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Cellular Responses of *Astrangia poculata* (Ellis and Solander, 1786) and Its Symbiont to Experimental Heat Stress

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Abstract: Climate change has had devastating effects on tropical coral reefs; however, much less is known regarding how heat stress affects temperate coral. This research focuses on *Astrangia poculata* (Ellis and Solander, 1786) collected from Narragansett Bay, RI, during the summer and winter seasons and understanding the effect of experimental thermal extremes (i.e., 26 °C) on seasonally different populations. Photosynthetic efficiency (Fv/Fm), symbiont density (via an inverse relationship with pixel intensity), and oxidative stress via reactive oxygen species (ROS) concentrations were measured on symbiotic and aposymbiotic *A. poculata*. Higher Fv/Fm rates were observed in summer- vs. winter-collected corals ($p \leq 0.05$). Lower symbiont density within symbiotic and aposymbiotic *A. poculata* were observed at elevated temperatures, and higher intensities as well as symbiotic coral were observed in winter compared to the summer collections ($p \leq 0.05$). No differences in ROS were observed in host tissue cells, suggesting that ROS produced in the algal symbionts was not translocated into host tissues. Overall, higher ROS concentrations were observed in summer- vs. winter-collected corals ($p \leq 0.05$) in both symbiotic states. ROS concentrations were higher in symbiotic compared to aposymbitoic colonies ($p \leq 0.05$), albeit no differences were observed between temperature treatments, suggesting that antioxidants mitigate the deleterious effects of ROS on host tissues.

Keywords: *Astrangia poculata*; temperate coral; heat stress; climate change; coral reefs; oxidative stress; reactive oxygen species; imaging flow cytometry

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1. Introduction

Climate change studies have been at the forefront of marine science for the past several decades due to increases in atmospheric carbon dioxide (CO_2) [1–3]. Increases in CO_2 exacerbate the greenhouse effect, trapping more solar radiation and increasing global atmospheric and oceanic temperatures [4–7]. Increases in ocean temperature in turn affect a multitude of organisms, including coral reefs, which are considered fragile ecosystems as they require specific temperatures, nutrient concentrations, salinities, and light levels to survive [8]. When exposed to increased temperatures, corals undergo a process called bleaching in which they lose their mutualistic photosynthetic dinoflagellates

(Symbiodiniaceae) and/or symbiont pigmentation, causing the pale white skeleton to be exposed [9]. A lack of symbionts leaves the coral under the threat of mortality, as corals rely on symbiosis to supply most of their energy requirements [10–12]. Not all coral populations, however, suffer the same level of bleaching and mortality due to heat stress. For instance, both the host coral and/or the symbiont(s) that are harbored can withstand different temperatures [11,13–15]. Many authors suggest that both the host and symbiont may adapt (either individually or via symbiosis) to repeated exposure to stressful temperature and/or bleaching conditions, facilitating a better physiological advantage with regard to rising seawater temperatures [11,16–22].

Thermal stress events can also reduce photosynthetic efficiency [13,23–27]; high temperatures can damage thylakoid membranes and Photosystem II (PSII) and interrupt the Calvin cycle in Symbiodiniaceae, resulting in reduced rates of photosynthesis and causing increases in the irradiance absorbed and an over-accumulation of reactive oxygen species (ROS) [11,13,23,28–33]. There are three primary types of ROS which include (1) superoxide anion (O_2^-), (2) hydrogen peroxide (H_2O_2), and (3) hydroxyl radical HO. In the absence of stress and normal oxygen metabolism, ROS are produced in the mitochondrial matrix [34] and help regulate cell differentiation and multiplication [34]. When stressed, a buildup of ROS can occur (called oxidative stress), causing these molecules to become unstable and highly reactive with other molecules, damaging DNA, RNA, cellular proteins, membrane oxidation, protein denaturation, and the degradation of PSII [32,34–42] and often resulting in the loss of mitochondrial membrane potential and cell death (apoptosis; [34,43]). In a coral holobiont, oxidative stress causing an excess of ROS usually results in the loss of algal symbionts [37–39]. Studies have shown correlations between the decrease in photosynthetic efficiency and increased ROS concentrations, leading to oxidative stress negatively impacting coral–algal mutualism [31,39,44]. The accumulation of ROS can also leak into host tissues, triggering additional immune responses [32,36,42] and overall increased health-related stress. Downs et al. [45] proposed the "Oxidative Theory of Coral Bleaching", where they posited that tropical host corals receive significant concentrations of hydrogen peroxide (H_2O_2) predominantly produced by their symbionts. Once transferred from the symbiont to the host, H_2O_2 is either neutralized by enzymatic and non-enzymatic processes or via a series of chemical reactions (associated with Haber–Weiss chemistry; see [46]), and H_2O_2 becomes far more toxic, triggering a coral response usually resulting in the removal/loss of the symbiont algae [45].

The increase in ocean temperatures is more relevant to corals in tropical environments, where bleaching frequently occurs [13,47,48], as opposed to mesophotic or deep coral habitats. In some instances, episodic summer temperatures in the United States are becoming more frequent, e.g., 38.39 °C in Florida Everglades National Park [49], and are far higher than normal, expected summer temperatures on this continent. Recent IPCC reports [50] hypothesize that oceanic temperatures, should they continue to increase over the next several decades, will have a profound effect on mesophotic and perhaps deep-water coral habitats, and will certainly have a deleterious effect on temperate coral species. The effects of climate change on temperate species are much less studied but have gained more attention in recent years, with studies on high light tolerance [51], response to increased CO_2 [52], heterotrophy mitigating the impacts of thermal stress [53], seasonal patterns in dinoflagellate symbiont corals [54], diel "tuning" in coral metabolism responding to light cues [55], season-dependent responses of octocoral–algal symbiosis [56], and microbiome seasonal stability in temperate gorgonians [56–58]. Species such as *Cladocora caespitosa* [59] and *Oculina arbuscula* [53] have been studied, but few studies have focused on *Astrangia poculata* (Ellis and Solander, 1786).

Astrangia poculata is a facultatively symbiotic coral existing as both symbiotic and aposymbitoic, where symbiotic densities are less than 10^5–10^7 cm^{-2} [60–62] and

aposymbiotic corals have chronically low symbiont densities [62,63]. *Astrangia poculata* has an exclusive mutualistic relationship with *Breviolum psygmophilum* [64–66], but this coral species primarily focuses on heterotrophic feeding rather than fully relying on its algal symbiont [51,61,66]. Jacques et al. [67] suggested that concentrations of *B. psygmophilum* within *A. poculata* are so low that although symbiosis exists, the impact of this symbiont on the host physiology is likely minimal. Dimond and Carrington [68] suggested that although aposymbiotic colonies are not completely azooxanthellate (i.e., devoid of zooxanthellae), the host does not receive any functional photosynthetic benefit from *B. psygmophilum*. That said, the symbionts do provide an advantage over aposymbiotic colonies, boosting better host growth rates and, probably, helping feed the host during stressful thermal adaptation/acclimation [67–69]. The distribution of *A. poculata* in the United States ranges from its northern limit in Cape Cod, Massachusetts [70], to as far south as the Atlantic coast of Florida and the northern coast of the Gulf of Mexico [71–73]. Aichelman et al. [69] described thermal stress on photosynthetic and respiration outputs from two populations of *A. poculata*; however, other cellular functions have yet to be examined. For instance, Dimos et al. [74] suggested that the monitoring of mitochondria may be important as the regulation of protein responses by a host coral directly influences free radical detoxification. Wuitchik et al. [75] suggested that the deterioration of symbiosis (called dysbiosis) is related to gene expression, the unfolding of proteins, and, for instance, TRAF3 (tumor necrosis factor receptor 3). Gaps in the knowledge of how symbiosis is associated with *A. poculata* continue to exist as few studies report how dysbiosis (i.e., energy deprivation resulting in coral bleaching) occurs in this species.

The goal of this study was to broadly assess symbiotic state and seasonality as functions of overall health against experimental temperatures of the most thermal extremes (26 °C) predicted for the year 2100 (+2 °C; see IPCC) [73] using three types of analyses: (1) photosynthetic efficiency (Fv/Fm), (2) symbiont density, and (3) oxidative stress (monitored via ROS). Photosynthetic efficiency is directly related to symbiont performance and, thus, host survival during stressful events. Pfab et al. [76] suggested that maximum photosynthetic rates increase proportionately with temperature increases up to a certain threshold, after which, Fv/Fm decreases sharply, caused by cellular damage. Symbiont density, according to Palacio-Castro et al. [77] and Martinez et al. [78], is also a necessary measure as coral resilience may be directly related to the total number of symbionts present in a host, shuffling symbiont concentrations, and/or types and subtypes of symbionts. Thirdly, oxidative stress caused by ROS is directly involved in both reducing photoinhibition (Fv/Fm) and symbiont density shuffling (i.e., bleaching) via oxidative damage, as supported by the "Oxidative Theory of Coral Bleaching" [79]. Tang et al. [80] suggested that oxidative stress is an important cellular function to monitor as most tropical coral reef studies correlate bleaching to heat stress caused by the toxic accumulation of ROS.

2. Materials and Methods

2.1. Coral Collection and Husbandry

Colonies of *A. poculata* were collected at Fort Wetherill State Park in Jamestown, Rhode Island, USA (Figure 1; 41°28′40.8″ N, 71°21′45.8″ W), using self-contained underwater breathing apparatus (SCUBA) (RIDEM permit #429, Type 1) during both summer (July 2019) and winter (February 2020) seasons. Symbiotic and aposymbiotic colonies were collected ~1 m apart to avoid sampling clones and removed using a hammer and chisel at multiple sites at depths ranging from 5 to 9 m. Collected colonies were immediately transferred to mesh bags and, upon surfacing, separately placed into plastic bags filled with seawater and chilled on ice while being transported back to the mesocosm facility located at the Annis Water Resource Institute (AWRI) in Muskegon, MI, USA. Following Wuitchik et al. [75], all colonies upon arrival regardless of environmental

temperatures were placed into recirculating aquarium systems (Figure 2) and acclimated to control conditions (18 °C) at rates of ±1 °C day^{-1} [76] to help ensure that each coral and its associated microbial and symbiont population were homeostatically equal. Each system was filled with artificial seawater (Instant Ocean, Spectrum Brands, Blacksburg, VA, USA) with salinities between 32 and 37 ppt and maintained at a maximum intensity of ~60 µmol m^{-2} s^{-1} using 300 W full-spectrum (violet/indigo (420 nm), royal blue (450 nm), blue (470 nm), green (520 nm), red (660 nm), neutral white (6500 K), and cool white (12,000 K) LED lights (Bozily, Inc., Beijing, China) programmed to simulate diurnal patterns (07:00 to 19:00 h) and calibrated with an Apogee SQ-420 Smart Quantum Sensor (Apogee Instruments, Logan, UT, USA). Corals were fed brine shrimp (*Artemia nauplii*) three days per week, 40% total water changes were performed twice per week, and water chemistry measurements (nitrate, phosphate, magnesium, alkalinity, and calcium) using Red Sea (Red Sea Fish Pharm LTD, Herzliya, Israel) and Hanna Instruments kits (Hanna Instruments, Smithfield, RI, USA) were assessed once per week to maintain parameters.

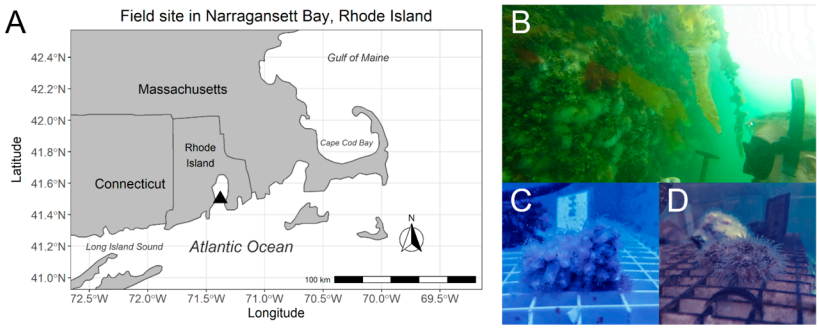

Figure 1. (**A**) Map of Narragansett Bay, RI, USA. Black triangle indicates *Astrangia poculata* collection site in Narragansett Bay (41°28′40.8″ N, 71°21′45.8″ W). (**B**) Underwater photograph of the local environment where *A. poculata* was harvested. (**C**) An aposymbiotic colony of *A. poculata* vs. (**D**) a symbiotic colony of *A. poculata*.

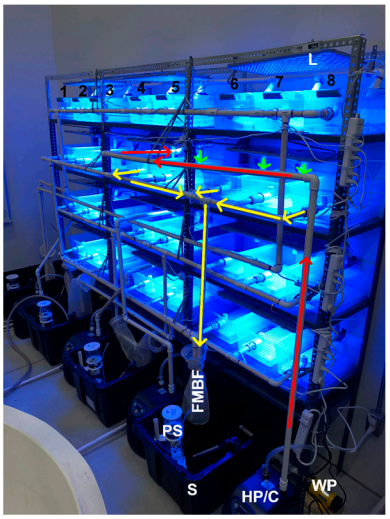

Figure 2. The experimental aquarium system consisted of 8 tanks (numbered above). Water originating from sumps (S) and consisting of bioballs and protein skimmers (PS) was pumped (red arrows) to each individual tank (green arrows), where it was circulated; it was then moved from the tank back to the sump (yellow arrows), first passing through 10 µm fine mesh bag filters (FMBFs). All water temperatures were maintained by heat pumps/chillers (HPs/Cs) and digitally monitored.

2.2. Experimental Design

Following the methods of Wuitchik et al. [75–78], our modified protocol involved fragmenting collected corals (hereafter called "frags") using a Gryphon C-40 bandsaw (Gryphon Corporation, Sylmar, CA, USA) and then glued to acrylic glass discs using IC-gel (Bob Smith Industries, Atascadero, CA, USA). The frags were then randomly placed between the two custom-built tank systems (Figure 2) and acclimated to tank conditions for a minimum of 2 weeks [79–82]. This acclimation regime was performed to help ensure that any elevated temperatures resulted in frags having a relatively equal growth period for the microbiome rather than directly using nature-collected samples where different frags could experience different temperature regimes, resulting in an unfair microbiome advantage [83–86]. Overall, 77 frags resulted from the summer-collected coral (44 symbiotic, 33 aposymbiotic) vs. 40 frags from the winter collection (20 symbiotic, 20 aposymbiotic). To help ensure that aposymbiotic coral lacked any significant concentration of symbionts or, rather, had an absolute minimum number of symbionts, we tested each coral frag by utilizing the photo quantification method outlined by Winters et al. [87]. It must be noted clearly that "aposymbiosis" in *A. poculata* is defined as having minimal symbionts since this species is recognized to never be completely devoid of its algae [88], even when held in the absence of light for extended periods of time [68,75]. It is also well recognized that other artifacts such as endolithic algae and/or other non-symbiotic algae may be present and need to be accounted for when measuring Fv/Fm. In this study, the presence of any optical artifacts prior to the beginning of the experimental study resulted in those coral frags being treated (i.e., placed in the dark for longer periods of time) to help further reduce both symbiont density and/or any other algae (see Supplemental Figure S1 for more details) [89]. For experimental analyses, corals in system 1 were exposed to ambient temperatures (18 °C) and system 2 corals were exposed to elevated temperatures at 26 °C. Increases in experimental temperatures occurred over a period of two weeks at a rate of +0.5 °C per day [76]. In our experimental design, sampling occurred every second day for a total of six sampling periods. Frags were sampled to measure (1) photosynthetic health using pulse–amplitude-modulated (PAM) fluorometry [90], (2) symbiotic density via photo quantification [87], and (3) oxidative stress via ROS concentrations using imaging flow cytometry (IFCM).

2.3. Maximum Quantum Yield (Fv/Fm)

Measurements of photosynthetic health (i.e., maximum quantum yield; Fv/Fm) throughout the thermal stress treatment duration were taken using pulse amplitude modulation (PAM) via a DIVING-PAM (Walz, Germany). PAM fluorometry is a well-established method, having been used for more than two decades [91,92] to characterize coral reefs and their symbionts and identify the efficiency of photosystem II (PSII) reaction centers, which correlates to overall photosynthetic performance [93–96]. Following Ralph [97], the DIVING-PAM settings were as follows: measuring light = 8, saturation intensity = 8, saturating width = 0.6 s, gain = 2, and damping = 2. The operational aspects of PAM included a 3 μs light pulse synchronized to a lock-in amplifier used to remove all signals not associated with the locked-in signal. Light sources included light measuring (<0.4 μmol photon m^{-2} s^{-1}), actinic light (1–2000 μmol photon m^{-2} s^{-1}), saturation pulse (>6000 photon m^{-2} s^{-1}), and far-red light set to 730 nm [97]; the measuring light setting needed to be low to prevent photosystem activation but sufficient to actually measure fluorescence. Following Ralph [97], we also noted that once the optical geometry had been established and the PAM had been adjusted with offset to zero, the fluorescence needed to be ≥300 and ≤1000 units. During each experimental stress treatment (i.e., 18 °C vs. 26 °C), Fv/Fm measurements were taken after the frags had been dark-adapted for 30 min [69,98]. Chlorophyll fluorescence at ambient temperatures almost exclusively emanated from

photosystem II. When dark-adapted samples were illuminated by a pulsed weak red light source, an initial/constant fluorescence (F0) indicated that photosystem II reaction centers were fully oxidized [98]. When applying a saturated pulsed white light source, a reduction/closing of photosystem II reaction centers occurred, increasing fluorescence to maximal values (Fm). Variable fluorescence (Fv) was observed during illumination (i.e., the fluorescence change from F0 to Fm; see [99]). The ratio of Fv to Fm when samples were dark-adapted was associated with the photosynthesis quantum yield representing the measure of maximum potential quantum yield [99,100]. Following Chan et al. [101] and Camp et al. [102], measurements of Fv/Fm were taken in triplicate for each coral frag prior to any experimentation to establish a baseline (i.e., control) and after each stress to obtain average Fv/Fm values (summer symbiotic: n = 43, winter symbiotic: n = 20, summer aposymbiotic: n = 34, winter aposymbiotic: n = 20). It is important to note here that when measuring Fv/Fm values in the aposymbiotic frags, the values could not be zero as (1) Burmester et al. [103] suggested that *A. poculata* is well known as a species to never be completely devoid of any of its symbionts *B. psygmophilum* and (2) optical artifacts, endolithic algae, and other non-symbiotic algae may exist. As such, control measurements needed to be taken to avoid potential artifacts of basic chlorophyl fluorescence signals (minimal and maximal fluorescence, F0 and Fm, respectively) eliminating any background fluorescent signals.

2.4. Symbiotic Density via Photo Quantification

Photo quantification measurements followed the methodology described by Dimond and Carrington [68] and Winters et al. [87], who non-invasively quantified algal symbiont density; this method identifies an inverse relationship, with pixel intensity vs. symbiont density correcting for variations in frag light intensity associated with different acclimation periods. Using photo quantification measurements has been well described by various authors such as Ferris et al. [104], who worked on coral reef scapes; Chow et al. [105] who worked on coral bleaching; DeFilippo et al. [106], who characterized surface lesions on *A. poculata*; Li et al. [107], who studied the stony coral *Montipora capricornis*; Sunoj et al. [108], who studied microalgae; Salgueiro et al. [109], who studied *Chlorella vulgaris*; and Sarrafzadeh et al. [110], who studied *Chlorella vulgaris*, *Botryococcus braunii*, and *Ettlia* sp. In our study, a Kodak grayscale was placed inside a 14 L aquarium tank and photographs were taken in triplicate using a GoPro HeroTM camera (GoPro Inc., San Mateo, CA, USA). Custom MATLAB files (Alex Blekhman 2005©) were used for photographic corrections by calibrating each file to a KodakTM grayscale and then selecting ten points on each frag to generate red intensity values (summer symbiotic: n = 43, winter symbiotic: n = 20, summer aposymbiotic: n = 34, winter aposymbiotic: n = 20), which inversely corresponded to symbiotic density [87].

2.5. ROS Concentrations via Imaging Flow Cytometry (IFCM)

After PAM and photographic measurements were completed, coral fragments were carefully removed from their acrylic glass discs and placed into 50 mL Falcon tubes with 3.5 mL of 0.22 μm filtered artificial seawater (FAS). Fragments were agitated using a benchtop vortexer to produce three aliquots (1 mL) of tissue slurry and subsequently washed with 0.75 mL FAS three times at 3000 rpm using an Eppendorf 5425 centrifuge (Eppendorf, Hamburg, Germany). A general fluorogenic oxidative dye called chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA, 2.9 μL; Thermofisher Scientific, Waltham, MA, USA) commonly used to assess cellular oxidative stress via ROS production was then added to each of the samples, as per recommendations from the manufacturer (10 μM concentration). Each sample was then agitated at 300 rpm (Thermomixer R; Eppendorf, Hamburg, Germany) for 40 min in the dark at the experimental

treatment to ensure the homogenization of the stain. Following agitation, samples were washed twice with 0.75 mL (0.01 M) phosphate-buffered saline (PBS, pH 7.4; Sigma Aldrich, St. Louis, MO, USA) before analysis. It should be noted that the stain CM-H2DCFDA can produce several artifacts, increasing its fluorescence due to oxidation in incubated media; incubated media are defined as growth media held at a specific temperature for a set time. In our study, we avoided "incubating" any samples, avoiding these artifacts. We also used controls (non-stressed coral) to compare to our experimentally stressed coral to establish a baseline fluorescence and help reveal the relative increase in oxidative stress in samples when they were exposed to stressful conditions (elevated temperatures). This helped to ensure that the relative change in ROS observed was caused by stress, instead of just fluorescence intensity.

Processed samples were analyzed with an Amnis Imagestream X Mark II imaging flow cytometer (IFCM; Luminex, Seattle, WA, USA). IFCM settings were as follows: 40X magnification, 60 μm field of view, 0.5 mW 488 nm laser intensity, and a low flow rate/high sensitivity setting for higher quality imaging. Two collection gates were generated based on preliminary analyses to separate algal and host tissue congregations (see Supplemental Figure S2). Algal collections were associated with high intensities in channel 5 corresponding to autofluorescence (640–745 nm) and host tissue collections were associated with high intensities in channel 2 corresponding to FITC fluorescence (505–560 nm). In addition, individual pictures of cells within these gates confirmed whether these cells were associated with symbiotic algae or host tissue cells (Figure 3). All aliquots (30 μL) were analyzed for 100,000 total cell events to keep every analysis uniform, and FITC intensity data were exported from their respective collection gates. All cell fluorescent events per fragment were averaged to acquire a mean fluorescence value and normalized against cells per μL (summer symbiotic: n = 42, winter symbiotic: n = 20, summer aposymbiotic: n = 25, winter aposymbiotic: n = 18). Some analyzed fragments did not collect any cells within the pre-determined gates; these inconclusive results were omitted from the dataset prior to analysis and are reflected in the sample sizes listed above.

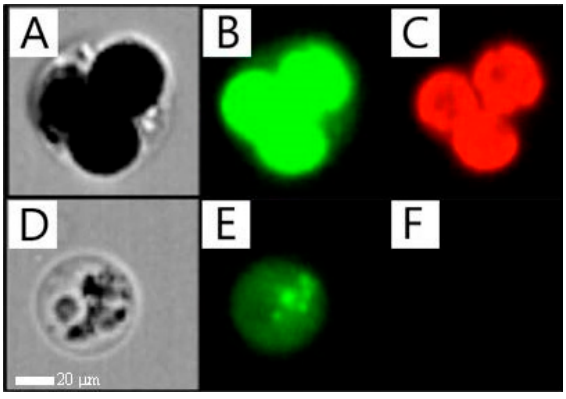

Figure 3. Imaging flow cytometry (IFCM) photographs (20 X) of individual cells from *Astrangia poculata* stained with CM-H2DCFDA. (**A**) Brightfield image dictating a photographed *Breviolum psygmophilum* cluster. (**B**) FITC channel (green) fluorescence of *B. psygmophilum*. (**C**) Auto-fluorescence of (red) *B. psygmophilum* in channel 5. (**D**) Brightfield image dictating a photographed host cell of *A. poculata*. (**E**) FITC channel (green) fluorescence of *A. poculata* host cell. (**F**) Auto-fluorescence was not shown within channel 5, indicating that it was a cluster of host cell tissue. Scale bar represents 20 μm.

2.6. Statistical Analyses

All statistical analyses were performed with RStudio (RStudio Team 2024) in R Version 4.1.0 (R Core Team 2024). Maximum quantum yield, symbiont density/pixel intensity,

and fluorescence data were analyzed using Kruskal–Wallis statistical tests to determine significant differences between treatments (e.g., 18 °C vs. 26 °C), summer- vs. winter-collected coral, and symbiotic state (symbiotic vs. aposymbiotic). Post-hoc pairwise Wilcoxon comparisons with Bonferroni corrections were used to further detect relevant differences between treatments, summer- vs. winter-collected coral, symbiotic state, and the interactions between these variables.

3. Results

3.1. Photosynthetic Health

Significant differences in Fv/Fm rates were observed between temperatures, symbiotic states, and seasons ($X^2 = 68.76$; $p < 0.001$; Figure 4A). Between symbiotic states, symbiotic fragments had significantly higher maximum quantum yield ratios in the summer ambient ($p < 0.001$, Table 1), summer elevated ($p < 0.001$, Table 1), and winter elevated treatments ($p < 0.001$, Table 1) compared to aposymbiotic fragments. No significant differences were found between temperature treatments only. Between seasons, there were no statistical differences between the aposymbiotic fragments. Summer Fv/Fm rates in symbiotic fragments were significantly higher for ambient ($p = 0.012$, Table 1) and elevated temperatures ($p = 0.002$, Table 1) compared to winter symbiotic fragments.

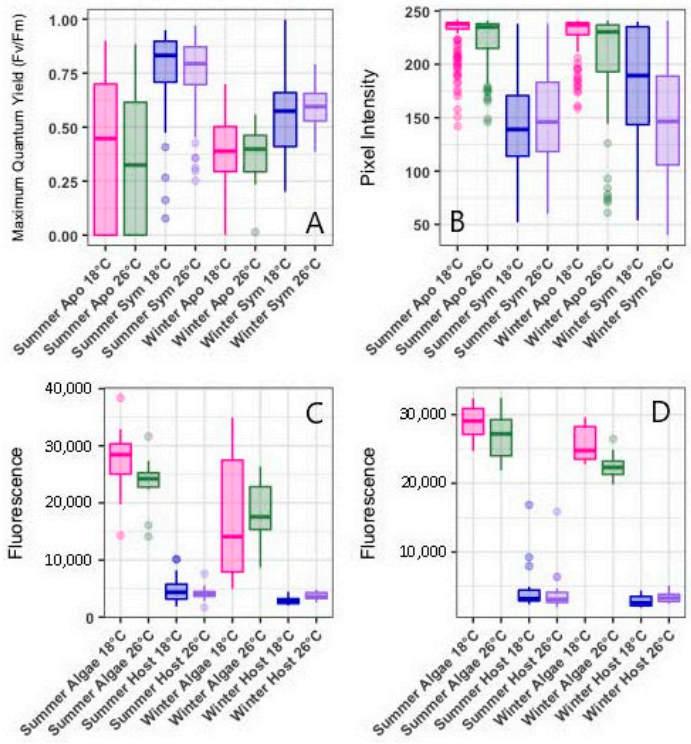

Figure 4. Boxplots of maximum quantum yield ratios (Fv/Fm) (**A**) and symbiont density (**B**) in aposymbiotic/symbiotic fragments between temperature treatments and seasonal collection, as well as ROS fluorescence in aposymbiotic (**C**) and symbiotic (**D**) *A. poculata* fragments in algal/host tissue cells between temperature treatments and seasonal collection. Colors are coordinated to their respective temperature treatment/symbiotic state or temperature treatment/cell type. Circles filled with a particular color reference outlier data, while whisker bars indicate the lower/upper quartiles from the boxplots.

Table 1. Pairwise Wilcoxon (with Bonferroni correction) results of maximum quantum yield, symbiont density, and ROS fluorescence in aposymbiotic and symbiotic *A. poculata* fragments between temperature treatments and seasonal collection. Bold *p*-values indicate significant differences between the targeted comparisons.

Maximum Quantum Yield						
Condition 1			***Condition 2***			***p-value***
Season	**Symbiotic State**	**Temperature**	**Season**	**Symbiotic State**	**Temperature**	
Summer	Aposymbiotic	18 °C	Summer	Symbiotic	18 °C	2.47×10^{-9}
Summer	Aposymbiotic	18 °C	Summer	Aposymbiotic	26 °C	1
Summer	Aposymbiotic	18 °C	Winter	Aposymbiotic	18 °C	1
Summer	Symbiotic	18 °C	Summer	Symbiotic	26 °C	1
Summer	Symbiotic	18 °C	Winter	Symbiotic	18 °C	1.32×10^{-6}
Summer	Aposymbiotic	26 °C	Summer	Symbiotic	26 °C	2.13×10^{-10}
Summer	Aposymbiotic	26 °C	Winter	Aposymbiotic	26 °C	1
Summer	Symbiotic	26 °C	Winter	Symbiotic	26 °C	4.56×10^{-6}
Winter	Aposymbiotic	18 °C	Winter	Symbiotic	18 °C	1.18×10^{-1}
Winter	Aposymbiotic	18 °C	Winter	Aposymbiotic	26 °C	1
Winter	Symbiotic	18 °C	Winter	Symbiotic	26 °C	1
Winter	Aposymbiotic	26 °C	Winter	Symbiotic	26 °C	2.07×10^{-7}
Photo Quantification						
Condition 1			***Condition 2***			***p-value***
Season	**Symbiotic State**	**Temperature**	**Season**	**Symbiotic State**	**Temperature**	
Summer	Aposymbiotic	18 °C	Summer	Symbiotic	18 °C	1.61×10^{-54}
Summer	Aposymbiotic	18 °C	Summer	Aposymbiotic	26 °C	1×10^{-3}
Summer	Aposymbiotic	18 °C	Winter	Aposymbiotic	18 °C	1
Summer	Symbiotic	18 °C	Summer	Symbiotic	26 °C	1.45×10^{-49}
Summer	Symbiotic	18 °C	Winter	Symbiotic	18 °C	7.98×10^{-7}
Summer	Aposymbiotic	26 °C	Summer	Symbiotic	26 °C	8.32×10^{-42}
Summer	Aposymbiotic	26 °C	Winter	Aposymbiotic	26 °C	1.36×10^{-1}
Summer	Symbiotic	26 °C	Winter	Symbiotic	26 °C	1
Winter	Aposymbiotic	18 °C	Winter	Symbiotic	18 °C	4.98×10^{-12}
Winter	Aposymbiotic	18 °C	Winter	Aposymbiotic	26 °C	4×10^{-3}
Winter	Symbiotic	18 °C	Winter	Symbiotic	26 °C	3×10^{-3}
Winter	Aposymbiotic	26 °C	Winter	Symbiotic	26 °C	2.50×10^{-6}
ROS Fluorescence (Algae)						
Condition 1			***Condition 2***			***p-value***

Season	Symbiotic State	Temperature	Season	Symbiotic State	Temperature	
Summer	Aposymbiotic	18 °C	Summer	Aposymbiotic	26 °C	6.12×10^{-1}
Summer	Aposymbiotic	18 °C	Winter	Aposymbiotic	18 °C	1.48×10^{-3}
Summer	Aposymbiotic	26 °C	Winter	Aposymbiotic	26 °C	2.17×10^{-1}
Winter	Aposymbiotic	18 °C	Winter	Aposymbiotic	26 °C	9.90×10^{-1}
Summer	Symbiotic	18 °C	Summer	Symbiotic	26 °C	3.12×10^{-1}
Summer	Symbiotic	18 °C	Winter	Symbiotic	18 °C	4.47×10^{-2}
Summer	Symbiotic	26 °C	Winter	Symbiotic	26 °C	4.50×10^{-4}
Winter	Symbiotic	18 °C	Winter	Symbiotic	26 °C	1.35×10^{-1}

3.2. Symbiotic Density (Associated with Pixel Intensity)

Significant differences in symbiont density were observed between temperatures, symbiotic states, and seasons ($X^2 = 71.33$; $p < 0.001$; Figure 4B). Overall, symbiont density was significantly higher (i.e., lower pixel intensity) in symbiotic fragments compared to aposymbiotic fragments for both ambient ($p < 0.001$, Table 1) and elevated ($p < 0.001$, Table 1) temperatures in summer colonies. No significant differences were found for similar conditions in winter colonies. Lastly, no significant differences were found between temperature treatments or between seasons.

3.3. Imaging Flow Cytometry (IFCM)

Significant differences in algal cell ROS fluorescence were observed between temperatures, symbiotic states, and seasons ($X^2 = 59.21$; $p < 0.001$; Figure 4C,D). Post-hoc analyses showed significantly higher ROS fluorescence in symbiotic fragments in ambient ($p < 0.001$, Table 1) and elevated ($p = 0.002$, Table 1) summer colonies as well as ambient ($p = 0.012$, Table 1) and elevated ($p = 0.007$, Table 1) winter colonies when compared to aposymbiotic fragments. No significant differences were observed between temperatures and seasons within symbiotic states. Similarly, analyses of host tissue cells did not result in significant differences between temperatures, seasons, and symbiotic states.

4. Discussion

In this study, the goal was to determine how *Astrangia poculata* may respond to episodic and/or elevated future predicted temperatures of the most thermal extremes for the year 2100 (+2 °C) [73]. By comparing and contrasting coral collected from two seasons with high vs. low temperatures (i.e., summer vs. winter) in symbiotic and aposymbiotic colonies against a thermal maximum (26 °C), this study observed how differences in seasonality influenced responses of *A. poculata* to experimental temperature exposure [111,112]. Tolerance to thermal stress was assessed by measuring photosynthetic efficiency (Fv/Fm), symbiont density (using pixel intensity metrics), and oxidative stress (via ROS concentrations) to better understand cellular stressors within this holobiont. The measurement of maximum quantum yield (Fv/Fm) is a well-documented indicator of the overall performance of photosynthetic processes within symbiotic algae [113–115]. These measurements can indicate whether symbiotic algae are stressed within certain experimental environments. Another indicator of photosynthetic health within corals can be shown within the photo quantification method by Winters et al. [87]. The photometric method utilizes RGB values to indicate whether these corals are stressed by differences in

RGB intensities (i.e., symbiotic density). Finally, reactive oxygen species (ROS) is an indicator of oxidative stress within the photo-physiology of symbiotic algae and can be detected within host tissues if temperatures are too stressful [113–115]. As photosynthetic processes begin to degrade within symbionts in extreme temperatures, increases in ROS concentrations further compound stress, affecting the physiology of this host–algae partner [116–118].

4.1. Photosynthetic Health

When examining the photosynthetic health of the symbiotic colonies, results from pulse–amplitude modulation (PAM) measurements generally showed higher Fv/Fm values in symbiotic colonies compared to aposymbiotic colonies, which was expected considering the significantly different concentrations in symbiotic algae. However, no differences between symbiotic states in ambient temperatures from winter-collected coral were observed. While aposymbiotic *A. poculata* have very low concentrations of *Breviolum psygmophilum* [119,120], winter conditions can reduce/shuffle symbiotic algae densities due to quiescence [81, 121], leading to reduced Fv/Fm values. Even though ambient conditions did not produce any differences, elevated temperatures from winter colonies caused higher Fv/Fm values in symbiotic colonies compared to aposymbiotic colonies. This result could indicate that the short-term exposure to warmer temperatures may have positively influenced symbiotic densities or overall photosynthetic health [81], leading to improved Fv/Fm values. No differences were observed between temperature treatments, indicating that these corals were able to withstand the higher thermal extremes within our experiments. Further research should exacerbate the upper maximum thermal limit of *A. poculata* to determine the future impacts of climate change on its photosynthetic health, considering how ocean temperatures have changed over the last 100 years. For instance, in the Florida Keys region over the last century, average temperatures rarely exceeded 28.9 °C; however, that average today is usually between upper 26 °C to 32 °C (~80 °F to low 90 °F) [122] and, in 2023, exceeded 38.3 °C (101 °F) [49], with degree heating weeks occurring earlier and lasting longer each year [123].

In this study, we assessed aposymbiotic conditions to determine long-term outcomes, should temperatures cause mass bleaching in tropical environments. For instance, we know that aposymbiotic *Astrangia* sp. can survive during mass bleaching heat events, whilst other coral (i.e., tropical coral) cannot, and this host remains unbothered. Here, aposymbiotic *A. poculata* colonies maintained consistent Fv/Fm values between summer and winter collections. However, symbiotic colonies generally had higher Fv/Fm values from summer collections compared to winter. Although summer collections took place near the peak of the in situ thermal range (24 °C; Harman, unpubl.) and winter collections took place near the bottom of the in situ thermal range (4 °C; Harman, unpubl.), all coral colonies were acclimated to the same temperature to help ensure that each microbiome was homeostatically equal; otherwise, as Brown et al. [124] suggested, the microbiome shifts rapidly between quiescence vs. emergence. Hence, there should have been no experimental design bias caused by the coral and their symbiont population affected by ambient seawater temperatures at the time of collection. However, it is still plausible that despite our experimental intent to ensure that all corals were equally acclimated to the temperature, the maintenance of these coral and their symbiont partners as a microhabitat at the time of collection may have been biased by prolonged environmental conditions that are difficult to replicate in a lab, i.e., months vs. weeks, having established a density of symbionts vs. endolithic vs. other non-symbiotic algae. Hence, these observational Fv/Fm differences may also be attributed to general trends in symbiotic density from quiescence in winter months [121,122], which can influence overall Fv/Fm values due to lower symbiotic densities alone [125]. In addition, summer-collected colonies had

significantly longer acclimation times to laboratory conditions compared to winter-collected colonies. The static conditions (i.e., temperature/light) and consistent heterotrophic feeding schedules may have also been a significant contributor to the summer vs. winter differences observed in this study. In this study, we also attempted to control for optical artifacts by increasing the length of dark-adapted time that a fragment was exposed to. Perhaps a better strategy could be a "baseline" fluorescence in the aposymbiotic coral in combination with airbrushing (complete removal of algae and/or artifact) subtracted from the experimental intact frags, producing a more accurate Fv/Fm.

Symbiotic *A. poculata* from summer collections had higher Fv/Fm results than expected. Many authors report that Fv/Fm in *A. poculata* should range between 0.5 and 0.7 [68,126]; however, our observations were between 0.6 and 0.8. We surmise that a few reasons may have been responsible for the higher-than-normal Fv/Fm values. These include the heterotrophic feeding during the experiments [89], which could have mitigated thermal stress and increased Fv/Fm values to between 50 and 70% higher [127]. Secondly, higher nitrate values recorded from the recirculating aquarium system (>4 ppm) may have influenced the Fv/Fm values, where the photobiology and growth rates of symbiotic algae benefitted from high nitrate levels [128,129]. Lastly, these Fv/Fm values may have been influenced by low irradiance, as we mimicked in situ conditions within experimental treatments (~60 μmol). Results from Aichelman et al. [69] show that saturating irradiance for *A. poculata* was found at 400 μmol photons m^{-2} s^{-1}. Furthermore, correlations between irradiance and Fv/Fm have been observed in other published works [130,131].

4.2. Symbiotic Density (Pixel Intensity)

Pixel intensity is related to symbiotic densities based on photo-pigment measurements. First developed by Winters et al. [87], these authors established a photo-quantification method by comparing chlorophyll a and c2 pigment measurements to pixel intensities. Winters et al. [87] observed a negative relationship specifically for the red channel between pigment (a and c2) and pixel intensity ($R^2 = 0.82$) data, indicating that higher pixel intensities had low chlorophyll a and c2 pigments.

Within summer collections, symbiotic *A. poculata* had significantly higher symbiont densities compared to aposymbiotic colonies, indicating lower pixel intensities within symbiotic colonies. This was to be expected given the general nature of symbiotic versus aposymbiotic *A. poculata*. However, no significant differences were observed between symbiotic states within winter collections, in both the ambient and elevated temperature treatments. It was proposed previously in this paper that the higher Fv/Fm values in symbiotic *A. poculata* colonies from elevated temperature treatments may have been due to higher symbiotic algae densities. Even with non-significant results, winter symbiotic colonies showed generally higher symbiont densities compared to winter-collected aposymbiotic coral colonies. With this in mind, these results could suggest that higher Fv/Fm rates may be largely due to improved photosynthetic efficiency from short-term exposure to warmer temperatures [21,132], where symbiotic densities may benefit from the influences hypothesized here. Hence, increased photosynthetic efficiencies are likely the result of repeated exposure occurring year after year. Bowling [133], Quigley et al. [20], and Wang et al. [22] similarly suggested that repeated stress exposures to the symbiont population(s) associated with coral help restructure symbiont lineages, resulting in more fit algal populations and, consequently, better photosynthetic efficiencies, leading to more heat-tolerant coral. Between temperature treatments, no significant differences were found in relation to increasing/decreasing symbiont densities. This, along with the Fv/Fm results observed in this study, continues to support the conclusion that the coral investigated in this study has a much higher thermal limit than was tested. Astrangia may have the physiological capacity to tolerate predicted ocean warming temperatures 1–4 °C above summer means

predicted in IPCC's 2013 and 2023 reports [7,134]. Lastly, no significant differences were found between summer vs. winter collections within the aposymbiotic colonies, suggesting that these maintain stable concentrations of low or relatively no symbionts throughout the seasonal cycle. Among symbiotic colonies, no significant differences were found, but there were higher pixel intensities in winter ambient treatments compared to summer ambient treatments. This suggests that quiescence (i.e., winter dormancy) directly influences not only the host, which during the winter retracts its polyps and becomes less responsive to stimuli, but also the symbiont, such that its population "shuffles" to produce a more meaningful symbiotic association. Similar observations were reported by Brown et al. [124] in their 3-month study of *A. poculata*. These authors suggested that microbiome changes associated with quiescence vs. cessation, i.e., winter vs. summer (respectively) conditions occur as a long-term regulation for survival. Fitt et al. [54], who studied tropical corals, suggested that distinct seasonal patterns exist in host coral and that the highest zooxanthellae densities are typically found in winter months, with the lowest occurring during late summer/fall, suggesting that temperature and light dictate the health of the microbiome as shuffling occurs.

4.3. ROS Concentrations

No statistical differences in ROS fluorescence between temperature treatments were observed in host tissue cells. The lack of significance suggests that these temperature treatments may not have sufficiently stressed the host coral *A. poculata*, where extreme thermal stress events should have caused elevated oxygen concentrations in the host vs. symbiont relationship, leading to an over-production of ROS and internal "damage" [55]; had this occurred, Levy et al. [55] and Lesser [135] suggested that disruption of the symbiont association would occur, followed by an apoptotic cascade, as noted by Tchernov et al. [44]. It should be noted, however, that CM-H2DCFDA is not specific to only one form of ROS and binds to any production of ROS constituents such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and $O_2^{\bullet -}$, and IFCM can be used to rapidly assess these fluorescent concentrations to detect differences in these oxygen radicals. Although these products occur in PSII and can be detected in host coral tissues if large concentrations of ROS are produced from thermal stress events [35], it is recognized that an artificial increase in ROS may occur if superoxide is inexplicably introduced [36]. Although, Levy et al. [55] suggested that the host environment protects the symbiont(s) from chronic photoinhibition (also see [130]), reducing any free-radical harm. As a consequence, symbiosis is protected by the host's anti-oxidative defense mechanisms and the symbiont's "photoacclimative plasticity" [55]. It is plausible that *A. poculata* is tolerant of temperatures of up to 26 °C, if not higher, and future studies need to explore the thermal limit of this species. It is also plausible that expelling the symbionts could itself cause oxidative stress, changing the ROS levels independently of the experimental conditions and influencing the results.

Overall, symbiotic colonies had a higher average fluorescence of cells per μL^{-1} compared to aposymbiotic colonies, largely due to more symbionts being analyzed by IFCM in symbiotic corals. No significant differences between temperature treatments and the summer vs. winter collections were observed, suggesting that ROS concentrations may remain relatively stable throughout seasonal/ocean temperature cycles and that influences of higher temperatures are negligible regarding ROS in this species. It can be speculated that with minimal symbiont densities, ROS concentrations in aposymbiotic colonies would remain stable regardless of temperature treatment and season. For instance, McGinty et al. [25] similarly did not find significant decreases in ROS concentrations in *Breviolum psygmophilum* as temperatures increased, indicating that antioxidants influence these decreases to mitigate stressful scenarios. Van de Water et al. [57], who studied temperate microbiomes of the Gorgonian red coral *Corallium rubrum* in the Mediterranean

Sea, suggested that despite major differences in ocean temperatures and precipitation, the microbiome regulated by the host coral in their study was remarkably stable. As such, it may be reasonable to suggest that winter symbiotic colonies of *A. poculata* show no changes in ROS due to a state of quiescence—a stable microbiome where very limited activities occur in the holobiont—and that the reduced photosynthetic processes and less metabolic expenditure [136,137] may influence ROS output. As such, we speculate that during quiescence, some type of imperceptible re-shuffling occurs [57], allowing the host to become more flexible and adaptive—a biological bet-hedge—to unstable and rapidly changing environmental conditions [138]. Biological bet-hedging is broadly defined here as an evolutionary strategy often exercised by organisms frequented by variable biotic and/or abiotic environmental cues caused by unpredictable environments [139]. Considering that ocean temperatures are very unstable but predicted to continue to increase [140,141], many organisms including coral are likely shuffling their microbiomes [57] and physiologies [142,143] as a mechanism of acclimation, including the bleaching of coral [144]. In our study, we speculate that *A. poculata* uses quiescence as a mechanism for physiological and microbial/microbiome shuffling—a biological bet—as a mechanism for acclimation to global ocean sea surface temperatures.

5. Conclusions

This study examined how two seasonally different populations of *A. poculata* and its symbiont partner *B. psygmophilum* responded to an experimental thermal extreme based on predictions of what elevated temperatures are expected to be by the year 2100 (+2 °C) [73]. Overall, this study suggests that *B. psygmophilum* associated with summer- and winter-adapted *A. poculata* colonies are a thermally tolerant dinoflagellate. Further, *B. psygmophilum* will likely be thermotolerant to the temperatures predicted to occur by the end of the century due to climate change. Future studies should examine the upper thermal limit of *A. poculata* as a host species, even at temperatures that currently seem irrelevant (i.e., 30 °C, 32 °C, or even 34 °C), but may very well be normal in the future in some circumstances. It is plausible that *A. poculata* is a thermally tolerant coral capable of maintaining consistent maximum quantum yield ratios and symbiont densities from thermal stress exposure. Static oxidative stress responses via ROS concentrations were observed throughout the experimental study, implying the stability of this species in responding to stress. However, in contrast to other studies such as that by McGinty et al. [35], it is proposed that prolonged laboratory conditions influenced the current results. In the context of oxidative stress from algal symbionts, Dimond and Carrington [104] suggested that *B. psygmophilum* only provides partial benefits to this coral species and that the coral host plays an important role in controlling its physiological responses to environmental stressors. As such, it is plausible that oxidative stress does not have the same negative impact as it does on tropical coral species with obligate symbiosis. Further work should include a more comprehensive look into the cellular biology of *A. poculata* regarding oxidative stress. While summer-adapted colonies demonstrated tolerance, winter-adapted colonies exposed to these higher temperatures suggested reduced metabolic and cellular functioning due to a quiescent state.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/10.3390/w17030411, Figure S1: Preliminary pixel intensity analysis of aposymbiotic (A) and symbiotic (B) *A. poculata* fragments. Figure S2: Plot comparing fluorescent intensities of cellular material between Channel 2 (FITC; green in color) and Channel 5 (auto-fluorescence; red in color) of a processed *A. poculata* sample.

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