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The effects of thermal stress on fluorescent protein expression in an Indo-Pacific scleractinian coral species, Acropora tenuis

Anna Knochel
SIT Study Abroad

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By Anna Knochel
Project Advisors: Gergely Torda Ph.D., Aurelia Moya Ph.D.
ARC Centre of Excellence for Coral Reef Studies, James Cook University
Townsville, QLD

Academic Director: Tony Cummings
Home Institution: Rice University
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Abstract

The scleractinian coral species that so heavily define tropical coral reefs are increasingly threatened by anthropogenic global warming. Rising sea surface temperatures in combination with light stress causes the photosynthetic breakdown of the coral's algal symbiont, *Symbiodinium*. Corals have developed a number of physiological responses to handle acute stressors, such as the production of ultraviolet-protecting amino acids, heat shock proteins, the ability to shift symbionts, and the production of fluorescent proteins. The latter has been thought to play a photoprotective role in the coral holobiont, and studies have shown evidence that corals orient these pigments to divert harmful light away from their symbionts in shallow reefs that are at great risk of environmental stress. The biological role these proteins play is still largely speculative. This study is part of a larger study examining coral physiological responses to thermal stress.

In total, 170 *Acropora tenuis* colonies were tagged in reefs surrounding Pelorus and Orpheus Islands before the onset on the bleaching event of 2017 that affected the Central Sector of the Great Barrier Reef. In addition to studying the genetic differences between resilient and susceptible individuals, the fluorescent protein expression of these colonies was examined during the height (March) and end (April) of the bleaching event. Of 25 colonies that underwent analysis, 21 experienced extreme thermal stress and were nearly completely bleached in April and two colonies not only survived the bleaching, but increased their *Symbiodinium* counts. Fragments collected were split into top and bottom portions for analysis. Top fragments experienced greater rates of bleaching than bottom

fragments at both the March and April time point, suggesting that exposure to light in addition to heat is more stressful than heat alone. Total protein content linearly decreased weakly, but significantly, with the loss of *Symbiodinium* (r^2 = 0.3226; P-value = 4.292e^-10). Fluorescent protein expression between March and April in all 25 colonies did not significantly change (r^2 = 0.0002365, P=0.3142), but the ratio in concentration of cyan fluorescent protein (475 nm) to green fluorescent protein (505 nm) was higher in top fragments of colonies that remained bleached than in top fragments of colonies that remained healthy throughout March and April ($P < 0.05$). The same ratio difference was not detected in the bottom fragments ($P > 0.05$). This pattern suggests variability in the effects light and heat have on coral's physiological response to stress and that corals may downregulate energetically costly FPs during bleaching.

Key Words: Fluorescent proteins, CFP, GFP, *A. tenuis,* Spectrophotometer

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Acknowledgements

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I would like to thank Dr. Mia Hoogenboom for allowing me to process samples in her laboratory and Dr. Allison Paley for patiently guiding me through laboratory procedures until I felt confident enough to do them on my own. Additionally, I would like to thank Tess for her willingness to assist me in the laboratory so I could finish my project on time.

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Introduction

Increasingly, coral reefs are facing a barrage of anthropogenic threats, none of which are more insidious than rising sea surface temperatures caused by human-driven climate change. The foundational species of coral reefs contributing to the growth and maintenance of the reef framework through calcium carbonate secretion belong to the order Scleractinia. These colonial animals form endosymbiotic relationships with a multitude of other organisms, including archaea, fungi, bacteria, and algae. The symbiotic relationship between coral and their alga (*Symbiodinium* spp.) is considerably important to the health of the coral holobiont as well as to the functionality of the reef. Unfortunately, this symbiosis is incredibly sensitive to environmental stressors, especially heat stress.

Symbiodinium receive protection from predators, preferential location in the water column, and waste products from corals. The coral host benefits from the algal symbiosis by obtaining photosynthetically fixed carbon compounds for skeletal formation, waste disposal, and possibly photo-enhanced light calcification (Falkowski 1984). Corals rely on their photosynthetic algal partners for over 95% of their total energy requirements and compounds necessary for metabolic processes and skeletal formation (Falkowsi et al. 1984, Sutton & Hoegh-Guldberg 1990, Muscatine et al. 1981). Additionally, there is evidence to suggest the genus *Acropora* relies on its symbionts for metabolic processes. The amino acid cysteine biosynthesis that is necessary for some metabolic functions was not found in the coding region of the *Acropora* genome, suggesting that this genus may be more heavily dependent on its algal symbionts for metabolic processes than others

which could explain the fragility of these corals to environmental stress (Shinzato et al 2011). This symbiotic relationship is crucial to colony survival and is the basis for the entire coral reef ecosystem.

Bleaching is caused by a number of stress factors, including overexposure to heat, light, drastic changes in salinity, sedimentation, and chemicals. Since the first global bleaching event of 1998, rising sea surface temperatures has been the culprit behind a majority of bleaching events around the world. The coral-*Symbiodinium* symbiosis is fragile to heat stress and can become especially stressed during the summer when bleaching thresholds are more likely to be surpassed. Heat stress causes photosynthetic breakdown within *Symbiodinium* cells, most likely beginning in photosystem II, and results in the accumulation of toxic reactive oxygen species within the cells (Warner et al. 1999). Corals expel their symbionts as a mechanism for short-term survival and consequently lose their main energy source. Corals that have lost a large proportion of their pigmented symbionts appear white, exposing their skeleton through transparent tissues.

Bleaching is expected to increase in occurrence, duration, and intensity in the next several decades (Hughes et al. 2017). Sea surface temperatures have risen by a mean 0.6^{o}C in only 60 years (Bindoff et al. 2007). There are some projections that predict annual bleaching may occur on the GBR by 2030 if carbon emissions are not reduced (Hennessy et al. 2007). The distributions of scleractinian coral species will depend on inter and intraspecific variation in mechanisms designed to survive high stress conditions. Bleaching events in the past on the GBR have shown which species are more likely to be "winners" under future climate change conditions and which are the most susceptible to

increasing amounts of stress (Baird & Marshall 2000). Massive species such as *Porites* and *Platygyra* historically have been less vulnerable to stressors such as heat and cyclonic activity while tabular, branching, and plating *Acropora* spp. and *Pocillopora* spp. are usually the first to bleach during episodes of heat stress (Van et al. 2011). Determining which species and genera are more likely to survive as spatial and environmental frameworks become limited is critical to understanding how composition of reef communities will shift in the future.

Recent research has shown acclimation and adaptation mechanisms within corals.

Symbiont Shuffling. Different clades of *Symbiodinium* are variable in their metabolic and photochemical efficiencies and abilities to withstand certain stressors. There are over 250 types of *Symbiodinium* grouped into clades A-H and their genetic differences correspond with physiological response in accordance with photo-acclamatory response to light, growth, and thermotolerance (Coffroth & Santos 2005; Iglesias-Prieto et al. 2004, Robinson & Warner 2006). Types known to be particularly resilient to heat stress belong to Clade D (Baker 2004). Studies have shown that surviving corals shift their symbionts to predominantly Clade D after bleaching (Silverstein et al. 2014).

Corals vary in their selectivity of differing *Symbiodinium* Clades, with some species willing to host a variety of types while others are very specialized in their choice of symbiotic partners. Advanced molecular techniques have revealed that many corals thought to be specialists are actually generalists that host a great genetic diversity of algal symbionts. (Silverstein, Correa, Baker 2012). The coral's ability to shift *Symbiodinium*

assemblages is a crucial factor in acclimatizing to a changing environment under longer durations and occurrences of stress (Berkelmans & van Oppen 2006).

Bleaching provides the opportunity for the proliferation of taxa that are normally unfavorable during stress-free conditions. These opportunists have been defined as "disaster taxa" (Correa, Baker 2011), symbionts that normally live marginally in host tissues but become abundant during periods of extreme environmental stress. Corals tend to switch stress-sensitive symbionts for stress-resilient types during periods of high stress and keep stress-sensitive types under ambient conditions because members of disaster taxa have high-energy costs during stress-free conditions. Clade D, though known for its ability to withstand heat stress, is also known as a "selfish opportunist." This *Symbiodinium* type is unfavorable for corals to host because it does not translate as much of its photosynthetic products to the coral as other types do (Stat & Gates 2011).

Morphological Plasticity. Scleractinian corals are diverse in their life history strategies and energy investments. Large, branching, tabular coral species such as *Acropora, Pocillopora, Turbinaria, and Montipora* invest in quick growth, live in shallow reef environments, and are broadcast reproductive spawners. (Darling et al. 2012). This competitive life strategy is very successful under shallow, high-light environments with few disturbances. Corals that appear to have a stress-tolerant life history include slow growing species that reproduce through broadcast spawning and have high fecundity rates. Species belonging to *Orbicella* and *Montastrea* in the Caribbean and massive *Porites* and *Diploria* are normally very resilient to bleaching stress (Darling et al. 2012). Massive species that follow weedy, stress-tolerant, or generalist life histories tend to display partial coral mortality after bleaching events rather than the whole-colony

mortality often observed in competitive *Acropora* species (Baird & Marshall 2002, Darling et al. 2012). Due to increasing severity and occurrence of stressors, it is predicted that competitive, fast-growth life histories that otherwise compete intensively and successfully under normal conditions will fare poorly under stressed conditions, and weedy, stress-tolerant species may become dominating on the reef, contributing to an overall "flattening" of the reefscape (Darling et al 2012.)

Genetics. Shinzato et al 2011 found through analyzing the *Acropora* genome that corals can produce an amino acid (Mycosporine amino acids) independent of their symbionts that has ultraviolet protective abilities. Intraspecific variability in coral polymorphisms shows differences in genetic expression intraspecifically (Gittins et al 2015). Furthermore, Paley et al 2014 found that bleaching intensity differed greatly among different color morphs of *Acropora millopora*. Though stress-resilience is often attributed to the physiological response of the *Symbiodinium*, it is equally valid that corals themselves have distinct genotypes that produce phenotypic traits that vary in response to environmental factors. Subjecting coral colonies to experimental heat stress and subsequently selectively breeding stress-tolerant individuals has been proposed as a mechanism for restoring degraded reefs (Van Oppen et al. 2015). As an example, since heat stress combined with light intensity causes the production of noxious oxygen species by *Symbiodinium*, interspecific and intraspecific genetic variation in the ability to neutralize these species may determine bleaching severity. Indeed, two quantitative trait loci in *Acropora millepora* were identified for neutralization of reactive oxygen species (antioxidant capacity) and environmental stress tolerance (Jin 2016). Manipulations or

insertions from known genetic markers could be used to possibly bolster coral resilience against heat stress in the laboratory.

Fluorescent Proteins. Reef-building corals are generally restricted to shallow tropical seas and must have developed mechanisms to contend with high levels of UV irradiance. Corals have been postulated to have the ability to shift damaging light away from their *Symbiodinium* by placing fluorescent protein pigments above these algae in shallow water (Sahil et al 2000). Conversely, at deeper depths with minimal light penetration, corals place these pigments adjacent to or below their *Symbiodinium* in order to enhance light acquisition (Sahil et al 2000). Proposed and tested photoprotective hypotheses have indicated FP's may absorb and scatter damaging UV light that would otherwise damage photosystem II, a mechanism critical to the photosynthetic process (Gilmore et al. 2003) Coral FP's display functions other than photoprotection. FP's have been shown to deactivate reactive oxygen species and possess other antioxidant properties, which serves to mitigate toxicity stress to corals during bleaching (Bou-Abdallah et al. 2006; Palmer et al. 2009).

There are four basic colors of fluorescent proteins: Cyan (CFP), Green (GFP), Red, and a non-fluorescent purple/blue chromoprotein (Alieva et al. 2008). Cyan and green fluorescent proteins have the same chromophore, a molecule that absorbs light and re-emits it at a different wavelength, but are evolutionarily distinct, suggesting natural selective processes acting on these proteins resulted in two biologically distinct proteins (Henderson et al. 2005). Green fluorescent proteins emission maximum is greater than 500 nm while cyan fluorescent proteins emission spectrum maximum is below 500 nm. Blue-shifted variants are found ~477 nm (Alieva et al. 2008).

There may be a number of discrete biological indicators that precede the onset of visual bleaching and may also be determinants of organismal stress-resilience. In previous studies, different color morphs characterized by differing dominant fluorescent protein concentrations resulted in variable bleaching susceptibility, showing coral-mediated mechanisms in response to thermal stress (Paley et al. 2014). A study within the same thesis as Paley et al. 2014 found that changes in fluorescent protein type expression occurred in tandem with declining coral health. The switch from production of a highly fluorescent protein to a weaker fluorescent protein in correlation with degrading health suggests that continual production of certain fluorescent proteins are more energetically costly than others (Paley). An unpublished study from last year suggested a switch in CFP and GFP concentration in samples of *Stylophora pistillata* during a period of extreme thermal stress. This study aims follow up on the previous year's data to possibly identify changes in fluorescent protein pigment assemblages in *Acropora tenuis over* the period of the 2017 bleaching event to further our understanding of fluorescent protein expression.

Methods and Materials

2.1 Data Collection. In total, 170 *Acropora tenuis* colonies were tagged in groupings in four different locations surrounding Orpheus and Pelorus Island, two islands within the larger Palm Islands group in the central sector of the Great Barrier Reef (18°37'S 146° 15'E). Colonies were monitored at from February until May on a monthly basis to follow the bleaching event as it progressed. Two time points of collected samples were used in this study, March and April. Using scuba and a dive knife, two fragments from each

colony were broken off from each colony and placed in labeled plastic bags. A third fragment was collected if the colony was bleached and stored in paraformaldehyde for microscopy not utilized in this paper. Samples were processed immediately upon resurfacing. Each fragment was wrapped in aluminum foil with a paper tag indicating which colony number it came from and then stored in a portable dry-shipper (liquid nitrogen container). Samples were kept frozen on dry ice in transport back to the Coral Physiology Lab at James Cook University.

2.2 Sample Processing. Fragments from 25 colonies were spliced into upper and lower halves in order to eliminate biases resulting from light mediated spatial bleaching patterns. Each split fragment was labeled as either top or bottom and placed in its own plastic bag and filled with 10ml of filtered seawater. Fragments were tissue blasted completely using a high-pressure air gun to create tissue slurry in the bag that was then emptied into larger tubes for homogenization (Approximately for 30 seconds with IKA-Labortechnik). For the purpose of evaluating bleaching severity, 900uL of each slurry sample was pipetted into an eppendorf tube with 100uL of formaldehyde for *Symbiodinium* counts. The slurry was then centrifuged for 5 minutes at 3500 rpm and 4°C to remove tissue and cellular particulates and immediately stored at -30°C.

2.3 Surface Area Estimation Surface area of each nubbin was calculated to standardize *Symbiodinium* counts. Tissue blasted fragments that had been previously soaked in 20% bleach solution for two minutes were dipped into hot wax and weighed. After the first mass measurement was taken from the first wax dip, the fragment was then dipped in wax a second time, dried, and then weighed. The difference in weight between the first

and the second dipping was used to find surface area estimation based on a calibration curve formulated with plastic cylinders of known surface area and weight ranging from 8.41 cm^2 to 135.89cm^2. Using the following regression equation:

Equation 1.1 $SA(cm^{-2}) = M \times D$

where M is the variable from the regression equation (cm^{-2} mg^{-1}) and D is the difference in weight in *mg* between wax dipping. The calibration curve is $SA(cm^{-2}) = 0.0344*D$.

2.4 Symbiodinium Counts. Symbiodinium cell counts were conducted using an improved Neubauer haemocytometer with two counting chambers in order to assess the bleaching severity of the bleaching state of the sample. Tissue slurry was pipetted into the chambers using glass pipettes and examined under a microscope at 40X magnification. Two replicate cell counts on each haemocytometer plate gave four cell counts, which were averaged for each sample. The average concentration of *Symbiodinium* for each branch was calculated by the equation:

Equation 1.2 $Zooxenthellae = N \times 10^{-4} \times D/SA$

where N is the mean of the four cell counts, D is the volume of water used in the bag during airbrushing (a dilution factor of 10mL), and SA is the surface area of the nubbin in cm^{-2}.

2.5 Protein Quantification. Total protein concentration was calculated using a Peterson-s Lowry total protein standard assay utilizing a bovine serum albumin (BSA) serial dilution concentration of 2.0 mg ml^{-1} 1.0 mg ml^{-1}, 0.50 mg ml^{-1}, 0.25 mg ml^{-1}, 0.125 mg ml^{-1}. A plate map describing the location of every sample and standard was drawn up before plating the microplate. Twenty uL of each concentration of BSA standard and samples were pipetted over ice in triplicate into a clear 96-well standard microplate along with180 uL of Red 660 reagent using a multichannel pipette. Samples and standards were read in a SpectraMax M2 spectrophotometer with endpoint absorbance set at 660 nm. Additionally, 20 uL of sample was placed in triplicate in a 384-black/clear bottom Greiner fluorescence plate over ice. Samples were read on the SpectraMax M2 spectrophotometer set to read fluorescence within wavelengths 400-700 nm and read in increments of 5 nm at an excitation wavelength at 280 nm.

2.6 Statistical Analysis

Linear regression analyses in R were used to find significance and strength of correlation between: total protein content and *Symbiodinium* counts; cyan fluorescence and *Symbiodinium* counts. Welch Two-Sample T.Tests performed in R were used to find significant differences in the *Symbiodinium* counts from March to April time points and to find significance in differing CFP/GFP ratios.

Results

3.1 Symbiodinium Change Over Time

The relative numbers of *Symbiodinium* remaining in its host's tissues measures bleaching intensity. Bleaching progressed, as correctly hypothesized, from the March to

April time point for both bottom and top fragments (Figure 1) relative to the top fragments, bottom fragments were less bleached overall in both the March and April time point.

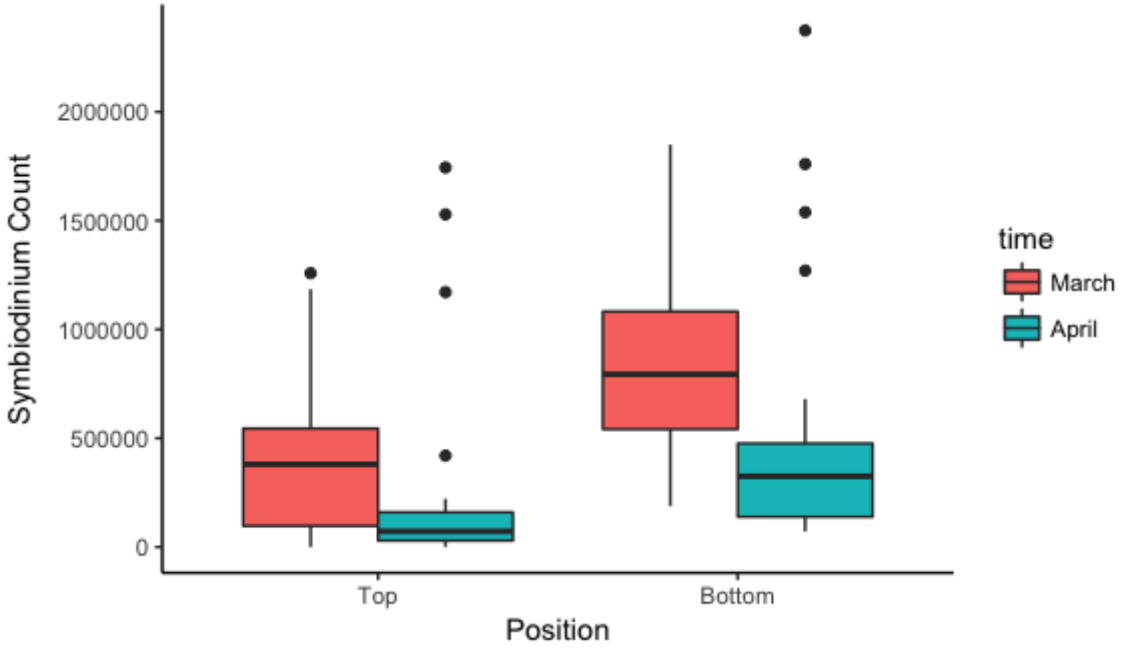

Figure 1. *Symbiodinium* density (cm^{-2}) of top and bottom fragments shown with respect to time points in March (red) and April (blue).

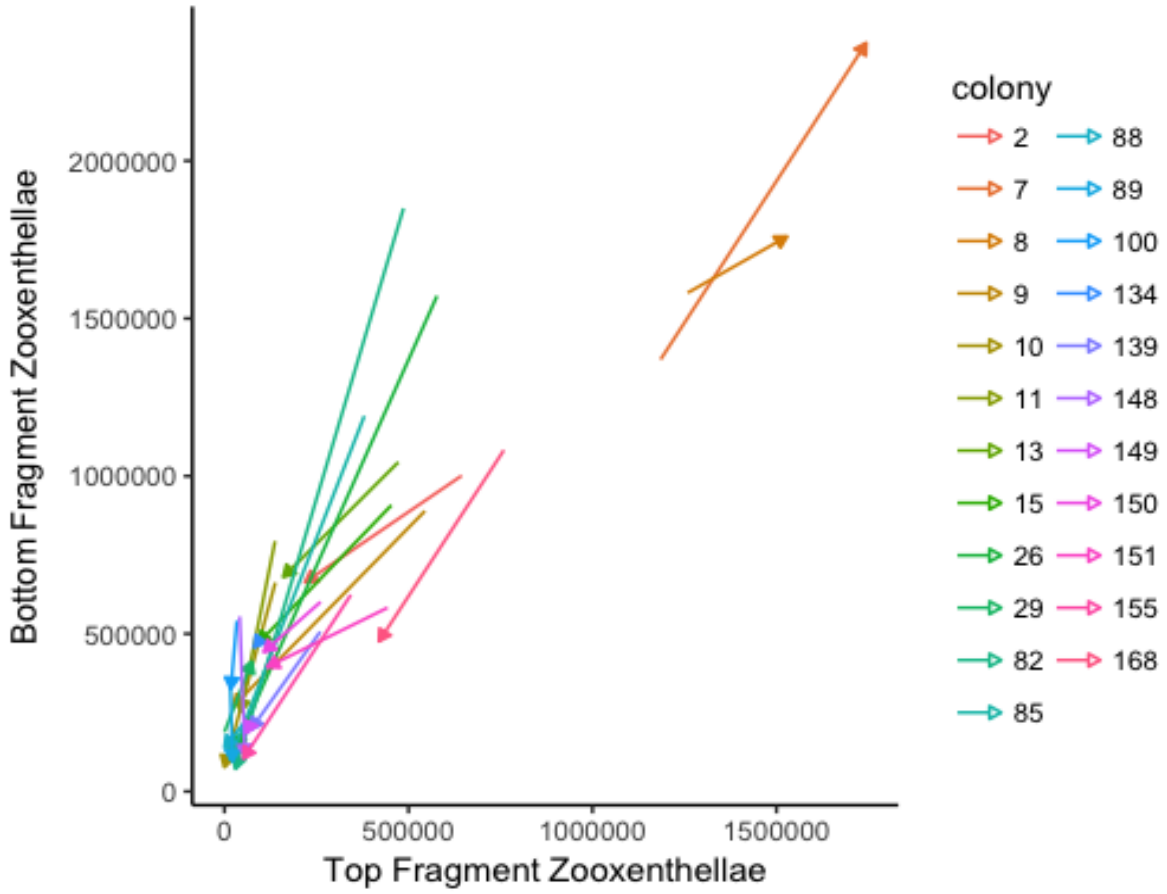

Figure 2. Change in bottom zooxenthellae (*Symbiodinium*) counts as a function of change in top fragment cell counts between two time points, March and April (n=23). The direction of the arrow correlates with time from March to April. Twenty-one colonies experienced decreasing cell counts while two experienced increasing cell counts over the bleaching period.

Symbiodinium, otherwise known as zooxenthellae, counts decreased both in the top and bottom fragments for 21 colonies from March to April. A total of two colonies experienced increases in cell count in both top and bottom fragments from March to April. Two colonies (top and bottom fragments) were removed from Figure 2 due to their determination as outliers, most likely due to processing bias.

3.2 Total Protein and Fluorescent Protein Temporal Variation

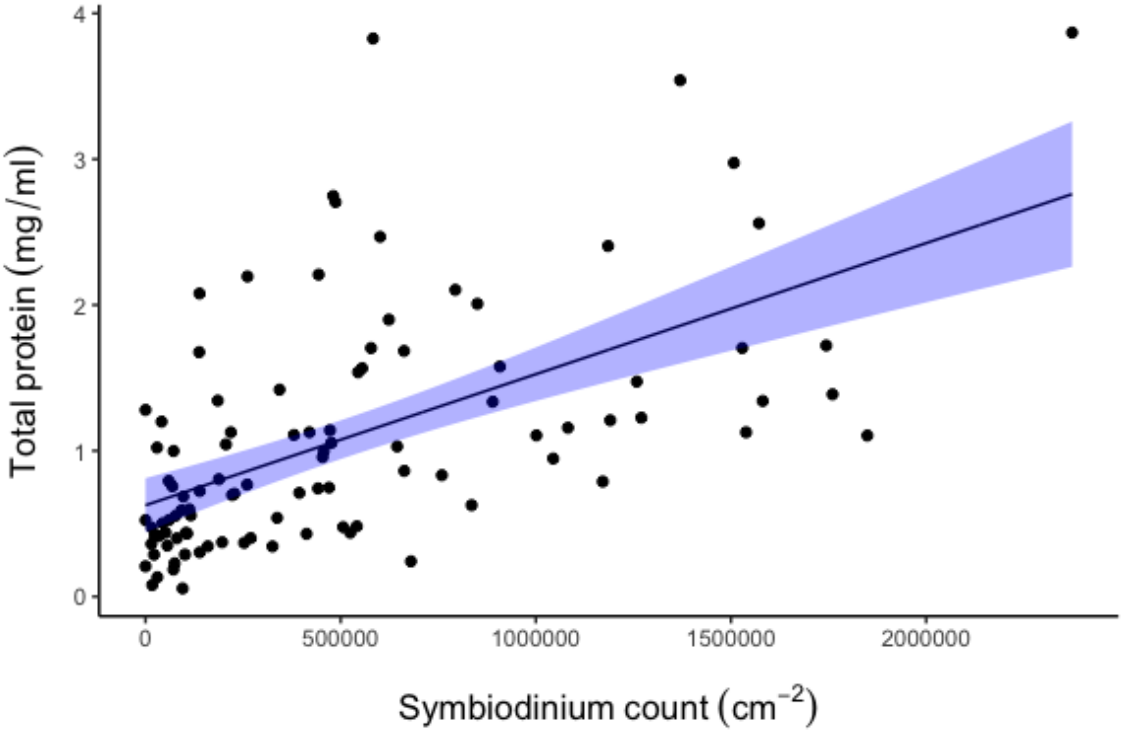

Figure 3. *Symbiodinium* concentration per cm^{-2} correlated with total protein content in mg ml^{-1}. Line shows linear regression line with 95% confidence intervals. ($r^2 = 0.3226$; $p = 4.29 \times e^{-10}$)

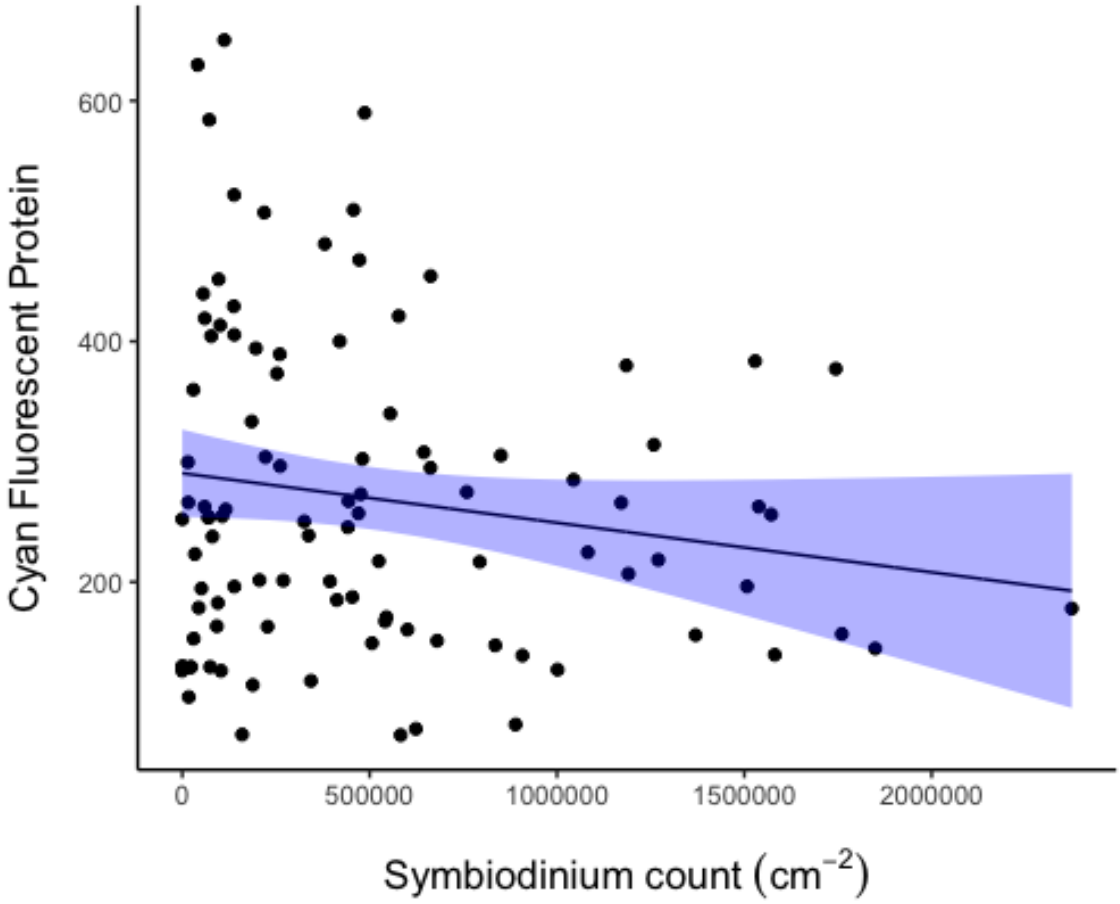

Figure 4. Intensity of CFP emission (measured as RFU mg^{-1} protein) at 475 nm decreases as *Symbiodinium* counts increase. Line shows linear regression line with 95% confidence intervals. (r^2 = 0.126; P-value = 0.000171)

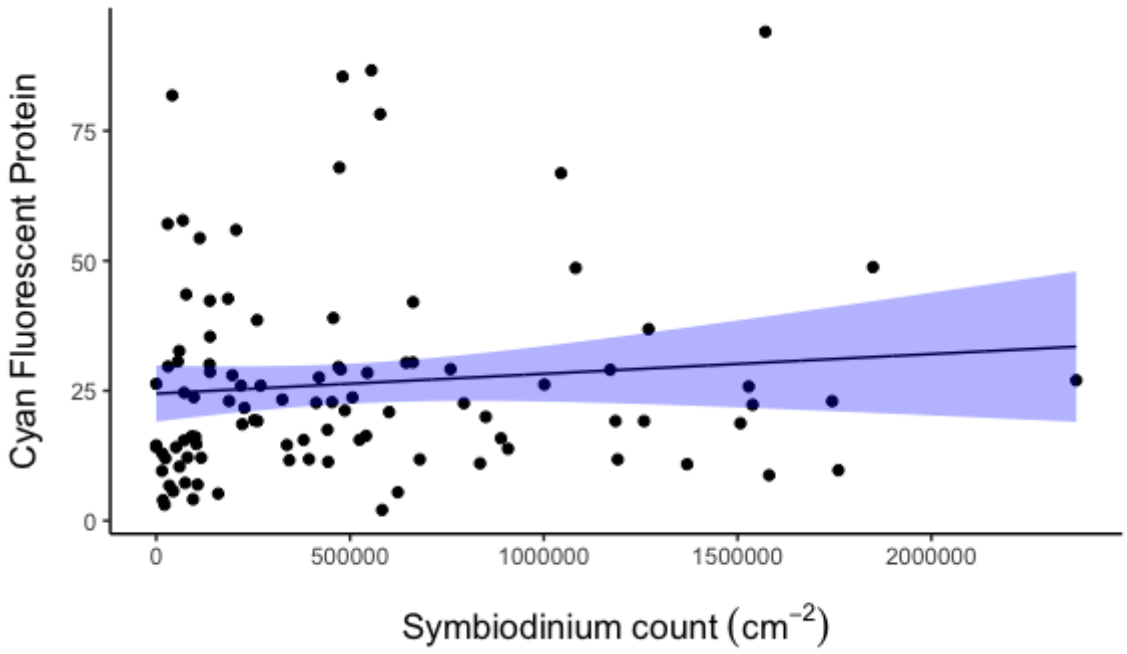

Figure 5. CFP expression as a function of *Symbiodinium* counts but fixed by Surface Area of the nubbin (measured as RFU cm^{-2}) as opposed to total protein content. Line shows linear regression line with 95% confidence intervals. (r^2 = 0.0002365, P = 0.3142)

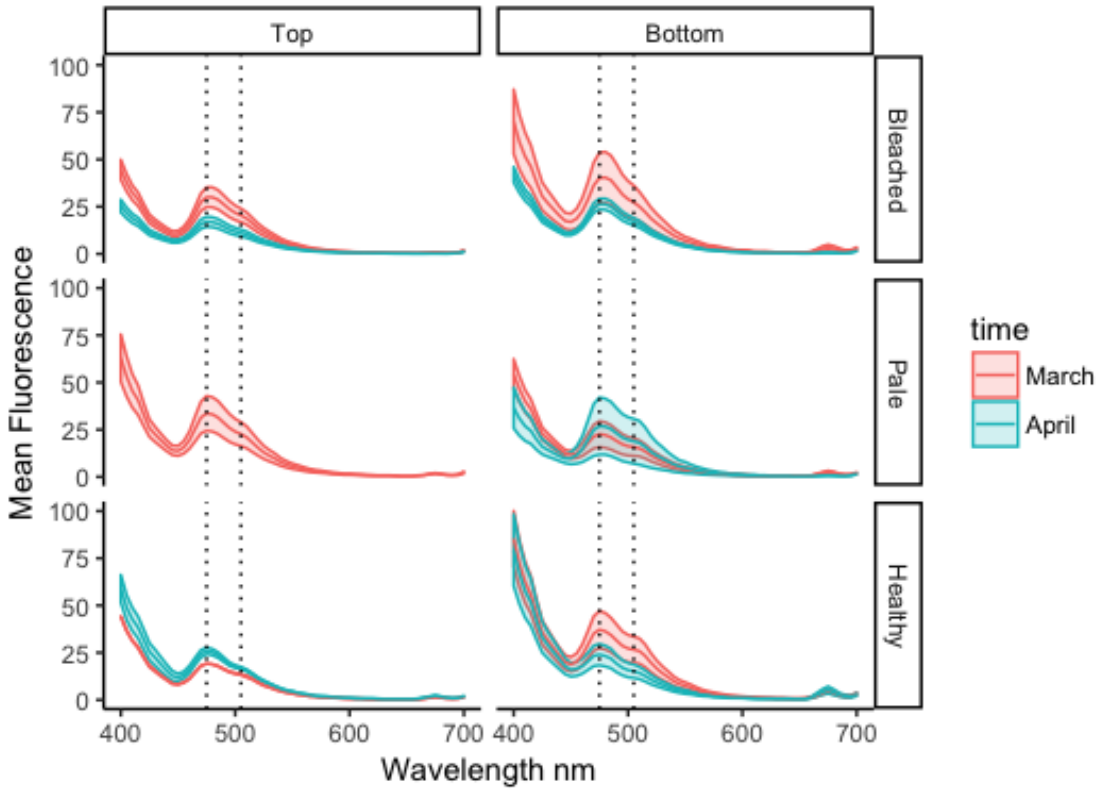

Figure 6. Fluorescence measured in RFU per cm^{-2} of top and bottom fragments of bleached, pale, and healthy corals separated into time points. Bleached cell counts (<500,000); Pale cell counts (500,000 – 999,000); Healthy cell counts (>1,000,000).

Total protein content (mg ml^{-1}) significantly, but weakly increased with increasing numbers of *Symbiodinium* (Figure 3; $r^2 = 0.3226$; P-value = 4.292e^-10). Cyan Fluorescent Protein (CFP) emission intensity at 475 nm was compared with increasing numbers of *Symbiodinium* cell counts. A significant, but weak, correlation was found showing increasing CFP concentration (when standardized by total protein content) with decreasing *Symbiodinium* density across all samples, including top and bottom fragments from both time points (Figure 4; $r^2 = 0.126$; P-value = 0.000171). However, this relationship becomes statistically non-significant and even weaker when CFP is standardized by surface area of the coral fragment (Figure 5; $r^2 = 0.0002365$, P = 0.3142).

Additionally, no distinguishable pattern in fluorescent expression can be found based on results shown in Figure 6.

3.3 Temporal Patterns of CFP/GFP Ratio

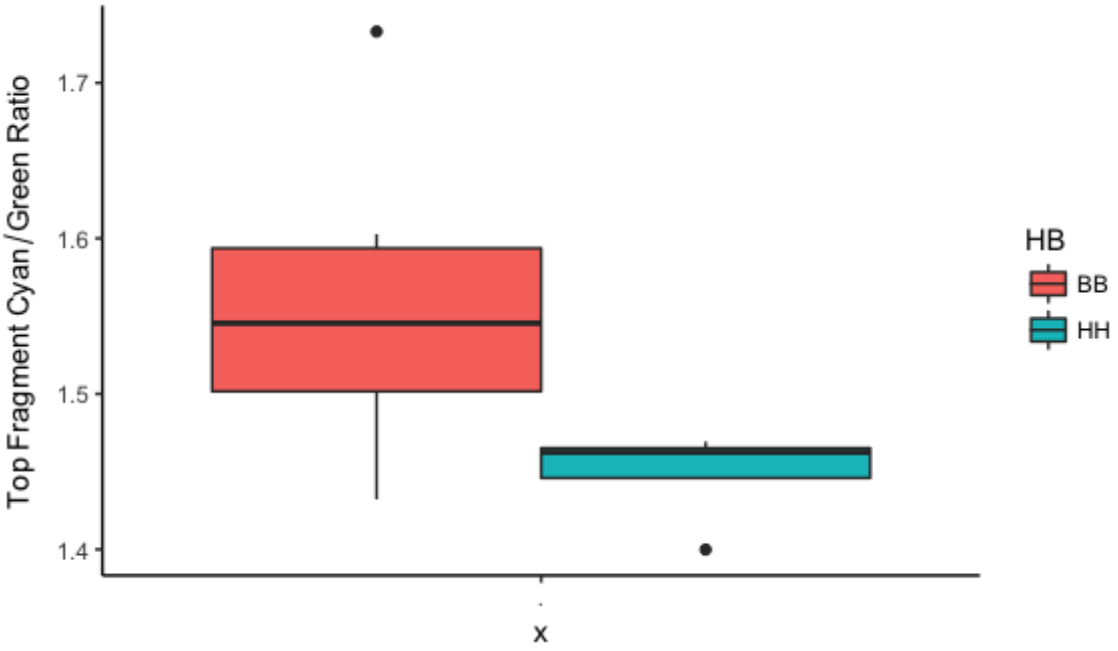

Figure 7. Ratio of CFP to GFP in top fragments of colonies that remained healthy and bleached in March and April (P = 0.04881). Fragments that contained >800,000 *Symbiodinium* cm^{-2} in both March and April were designated as HH and fragments that contained <20,000 *Symbiodinium* in both March and April were designated as BB.

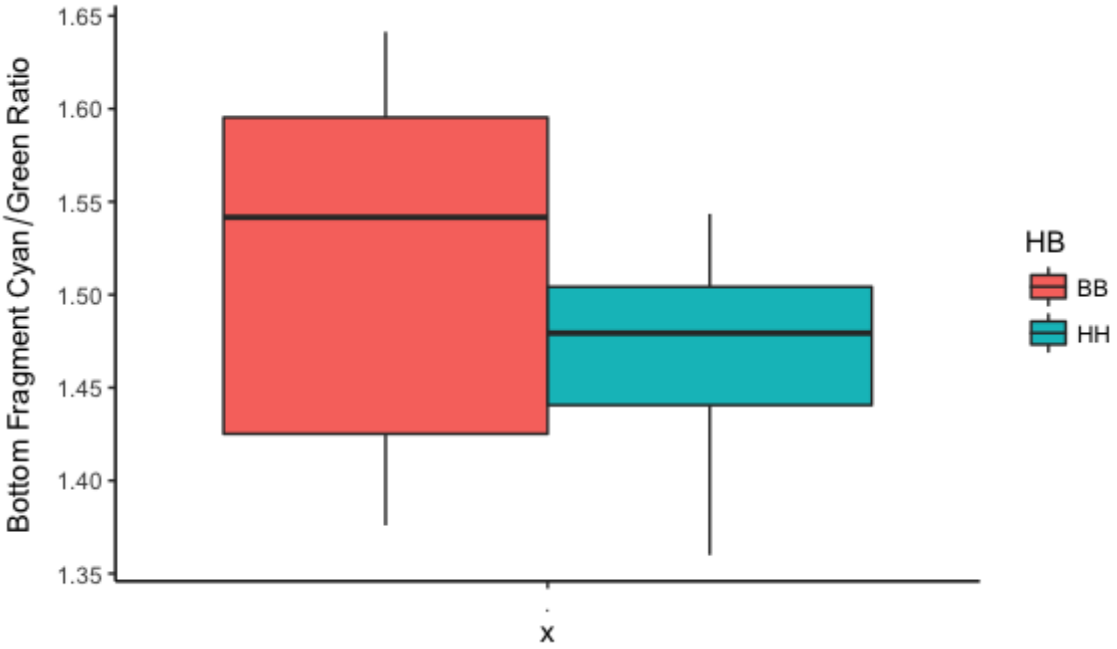

Figure 8. Ratio of CFP to GFP in top fragments of colonies that remained healthy and bleached in March and April (Not significant; P > 0.5).

CFP/GFP ratios were significantly different in top fragments from colonies that were bleached as opposed to top fragments in colonies that remained healthy during the bleaching event (Figure 6). The mean ratio in bleached top fragments was 1.558806 and the mean ratio in healthy top fragments was 1.448552 (Welch Two-Sample T.Test; P = 0.04881). This significant difference in the ratio in top fragments was not detected in the bottom fragments (Figure 7). The mean ratio in bleached bottom fragments was 1.465512 and the mean ratio for healthy bottom fragments was 1.516692 (Welch Two-Sample T.Test; P = 0.4126).

Discussion

Spatial Bleaching Patterns

Bleaching status of a coral is dependent on the stress incurred and the numbers of *Symbiodinium* lost from its tissues. Greater losses of *Symbiodinium* correlate with increasing amounts of stress and increasing bleaching severity. Based on the *Symbiodinium* counts of our 25 samples, the bottom fragments contained higher densities of cells than the top fragments in both March and April (Figure 1). This pattern of bleaching was consistent across all bleached colonies, with most samples showing more severe bleaching in the tips than in the bottoms of the fragments. Tops of the fragments were exposed to both heat and light stress while bottom fragments only experienced heat stress because upper portions of the colony shaded them. This spatial bleaching pattern is a strong indication that exposure to higher levels of solar radiation in combination with heat stress results in more severe bleaching than heat stress alone. Downs et al. 2013 demonstrated that light stress causes significantly more oxidative damage than heat stress alone and that light stress causes a buildup of damaged proteins. Oxidative damage was shown to be greater in samples exposed to a combination of heat and light stress than heat stress alone, thus indicating that heat and light stress cause photosynthetic degradation through different mechanisms.

In 21 of 25 colonies, densities of *Symbiodinium* decreased in both the top and bottom fragments as time progressed between March and April, respectively the height and end point of the bleaching event. The loss of *Symbiodinium* between March and April for both top and bottom fragments was highly significant. Two colonies showing bleaching anomalies inconsistent with our visual-based bleaching assessment were

removed as outliers likely due to laboratory processing bias. A total of two colonies surprisingly increased the densities of their *Symbiodinium* community in both the top and bottom fragment, suggesting intraspecific variation in physiological response to cope with environmental stress. Possible explanations for why these two colonies were not only able to cope with the thermal stress that bleached other colonies, but actually increase their photosynthetic *Symbiodinium* counts could be due to a switching or proliferation of stress-resilient *Symbiodinium* types or a genetic trait that would enable the individual to better cope with oxidative stress. (Berkelmans & van Oppen 2006, Baker 2004, Jin et al. 2016). The genetic structure of "winners" and "losers" in this study will be further analyzed at a later stage for differences in their genetic markers in order to assess environmental effects on gene expression. Additionally, the *Symbiodinium* will be genetically typed to determine whether *Symbiodinium* types differed between bleached and healthy colonies and determine if surviving bleached colonies recovered and changed symbiont types.

CFP/GFP Ratio

The aim of this study was to find a distinct change in the intensity of the fluorescent protein expression between CFP and GFP based on preliminary results from a study on *Stylophora pistillata* last year. Analysis of bleaching *S. pistillata* colonies indicated a switch in the concentrations of CFP and GFP over the duration of the bleaching event (Figure A). The ratio of CFP/GFP in healthy colonies changed significantly once those colonies bleached, creating an "X" pattern in the expression of these two different proteins. The concentration of CFP rose in comparison to the

concentration of GFP, which fell. This could imply that physiological stress response in corals occurred or that GFP degrades more quickly than CFP. We hypothesized based on this unpublished work that the expression of CFPs would increase while expression of GFP would decrease in our study species, *Acropora tenuis* during the months of thermal stress. The results from this study conclude a significant, but weak, difference in the CFP/GFP concentration ratios in top fragments of healthy and bleached colonies. However, this result came from a very small sample size comparing colonies that remained bleached to colonies that remained completely healthy throughout the period of thermal stress.

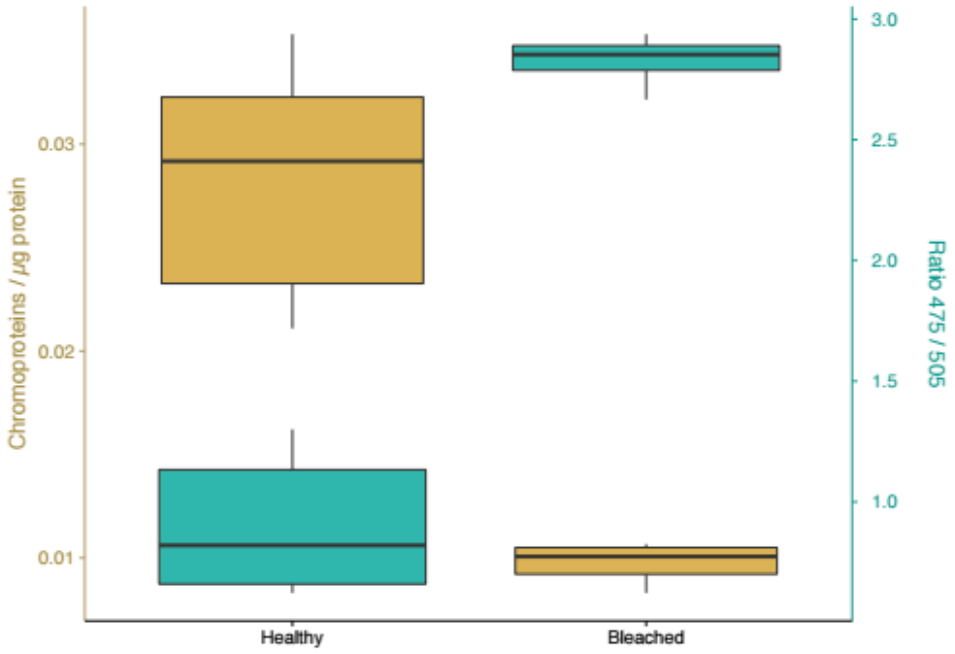

Figure A. Chromoprotein concentration per ug of total protein and CFP/GFP ratios of *S. pistillata* when the corals were healthy and subsequently bleached. This graph is the basis for the current study.

We offer an explanation to explain fluorescent protein type switching under stressful conditions. As bleaching progresses, corals lose their photosynthetic symbionts. Theoretically, it would be advantageous for corals not to invest their energy into processes that would not increase their resilience to stress. If we assume that FPs do play a photoprotective role for *Symbiodinium* under normal environmental conditions (Salih et al. 2000), corals may stop producing these fluorescent proteins during periods of bleaching because there is no longer a need to protect symbionts that are no longer in its tissues (Paley et al. 2014). If corals stop producing fluorescent proteins during stress, it is unclear at which stage during bleaching that fluorescent protein production may cease. It is likely that corals may continue to produce fluorescent proteins until the stress becomes severe, categorized by the loss of nearly all *Symbiodinium*. Production of assumed unnecessary fluorescent pigments may cease at this point. Breakdown rates between FPs may differ, and CFP may not degrade as quickly as GFP. This would result in relatively similar concentration of CFP and GFP concentrations at the onset of bleaching and decreased concentration of GFP towards the end of bleaching. Since CFP concentration did not significantly change during the bleaching event across colonies, we may speculate that either FPs were being produced at the same rates or that both proteins take a long time to degrade.

Some FP types may play a greater photoprotective role than other FP types under severely stressful conditions. Under normal circumstances, both CFP and GFP may be expressed at normal production levels with natural fluctuations (Paley et al 2014.) but thermal stress could trigger a shift in the upregulation of different FPs. Corals may upregulate CFP expression in the *early* stages of a warming event as a preventative

measure. However, further evidence of upregulation of CFP *during* thermal stress could indicate the existence of a fluorescent protein-specific stress-response mechanism in corals to protect remaining *Symbiodinium*. The upregulation, and favoritism, of one type of fluorescent protein over another during extreme stress may be analogous to observed *Symbiodinium* switching during thermal stress. (Berkelmans & van Oppen 2006, Silverstein et al 2014, Baker). However, we would need further evidence showing upregulation of a certain FP type during a period of extreme thermal stress.

Though our data shows a significant difference in the ratio of CFP to GFP in bleached top fragments of colonies during the bleaching event, the ratio in the bottom fragments is widely variable and there was no significant change in the ratio. This difference could be attributable to the differences in bleaching observed between top and bottom fragments. As previously discussed, evidence has been found to indicate that different stressors (heat and light) may negatively compromise *Symbiodinium* by different mechanisms (Downs et al. 2014). However, we cannot affirm that the local population of *Acropora tenuis* at Orpheus and Pelorus Islands switches expression of two of its FPs within the duration of high thermal stress. The samples of *S. pistillata* gathered last year that resulted in the "X" configuration were possibly collected under a more severe stress event than what our *A. tenuis* colonies experienced this year. This FP switching may be dependent on the severity of the stressor as well as dependent on species. In any case, the results from our study indicate probable inter and intraspecific variability in coral's physiological responses to stress through fluorescent protein expression.

Total Protein Standardization

This study can confirm a significant, but slightly weak, decrease of total proteins as *Symbiodinium* density decreases (protein loss correlated with bleaching), furthering evidence to suggest that loss of symbionts results in loss of energy necessary for metabolic processes. Additionally, we offer evidence to suggest that the method of standardization for fluorescent proteins should not be dependent on the total protein content of the sample. Our data shows that based on method of standardization, a significant relationship may be found that is otherwise statistically non-significant. When CFP expression was standardized by total protein content (RFU mg^{-1} protein) of the sample and mapped against *Symbiodinium* density, a weak, positive, significant correlation was found (increasing protein concentration with increasing coral health). However, when CFP was instead standardized by surface area (RFU cm^{-2}), this supposed positive correlation became statistically insignificant and the linear regression model become negative. The basis of our understanding for why this occurred is due to the negative change in total protein content over the course of the bleaching event. Standardizing fluorescent protein content by a metric that isn't stable may have produced the differences in the significant and non-significant relationship between CFP and bleaching progression. This is important and should be considered when evaluating past studies that have standardized FP data by total protein content. (See Palmer et al. 2009, Alieva et al. 2008). Based off the differences in statistical significance and concentration between fluorescence measured as RFU mg^{-1} of protein and RFU cm^{-2}, we conclude that less error is introduced when standardizing by the latter metric.

Data Anomalies

There was a discrepancy between in-field visual bleaching assessments of fragments from the time point in March versus the *Symbiodinium* count results from those samples. The visual data shows healthy bottom portions of several fragments while the *Symbiodinium* estimates for those fragments show relatively low counts that would be expected from pale or even bleaching colonies. Since this study only includes 100 samples from a total of 25 colonies, and the removal of these outliers does not create a visual difference in our data, besides the change in intensity of bleaching progression from March to April in the bottom samples, we kept the outliers in this data set. This project preliminarily examining fluorescent protein concentration using 25 colonies is part of a larger project that includes approximately 130 other colonies. Once all colonies have been processed, it will become more prudent to eliminate outliers because the sampling size will be much larger. Additionally, a greater sampling size will allow greater inference into determining the cause of this discrepancy and whether they are true biological anomalies or results from laboratory processing errors, such as biased tissue blasting techniques.

Conclusion

The processes underlying stress response in the coral holobiont are still widely unknown. Past studies have strongly indicated that fluorescent proteins act as antioxidants and are modulated for the photoprotection of *Symbiodinium*. (Palmer et al. 2009, Salih et al. 2000). Though a significant difference was detected in the CFP/GFP ratio in bleached versus healthy top portions of *A. tenuis*, the difference in ratio in the bottom samples was non-significant. Any conclusions we can obtain from this data are purely speculative due to our low sample size. Overall, CFP concentration (when

standardized by a method that introduces less bias) did not change with decreasing *Symbiodinium* counts nor did it differ significantly between bleached, pale, or healthy colonies. Past studies have indicated that high concentrations of FPs correlate well with higher stress tolerance (Paley et al. 2014), though there is likely variation among different coral species. Overall, fluorescent protein expression may be dependent upon a number of variables, including the severity of stress and the species being studied. *A. tenuis* may not be the best candidate for studying fluorescent protein profiles due to its low concentration of FPs.

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