiotic function in a changing environment (Jinkerson et al. 2022).

In addition to heat stress response variation by strain, pre-exposure to DTV was shown to reduce overall resistance to heat stress, with replicates showing a reduced performance in photosynthetic efficiency and a higher overall loss of pigmentation

compared to non-DTV replicates. DTV-treated replicates showed little to no additional resilience, implying that prolonged exposure to high-temperature variation ultimately reduces *E. pallida's* ability to recover from high-stress events.

Sources of Error and Limitations

On the first day of our experiment, the incubators containing the *E. pallida* replicates failed and increased the temperature significantly for a short time. The anemones were exposed to this temperature for one dark cycle, potentially disrupting overall anemone health from the beginning. However, the temporary spike in temperature is believed to be inconsequential as PAM readings remained within expected ranges following the malfunction, and overall phenotype health was unaffected. The unexpected change in experimental design limited our ability to effectively maintain a constant temperature for the replicates, as we were now relying on the temperature of the tanks to transfer through the plastic trays, potentially creating inconsistent data. Additionally, the sample size provided for the experimental setup was varied, with some strains and treatments needing an even number of replicates. Increased sample size and more successful initiation of the experimental phase may have yielded more conclusive results.

Future Work and Implications

Future work on *E. pallida* would benefit from selecting variables within our experimental design and conducting individualized studies for each. A more honed-in focus on specific variables would allow for a more straightforward experimental design, mitigating error and producing more reliable data for analysis. Much is still unknown about thermal threshold variation within the *E. pallida* genus, and further research is required to assess the effects of gradual temperature change on anemone fitness. The rapid change in temperature across the short-term period of 11 days likely produced inconsistencies across replicates. Therefore, a longer-term future study that maintains slower increases in thermal stress is recommended to produce more conclusive results regarding variations between strain and treatment in *E. pallida*.

While this paper explores many factors controlling thermal threshold variation in *E. pallida*, there is still a gap within the literature about the catalysts behind each response and how they differ among strain types. Future work could solidify *E. pallida* as an indicator species for our environment with specific applications across shallow marine environments, allowing for comparison between other species as a baseline for mitigating the effects of ocean warming.

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