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Predation and thermal stress affect color change in the symbiotic sea anemone *Aiptasia*

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**PREDATION AND THERMAL STRESS AFFECT COLOR CHANGE
IN THE SYMBIOTIC SEA ANEMONE *AIPTASIA***

By

Samantha L. Hamlin

Accepted in Partial Completion
of the Requirements for the Degree
Master of Science

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MASTER'S THESIS

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IN THE SYMBIOTIC SEA ANEMONE *AIPTASIA***

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

by
Samantha L. Hamlin
November 12, 2009

Abstract

Bleaching is the disruption of the symbiotic relationship between anthozoans and zooxanthellae. The term bleaching refers to the host appearing lighter—sometimes becoming completely white—as a result of losing their symbiotic dinoflagellates, their photosynthetic pigments, or both. Research has demonstrated that many abiotic factors, such as temperature, ultraviolet radiation, and salinity, cause bleaching. However, we know little about the role that biotic factors, such as predation, may play in coral bleaching. Additionally, little is known about the combined effects of different stressors, and whether these effects are additive or not. If effects are synergistic and difficult to predict, then much more research is needed to understand how coral reefs will respond to climate change. Using *Aiptasia* spp. as a model for coral, I investigated whether predators affect the anemones' response to thermal stress with a fully crossed 2-factor experiment. Using digital photography and color analysis to measure red, green, and blue color change, I determined that predation caused anemones to become darker over time when compared to anemones that were not preyed on. Specifically, there was a significant predator effect when analyzing the change in blue and green color values. The magnitude of mean color change for the predator treatment was up to three times the change for the anemones that were not preyed on. These results suggest that predation actually makes the organism more resilient, thereby lessening the bleaching response. This would imply that a certain degree of coral grazing may be beneficial, which indicates that overfishing may have effects more far-reaching than previously suspected. Additionally, the effects

of temperature and predators were additive, which suggests that we can predict the combined effects of thermal and predation stress from experiments that manipulated only a single factor.

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Table of Contents

Abstract.....	iv
Acknowledgements.....	vi
List of Figures.....	viii
List of Tables.....	ix
Introduction.....	1
Methods.....	20
Animal collection and maintenance.....	20
Experimental design.....	22
Estimating color change.....	27
Tentacle Measurements.....	29
Statistical analysis.....	30
Estimating color change.....	30
Tentacle measurements.....	31
Results.....	32
Color Data.....	32
Tentacle width.....	42
Discussion.....	44
Synergy.....	44
Temperature.....	46
Predation.....	50
Summary.....	54
Literature Cited.....	56

List of Figures

Figure 1. Experimental timeline, including location, for each run	26
Figure 2. The color change of anemones for each temperature treatment.....	33
Figure 3. The color change of anemones for each predator treatment.....	34
Figure 4. The color change of anemones for each of the eight possible combinations of temperature and predation treatments.....	35
Figure 5. Comparisons of the mean change in blue color values for each temperature and predator treatment.....	39
Figure 6. Comparisons of the mean change in green color values for each temperature and predator treatment.....	40
Figure 7. Comparisons of the mean change in red color values for each temperature and predator treatment.....	41

List of Tables

Table 1. A summary of invertebrate and vertebrate corallivores by taxon.....	10
Table 2. Results from the nested linear mixed-effects analysis of the split-plot design for blue, green, and red color-change values.....	36
Table 3. Results from the nested linear mixed-effects analysis of the split-plot design for tentacle width.....	43

Introduction

All organisms in their natural habitat experience stress over the course of their lives. Hans Selye (1936) is commonly acknowledged as the first person to fully analyze, identify, and define stress in a biological context. While Selye's research focused on mammals, particularly from a medical perspective, he greatly impacted many fields in biology. As he wrote in 1965, "all living beings are constantly under stress." Selye defined stress as "all the non-specifically induced changes within a biological system." Researchers have continued to refine Selye's work. Munday (1961) argued that Selye's "induced changes" could occur at different levels of functioning: morphological, functional, histological, or biochemical, and could be a response to biotic or abiotic factors. Other researchers have defined stress in terms of the taxon being studied (e.g., plants or animals), or incorporated Darwinian fitness into the definition (Grime 1979, Sibly and Calow 1989).

Currently, stress is defined as the loss of homeostasis (Reeder and Kramer 2005). Buchanan (2000) has offered a more detailed definition that I will be using in this paper: "Stress... refers to the combined physiological reactions brought about by short- or long-term adverse and unpredictable changes in environmental conditions... [acting] in an attempt to minimize the detrimental effects of the stressor—the event initiating the stress response." These environmental stressors can be biological, including predation, disease, competition, or parasitism. They can also be physical, such as thermal stress, drought, environmental contaminants, or ultraviolet radiation. In addition to affecting an

individual, stress can affect an entire population, community, or ecosystem (Cnaani 2006, Hoffmann and Parsons 1991).

Stress can affect an individual at a variety of different levels—cellular, tissue, or organismal, which can affect the life history and fitness of an organism. Stress might cause an organism to redirect its resources to basic physiological processes in an effort to maintain homeostasis, away from growth or reproduction (Charbonnel *et al.* 2008). For example, in fish and marine invertebrates, stress resulting from things such as extreme fluctuations in temperature and osmotic pressure or decreased food availability has been observed to negatively affect reproduction, development, metabolism, growth, and immune function (Bates 2005, Cnaani 2006, Dahlhoff 2004). More specifically, fish have been shown to have an entire cascade of responses to stress that result in alteration of metabolism, tissue pH and ion balance, respiration, and immune function (Barton 2002). A tropical limpet, *Cellana grata*, has been found to experience tissue swelling, disruption to osmoregulation, and decreased respiration when exposed to hypoosmotic stress (Morritt *et al.* 2007).

In its natural habitat, however, it is unlikely that any organism will be subjected to only one stressor at a time—an organism is much more likely to encounter several different stressors concurrently, both biotic and abiotic. For instance, thermal stress may be accompanied by drought, elevated carbon dioxide levels, osmotic stress, or ultraviolet radiation (Morritt *et al.* 2007, Qaderi *et al.* 2006). This has certainly been seen in corals—thermal stress is frequently accompanied with increased ultraviolet radiation or

fluctuations in salinity (Brown 2000, Coles and Jokiel 1978, Lesser 1996, Mascarelli 1999).

An additional challenge when faced with multiple stressors is that stressors can work in a synergistic fashion—that is, “the combined effects of two or more variables cannot be predicted from the individual effect of each” (Harley *et al.* 2006). There are numerous examples of synergistic effects between multiple stressors in the literature, relating to many different species: thermal stress coupled with spawning leading to increased mortality in Pacific oysters, and parasitism and decreased nutrition increasing the snowshoe hare’s vulnerability to predation (Li *et al.* 2007, Murray *et al.* 1997). Relyea and Mills (2001) demonstrated a striking example in gray treefrogs. They found a large synergistic effect between a pesticide and a predator cue in gray treefrog tadpoles—the pesticide carbaryl was much more lethal when administered in concert with water-borne chemicals from a predator. Specifically, mortality from predator cue alone was approximately 2%. When tadpoles were exposed to carbaryl, mortality was 60%. But when tadpoles were exposed to both carbaryl and predator cue, mortality jumped to 97%.

These synergistic effects have also been seen in corals. Coles and Jokiel (1978) found that there was a synergistic effect between thermal stress, ultraviolet radiation, and low salinity. Specifically, increased ultraviolet radiation and decreased salinity accelerated the effects of thermal stress in *Montipora verrucosa*, decreasing its tolerable temperature range. Furthermore, as these salinity or radiation levels fluctuated with the temperature, they saw increased mortality and decreased growth and reproduction.

Thermal stress is a common abiotic stressor that can have myriad effects on the functioning of an organism, including development, longevity, and fecundity (Hance *et al.* 2007). Lutterschmidt and Hutchison (1997) argue that it is actually the most pervasive environmental stressor because of the immediate, direct effects that temperature can have on chemical reactants. Because heat can negatively affect the stability of chemical bonds, many different processes throughout the organism may be impacted: gene function, protein and enzyme function, organ function, and the integrity of cell membranes (Cossins and Bowler 1987, Harley *et al.* 2006).

These effects of thermal stress are of particular importance to marine organisms. Intertidal organisms may be faced with the effects of not only fluctuating seawater temperatures, but fluctuating air temperatures, as well. This has been seen in several species of intertidal sea anemones—a change in temperature affects energy expenditures and oxygen consumption, as well as behavior in different species of sea anemones (Shick 1991). Additionally, many marine organisms, particularly those living in the littoral zone, are already living at the threshold of their thermal tolerances (Harley *et al.* 2006). This is certainly true of hermatypic corals, or reef-building corals. Hermatypic corals are a symbiotic relationship between the animal, cnidarian host and zooxanthellae, their algal symbionts. Hermatypic corals are extremely sensitive to even slight changes in seawater temperatures (Harley *et al.* 2006, McClanahan *et al.* 2007).

A stress response seen in symbiotic cnidarians, such as hermatypic corals, is bleaching. Bleaching occurs in symbiotic organisms when they lose their algal symbionts or the pigment from the symbionts (Brown 1997, Fitt *et al.* 2001). Symbiotic cnidarians

have photosynthetic dinoflagellates called zooxanthellae (*Symbiodinium* spp.) living within their tissues. There is an intimate carbon cycle occurring between the host and the zooxanthellae. The zooxanthellae fix inorganic carbon, some of which is used for their own respiration and synthesis of more zooxanthellae, and a portion of this fixed carbon is translocated to the host, often in the form of amino acids and glycerol (Muscatine 1980). Carbon dioxide waste from the host's respiration is then used by the zooxanthellae for further use in photosynthesis. In like fashion, some of the oxygen produced by the zooxanthellae is used for the host's respiration. Additionally, the host provides physical protection for the symbiont within its tissues, and some of its waste, particularly ammonium, is used by the zooxanthellae (Muscatine 1990).

Bleaching occurs when this symbiosis breaks down. Environmental factors such as increased temperature and irradiance stress both the animal host and symbionts, mainly because of high oxygen concentrations produced during high rates of photosynthesis (Weis 2008). Both partners of the symbiosis have enzymatic mechanisms to address oxygen toxicity, but if these oxygen concentrations become too great, the mechanisms present in both partners of the symbiosis become overwhelmed, resulting in bleaching (Weis 2008). Oxygen toxicity is not the only cause of the breakdown in the symbiosis, though, and it is likely that there are different cellular mechanisms that ultimately lead to bleaching (Douglas 2003). Furthermore, there is considerable debate as to which partner actually mediates the bleaching response, regardless of the mechanistic pathway (Douglas 2003, Weis 2008). Bleaching is commonly seen in corals, and

numerous reefs have reported bleaching events, but it is also seen in other cnidarians, mainly anemones (Lesser 1990).

While it is unknown whether the host or the symbiont initiates bleaching, there have been numerous agents linked to bleaching events: reduced salinity, lowered sea levels, sedimentation, heavy metal exposure, eutrophication, oil pollution, cyanide poisoning, and ultraviolet radiation (Brown 2000). Heat stress is an important correlate with bleaching in cnidarians—it is actually “one of the first visible signs of thermal stress” (Jokiel and Coles, 1990). Bleaching has been linked to unusually high sea temperatures in reefs throughout the world, with Goreau (1964) describing the phenomenon in the 1960s and Glynn (1984) describing elevated sea temperatures due to an El Niño Southern Oscillation (ENSO) event in 1983 (Gates *et al.* 1992).

Approximately 800 species of hermatypic corals have so far been described, and any of these living in the shallow water of reefs are considered to be susceptible to bleaching (Brown *et al.* 2002, Bryant *et al.* 1998). Severe bleaching events have been recorded in most tropical areas, including the eastern Pacific, off the western coasts of Central and South America, the southern Pacific, the western Atlantic, the Caribbean, and in the Great Barrier Reef (Glynn 1984).

Of the possible biotic stressors, predation is surely one of the most potent. Predation stress can have an effect either through predation or the threat of predation. Tissue loss from predation is a powerful stressor that may redirect the prey’s resources towards repairing the damage and away from other vital processes, such as growth or reproduction. The presence of a predator may also create stress indirectly. Chemical cues

from a predator are enough to disrupt the prey's biological processes as it attempts to avoid predation; an individual exposed to the threat of predation may exhibit decreased activity and feeding behaviors and delayed reproduction (Boonstra *et al.* 1998, Harvell 1990, Kats and Dill 1998). For example, decreased growth and metabolic rate are frequently observed in prey in the presence of predators; this has been observed in numerous invertebrates, including isopods, mayflies, and snails (reviewed in Relyea 2003). In addition, many prey species have developed a variety of defenses against predators, many of which have energetic costs, including reduced growth and reproduction; these defensive adaptations may be morphological, physiological, life historical, or behavioral (Harvell 1990, Kats and Dill 1998).

The chemical cues that are detected by prey can range from the actual scent of the predator to the scent of prey consumed by predators (Wisenden 2000). An excellent example of the latter can be found in the predatory relationship between the nudibranch *Aeolidia papillosa* and its prey, the sea anemone *Anthopleura elegantissima*. Howe and Harris (1978) found that when the nudibranch ate the sea anemone, an alarm pheromone (anthopleurine) was released by the anemone, which persisted within the tissues of the nudibranch. For at least five days after eating the anemone, the nudibranch elicited an alarm response (without actual contact—the nudibranchs were always caged) in other sea anemones, presumably as the nudibranch continued to release anthopleurine into the environment. The anemones' alarm response was significantly higher when exposed to nudibranchs that had eaten conspecific anemones versus starved nudibranchs.

Predation on corals—corallivory—has been widely observed. The most well-known coral predator is *Acanthaster planci*, the crown-of-thorns sea star. This carnivorous sea star is present on coral reefs throughout the Indo-Pacific (it is absent from the Atlantic), and illustrates classic boom-bust population dynamics (Madl 2002, Moran 1986). It is always present on the reefs, to varying degrees, with periodic outbreaks in which the size of an aggregation may climb well into the hundreds of thousands (Moran 1986). *Acanthaster planci* outbreaks have been extensive on the Great Barrier Reef—there have been three outbreaks since the 1960s—and have consequently been the focus of much of the research (Madl 2002). *Acanthaster planci* has the potential to completely restructure a reef community; most estimates are that one individual can consume 5-8 m² of live coral tissue per year. During an outbreak, this may mean widespread coral mortality—up to 90% (or more) of a reef can be destroyed (Madl 2002, Moran 1986).

However, not all corallivores' consumption is so exhaustive when feeding on corals—even *A. planci* predation is often partial when the population decreases between outbreaks (to approximately 6 km⁻² or less; Cameron 1977, McClanahan *et al.* 2005). There are estimated to be over 160 different invertebrate and vertebrate species that are either facultative or obligate corallivores (Table 1, Antonius 1977, Baums *et al.* 2003, Bruckner and Bruckner 2000, Cole *et al.* 2008, Cole *et al.* 2009a, Cole *et al.* 2009b, Knowlton *et al.* 1990, Rotjan and Lewis 2006). These other corallivores can have varying impacts on a reef, but usually not to the same degree as *A. planci*; these other corallivores impart a more chronic predation stress, rather than the massive, acute damage from *A.*

planci (Cole *et al.* 2008). It is only recently that these other corallivores have been recognized as a selective force having ecological significance in a reef community. There is still much to be learned about their role in the community, but it appears these corallivores can alter coral diversity, abundance, and resilience (Cole *et al.* 2008, Rotjan and Lewis 2008).

Other interactive effects between predation and thermal stress have been observed in corals that resulted in bleaching. Rotjan and Lewis (2006) found that chronic parrotfish grazing impeded coral recovery, defined as the reacquisition of zooxanthellae, after a bleaching event. Their results also suggest the predation by parrotfish may have an effect on how the corals respond to other stressors, hypothesizing that predation stress may actually “contribute to spatial variations in the bleaching process.” This study of predation stress coupled with a bleaching response elicited by thermal stress further supports the conclusion reached by Coles and Jokiel (1978), that the “response of corals to a given ecological factor must vary with changes in other environmental parameters, and that these interactions are most important near the limits of tolerance for a given parameter.”

It is unclear, however, to what extent, if any, that predation stress affects an organism’s response to thermal stress. Any possible effect could basically be described as deleterious or beneficial. Frequently, stress is considered to be deleterious. This is certainly the case with predation. Predation has the potential to permanently decrease an individual’s fitness. However, as discussed above, even the presence of a predator can be enough to decrease prey fitness, possibly to the point of death (Slos *et al.* 2009).

Table 1. A summary of invertebrate and vertebrate corallivores by taxon.

Phylum	Order	Common name	Number of species	Reference
	Family			
Annelida		Bearded fireworm	1	Knowlton <i>et al.</i> 1990 Rotjan and Lewis 2008
Arthropoda		Crustaceans	9	Rotjan and Lewis 2008
Echinodermata		Sea stars	10	Antonius 1977
		Sea urchins	11	Knowlton <i>et al.</i> 1990 Rotjan and Lewis 2008
Mollusca		Gastropods	20	Baums <i>et al.</i> 2003 Knowlton <i>et al.</i> 1990 Rotjan and Lewis 2008
Chordata				
	Perciformes			
	Blenniidae	Blennies	2	Bruckner and Bruckner 2000
	Chaetodontidae	Butterflyfish	69	Cole <i>et al.</i> 2008
	Pomacentridae	Damselfish	8	Cole <i>et al.</i> 2009a
	Gobiidae	Gobies	3	Cole <i>et al.</i> 2009b
	Labridae	Parrotfish	8	Knowlton <i>et al.</i> 1990
		Wrasses	10	Rotjan and Lewis 2008
	Tetraodontiformes			
	Ostraciidae	Boxfish	2	Cole <i>et al.</i> 2008
	Monacanthidae	Filefish	8	Rotjan and Lewis 2008
	Tetraodontidae	Pufferfish	9	
	Balistidae	Triggerfish	7	

Conversely, stress may be beneficial, eliciting physiological responses such as stress proteins, which increase the individual's resilience to further stressors. Attempts to determine the effect of predation stress have become increasingly important for coral reefs in the context of overfishing—an increasing problem in reef ecosystems (Birkeland 1997, Sorokin 1993). In an overfished ecosystem, decreased numbers of corallivores could have a significant effect on corals. If predation is actually deleterious as a stressor, the removal of these corallivores would increase the health and resiliency of corals. However, if predation has a beneficial effect by increasing resiliency within individual prey, then overfishing would also have an indirect effect on coral bleaching. In this scenario, the removal of corallivores would actually decrease the corals ability to tolerate thermal stress, possibly leading to an increase in bleaching events.

It has become imperative to study these interactions between multiple stressors because bleaching is a common, generalized stress response in corals (Brown 2000, Glynn 1991, Jones 1997). Bleaching causes extensive damage, some of it irreparable, to reefs worldwide. Specifically, bleaching reduces growth and reproductive rates, impairs healing from mechanical damage (such as predation), increases susceptibility to disease, lowers lipid and protein production, causes tissue necrosis, inhibits skeletal growth, and may cause mass mortality (Brown 2000, Brown *et al.* 2002, Glynn 1991).

The term bleaching refers to the host appearing lighter due to the loss of their symbionts, or the concentration of photosynthetic pigments in the symbionts. The result is a host organism that becomes increasingly lighter as more symbionts or pigments are lost; complete bleaching results in an organism that appears white. However, bleaching is

not the only mechanism that can cause a host to appear lighter. Muscular contraction can result in the animal appearing darker. For instance, muscle contraction could result in a change in the animal's geometry, such as a long narrow tentacle contracting to become thicker and shorter; this would result in the tentacle appearing darker. Additionally, if the expulsion of water accompanied muscle contraction, then this would result in a decrease in volume and thus increase the density of zooxanthellae. In either situation, the overall effect would be a host that became darker, even though there was no actual change in either the number of zooxanthellae or pigment concentrations.

Traditionally, bleaching has been measured by calculating chlorophyll-*a* concentrations and algal cell counts. Measuring pigment concentrations has traditionally used spectrophotometry. This requires homogenization of the animal tissue to separate the endosymbionts, extraction of algal pigments (usually with acetone), followed by spectrophotometric measurements (Jeffrey and Humphrey 1975). This analysis is usually accompanied by quantification of animal host protein, yielding a measurement such as μg chlorophyll-*a* μg^{-1} animal host protein. More recently, researchers have also used gel filtration chromatography following pigment extraction (Dove *et al.* 2006). Fluorometry may also be used to measure chlorophyll-*a* concentrations; this method may be used for extracted pigments, separated zooxanthellae, or in some cases, the entire organism can be placed inside a fluorometer to measure chlorophyll fluorescence. Algal cell counts are done by homogenizing a tissue sample and separating the endosymbionts from the host tissue through centrifugation. A hemacytometer is then used to quantify the density as the number of zooxanthellae per mg of weight (wet or dry) or per mg of animal protein.

However, this method only provides information on density of zooxanthellae at one point in time and does not provide any information on expulsion rates. To measure expulsion rates using a cell count method, researchers will collect the water from the animal's container to count any algal cells that were expelled. A simple equation then allows for quantification of the percent of released cells (Hoegh-Guldberg *et.al* 1987, Perez *et al.* 2001).

For estimations of chlorophyll-*a* concentrations, fluorometry has demonstrated superior sensitivity in comparison to spectrophotometry or chromatography. It is a fairly simple and easy technique and has many applications for field work. It also offers more specificity than spectrophotometry (Smith *et al.* 1981). Cell counts are the most direct and accurate method of measuring zooxanthellae densities, and they don't require specialized equipment. These methods for measuring both chlorophyll-*a* concentrations and zooxanthellae density, have similar problems. Grinding and homogenizing the animal obviously prevents any future sampling, so the data provided by these methods is for one point in time—repeated sampling is not possible. Additionally, these methods are time-consuming and labor-intensive, and not very practical for a large number of samples. Also, because bleaching can be either the loss of zooxanthellae or a decrease in photosynthetic pigments, measurements of chlorophyll-*a* concentrations would be required to definitively determine bleaching has occurred. A decrease in the chlorophyll-*a* concentration would indicate that the organism has bleached; cell counts would determine the mechanism. However, cell counts alone do not account for bleaching due

to a loss of photosynthetic pigments. This means that both analyses would be required in order to confidently identify bleaching.

Recently, researchers have used color analysis as an alternative to traditional methods for measuring bleaching assay. These methods capitalize on the prominent role zooxanthellae have in the coloration of the host. Analyzing color in photographs can serve as a proxy for cell counts and pigment analysis and allow repeated samples to be taken over time. Johnson and Goulet (2007) used *Aiptasia pallida* to compare the results from different types of color analyses to the results from both cell counts and chlorophyll-*a* measurements. Johnson and Goulet (2007) analyzed red, green, and blue (RGB) intensity values and a principal components analysis (PCA) for RGB, in addition to several other imaging methods. They found the strongest correlation for both zooxanthella densities and chlorophyll-*a* concentrations with the blue intensity values ($r = -0.75$, $p < 0.0001$ and $r = -0.70$ ($p = 0.0001$), respectively. Of the RGB measurements, green was the next best indicator of zooxanthella densities and chlorophyll-*a* concentrations ($r = -0.69$, $p = 0.0002$ and $r = -0.62$, $p = 0.0012$, respectively). Red, however, while still significant, had one of the lowest correlation coefficients for zooxanthella densities and chlorophyll-*a* concentration ($r = -0.63$, $p = 0.0011$ and $r = -0.58$, $p = 0.0028$, respectively).

There are some considerations when using color analysis, however. This method requires carefully controlled lighting in a laboratory environment. As mentioned earlier, the correlation coefficients will change depending on the color of organism being analyzed. To address this issue, however, Johnson and Goulet (2007) determined that

PCA analysis allows for more appropriate analyses and predictions, of both zooxanthella densities and chlorophyll-*a* concentrations, that involve varying colors and different species. Also, while working in the laboratory, carefully controlled conditions—especially lighting—are required for this to be an effective assay. This is obviously not possible in the field, but the use of dampening devices can be used to deal with reflective glare, and color calibration techniques can be used to standardize colors. Furthermore, there is the consideration that while the correlation coefficients are significant, they are not as accurate as the traditional, more invasive techniques of cell counts and spectrophotometry. Finally, by discussing apparent color changes in the host, we are relying on visual perception, and it is important to note that the geometry of the host's tissues could affect its color. Color change, or more accurately, change in color intensity, may be the result of bleaching, or it may be reflecting other physiological processes.

Using color analysis as an assay for bleaching has several advantages, though. One of the reasons that Johnson and Goulet (2007) analyzed this technique was to determine if it would be suitable for use with aerial and satellite imagery. The ability to analyze photographs from aerial surveys of a reef ecosystem and determine possible bleaching would allow researchers to survey much larger areas in much less time. It is also a promising technique in a laboratory setting. This method is non-invasive, so repeated sampling can easily be done. It does not require the removal of the organisms from their environment, an increasing concern with declining health in coral reefs globally (Henry and Hart 2005). Analyses can be performed with just a digital camera

and basic imaging software, such as Adobe Photoshop. As Johnson and Goulet (2007) state, “sampling is rapid, easy, and does not require expensive equipment.”

Interpreting color intensity values from an RGB analysis requires some clarification, though. There are three values returned from an RGB sample, one for each wavelength. These values range between 0-255; the three color intensity values together produce the color we see. On this scale, 0 is pure black and 255 is pure white. This is contrary to intuition because a lower color intensity value corresponds with a darker color, and a higher color intensity value corresponds with a lighter color. This means that as the color is becoming lighter, the color intensity number is getting larger. As the color intensity is increasing, or becoming darker, the number is getting smaller. Additionally, when considering red, green, or blue individually, an increase or decrease in the intensity value is a change in the intensity of the wavelength, not an actual color change. For instance, as the red intensity values increase, it is losing the intensity of that wavelength. Visually, we may interpret this as a color change, but for digital color analysis, it is not. When all three color intensity values are combined to create color, there is the potential for color change. If the ratio of the three wavelengths to one another changes then the color changes, but if the ratio stays the same between the three wavelengths, the color stays the same, but the intensity changes. Most importantly, though, as the color intensity is decreasing—that is, it is becoming lighter—the color intensity value is getting larger. As the color intensity is increasing, or becoming darker, the color intensity value is getting smaller.

Controlled studies of corals and coral bleaching in a laboratory setting, however, can be quite difficult. Corals are quite sensitive to handling, requiring very specific growth conditions. Even when these conditions are met, they often have a slow growth rate. There are also several problems regarding the collection of corals; many biologists do not have access to corals, and many corals have protected status (Weis *et al.* 2008). Therefore, I am using the tropical sea anemone *Aiptasia* spp. as a model for coral. *Aiptasia* is extremely robust, easy to grow in a laboratory setting, and reproduces rapidly. More importantly, *Aiptasia*, like hermatypic corals, forms a symbiotic relationship with *Symbiodinium* dinoflagellates; *Aiptasia* also bleaches like coral. For these reasons, *Aiptasia* has been used as a model organism for coral for over 30 years (Weis *et al.* 2008).

Researchers have observed bleaching in these anemones in response to changes in temperature and light. Additionally, the duration of exposure to experimental treatments affects the amount of bleaching. Belda-Baillie *et al.* (2002) kept anemones in the dark at 35°C (10° above the acclimation temperature). After ten days, anemones were removed and immediately analyzed for chlorophyll-*a* concentration and zooxanthellae density. They observed that these anemones had fewer zooxanthellae and less chlorophyll-*a* than the control anemones, which were maintained at 25°C in a 12 h: 12 h photoperiod. Anemones kept in the same temperature and light treatment for 20 days and then immediately analyzed had bleached completely, a significant difference from the control anemones. Dunn *et al.* (2004) exposed anemones to several temperature treatments—26.5°C (the control), 29.5°C, 31.5°C, and 33.5°C with a 12 h: 12 h photoperiod. Once

anemones were randomly assigned to a temperature treatment, the temperature was increased over the course of 30 minutes. Once the treatment temperature was reached, they began removing anemones at different times, ranging from 0 minutes to 6 days, and immediately measured photosynthetic pigments and zooxanthellae densities. They observed a greater loss of zooxanthellae and photosynthetic pigments as the temperatures increased, and also as exposure time increased.

Previous researchers have also observed bleaching in response to cold shock. Most of these experiments were designed similar to Steen and Muscatine (1987) or Muscatine *et al.* (1991). In each of these studies, bleaching was observed in anemones exposed to 16° C or less, with individuals bleaching more with colder temperatures. As with experiments that elevated temperatures, in both of these studies longer exposure to decreased temperatures increased the expulsion of zooxanthellae.

I used *Aiptasia* to determine whether predation would affect the temperature at which corals bleach. I chose these two factors because temperature is an important stressor of tropical anthozoans, particularly in light of global climate change. I was also interested in predation because there has not been a lot of research on the effects of corallivory. I was interested if these two stressors would have an interaction with one another, or if they were independent. I hypothesized that predation would stress the anemones so that they are more sensitive to other stressors, specifically thermal stress. Because the anemones would be diverting resources to heal tissue damage resulting from predation, they would have fewer resources to address thermal stress. A second hypothesis was that predation stress may elicit protective stress responses, making the

anemones more resistant to thermal stress. Finally, predation may have no effect on the anemones' response to thermal stress. If predation has a negative effect on the anemones' response to thermal stress, then anemones exposed to predation would become lighter, indicating a bleaching response, than the anemones that were exposed to thermal stress alone. Conversely, if predation had a protective effect, then anemones that were exposed to predation would be darker than the anemones that were not exposed to predation. Finally, predation might not have had a significant effect on the anemones' response to thermal stress. In other words, there would be no synergistic interaction between these two main factors.

Methods

I used anemones of the genus *Aiptasia* and a fully crossed two-factor experimental design to study the effects of predation and heat stress on bleaching. Half of the anemones were preyed on by *Lysmata wurdemanni* (peppermint shrimp) and the other half were not. Following the predation treatment, the anemones were exposed to heat stress. This was done by putting the anemones in an incubator that was set to increase to one of four temperatures: 23° C, 26°C, 28°C, or 30°C, and then returning them to an ambient temperature of 23°C.

I took digital photographs of the anemones to detect a change in color and determine the extent of bleaching. Initial photographs were taken immediately prior to the predation treatments; I also photographed the anemones immediately following the temperature treatment, and then every other day for ten days. To reduce bias, these photographs were randomized and renamed for blind measurements in Adobe Photoshop CS3.

Animal collection and maintenance

Aiptasia spp. were cultured in the laboratory beginning February 2008. The experimental anemones came from two sources: clones of an individual from Walsingham Pond, Bermuda and anemones of unknown origin from a saltwater aquarist in Bellingham, WA. All anemones were kept in aquaria with filtered seawater collected from Shannon Point Marine Center in Anacortes, WA. The salinity was kept at 32 ‰. The aquaria were kept on a 12-hour light/12-hour dark cycle under Sylvania 3500K

fluorescent bulbs that provided light levels of 1800 lux. The aquaria were kept at the ambient temperature in the laboratory, which fluctuated between 20°C-23°C. The anemones were not fed during this time.

Prior to me rearing anemones in the laboratory, the anemones had been maintained under disparate conditions. The specimens collected from the local saltwater aquarist came from one of two tanks. In one tank, the lighting was provided by a four-foot coral life fixture with two broad-spectrum “Actinic” 65-watt tubes on an 11-hour light/13-hour dark cycle and two 65-watt 10,000K purple bulbs on a 13-hour light/11-hour dark cycle. The temperature was kept at a fairly constant 25.5°C. The second tank had one small wide-spectrum light on a 12-hour light/12-hour dark cycle, and was kept at approximately 20°C. For both tanks, artificial seawater was used, kept at a salinity of approximately 32‰, although the salinity varied more in the second tank. Anemones in both tanks were fed daily with a mixture of frozen brine shrimp and finely chopped mixed seafood. Additionally, a monthly supplement of Seachem Reef Builder was added to each tank.

The clones from Bermuda were kept at Shannon Point Marine Center. They were kept in covered finger bowls with filtered seawater in a 23°C incubator. These anemones were maintained under cool-white fluorescent bulbs providing light levels of approximately 3700-5920 lux on a 12-hour light/12-hour dark cycle. They were fed weekly, with freshly-hatched brine shrimp nauplii; the water in the finger bowls was changed the day after feeding.

The peppermint shrimp, commonly sold to saltwater aquarists to control infestations of *Aiptasia*, were purchased from a local supplier. Each shrimp was kept in an individual 400 mL beaker filled with filtered seawater. The water in the beakers was not filtered, but was aerated with an air stone. The water in each beaker was changed every 1-2 days. I kept 8-12 shrimp at a time; over the course of the experiment three shrimp died and were replaced. The shrimp were fed Nutrafin Max marine complete food daily, except prior to consuming anemones in the predator treatment.

Experimental design

I designed a fully-crossed, two-factor, split-plot experiment, with predator nested in temperature, to test whether predators can alter bleaching to temperature stress in *Aiptasia*. I used a split plot design because I only had a single experimental incubator in which to alter the temperature, but wanted to expose several anemones to a given temperature treatment. This design allowed me to balance the duration and power of the experiment. Anemones received one of four temperature treatments: 23°C, 26°C, 28°C, or 30°C, and one of two predation treatments: no predation or predation. Therefore, there were eight possible treatment combinations that an anemone could have received. I used a randomized block design; there were three blocks, with 32 anemones in each block (four anemones in each of the eight treatment combinations per block). Temperature was not replicated in a block, and the blocks were temporal. Because I had only one incubator available for each temperature treatment, I ran one temperature at a time; the order of temperatures was randomly assigned. Each temperature treatment was split to include the

two predator treatments. There were eight anemones in each treatment per block with half assigned to one of the no predator treatments and the other half assigned to predator treatment.

Anemones were haphazardly assigned to one of the temperature treatments; I was continually collecting anemones throughout the experiment, and at times had only enough anemones for one run. After the anemones were assigned to a temperature treatment, they were randomly assigned to a predation treatment.

Acclimation

Before beginning the predation and heat stress treatments, the anemones went through an acclimation process. Each anemone was placed in a glass finger bowl with approximately 150 mL of filtered seawater. I allowed approximately 24 hours for each anemone to attach. Once attached, the anemones were moved to an incubator at 23°C. They were kept on a 12-hour light/12-hour dark cycle. Philips fluorescent 20-watt bulbs were used in the incubator, providing light levels of 1800 lux. The anemones were kept in this incubator for approximately 72 hours.

I stopped feeding the shrimp when I placed the anemones in the finger bowls. Pilot studies and previous research demonstrated that the shrimp would not eat the anemones unless they were starved (Rhyne 2004).

Predation treatment

After the acclimation period, I took initial photographs, and then exposed half of the anemones to shrimp. I removed all eight anemones from the incubator. Four anemones were exposed to the peppermint shrimp. Two shrimp were placed in each dish with an anemone, and I waited until the shrimp had consumed approximately 20% of an anemone's tentacles. I left the shrimp in each finger bowl for 60 minutes because I determined through pilot studies that it usually took between 20-60 minutes for the shrimp to prey on an anemone. Additionally, the shrimp usually would not eat if I was observing them. Because of this, there were two instances of an anemone being almost completely consumed before I noticed and was able to remove the shrimp from the finger bowl. In these two instances, I continued with the temperature treatment and photographs.

Shrimp didn't always eat anemones within one hour, however. In these instances, I allowed three one-hour bouts on consecutive days for the shrimp to prey on all four of the anemones. If I still had anemones that had not been partially consumed at the end of one hour, I placed all of the anemones back in the 23°C incubator, while the shrimp continued to starve. After 12-24 hours, all of the anemones were again removed from the incubator. I then placed two shrimp into each dish that had an uneaten anemone, and waited one hour before removing the shrimp. I continued this repetitive procedure for up to three days, if necessary, until all four anemones had been partially consumed. Of the twelve runs performed, there was only one run in which the shrimp did not prey on all

four of the anemones within three days. In the one instance of no predation, I completely restarted the run with new anemones.

Temperature treatment

Immediately following completion of the predation treatment, I began the temperature treatment. The protocol that I used for the temperature treatments was adapted from Belda-Baillie *et al* (2002).

Once shrimp had partially consumed the four anemones, I put all eight anemones into an incubator (not the incubator used for acclimation) and exposed them to one of the four different temperature treatments: 23°C, 26°C, 28°C, or 30°C for 12 hours. During this time, the anemones were kept in the dark. Belda-Baillie *et al.* (2002) found that temperatures below 35°C were ineffective in making the anemones bleach if they were being maintained in a 12-hour light / 12-hour dark cycle. However, anemones kept in the dark at temperatures between 25°C and 35°C all bleached to varying degrees. After 12 hours, the anemones were removed from the incubator and photographs were taken. They were then placed back in the 23°C incubator, where they stayed for ten days, with photographs being taken every other day (Figure 1).

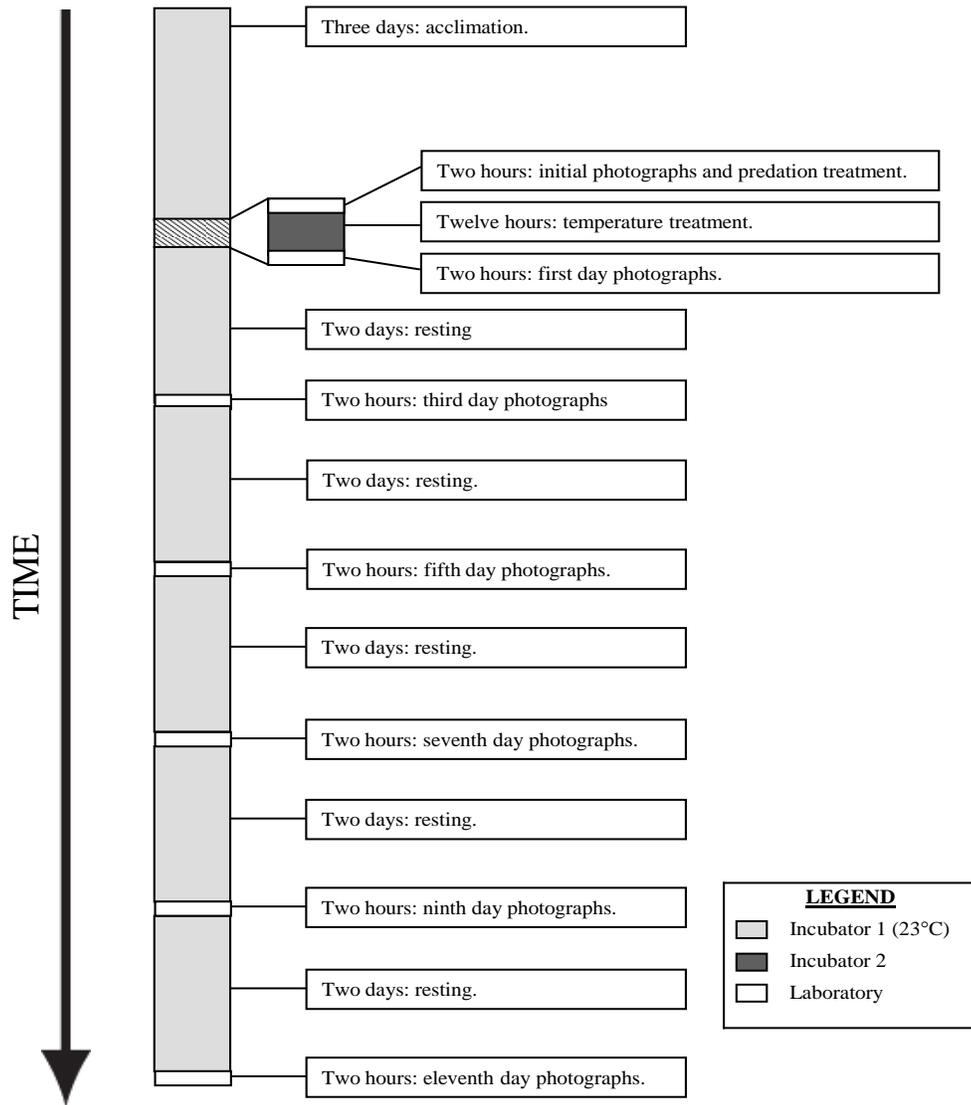


Figure 1. Experimental timeline, including location, for each run. Each run began with anemone acclimation, followed by exposure to one of eight possible treatment combinations, after which, photographs were taken at regular intervals for eleven days.

Estimating color change

Throughout the experiment, I took digital photographs of the anemones as a method of estimating bleaching, following the basic protocol of Johnson and Goulet (2007). To take the photographs, I used a Canon EOS 30D camera with a Canon Macro EF-S 60 mm lens mounted on a Bogen Mini Repro copy stand. The camera lens was 10.25 cm from the table surface. Each finger bowl with an anemone was placed on top of a Pantone neutral 18% gray card. Two utility lights, each with a General Electric compact fluorescent 6500 K bulb, were on opposite sides of the finger bowl, and kept in the same position for all photographs. I used the manual setting on the camera, with automatic white balance, an aperture of 3.5, and a shutter speed of 1/60. The photographs were taken in the raw format.

When I photographed an anemone, it would be attached to either the bottom or the side of the finger bowl. Because I was going to analyze the color in the tentacles of each anemone, I needed a photograph with well-focused tentacles. If the anemone was on the bottom of the finger bowl, this was fairly straightforward—after taking several photographs I would have at least one image in which the tentacles were in sharp focus. If the anemone was on the side of the finger bowl, I had to take more photographs. In these cases, I took several photographs at different focal planes, so that by looking at two or three different photographs of the anemone, I would be able to see most of its tentacles in focus. Additionally, at least one photograph in each series was taken with a ruler.

After the photographs were taken, I sorted them to select and save the ones that would be used for later analysis. I chose photographs in which the anemone's tentacles

were in focus and unfurled. If the anemone was on the bottom of the finger bowl, I usually saved only one photograph. For anemones that were on the side of the finger bowl, up to three photographs were saved for analysis.

The photos were initially saved with file names that indicated all of the identifying information about that particular anemone—the treatments it had received, and the day the photograph was taken. However, I wanted to analyze the photographs blindly, without this identifying information, to reduce bias. Therefore, prior to analysis, the photographs were randomized and renamed. Because this was a very time consuming process, I separated the photographs into two batches: the first batch contained initial photographs and the final photographs from the eleventh day; the second batch contained the photographs that were taken immediately following the temperature treatment on the first day, and every day thereafter, through the ninth day. This allowed my research assistant to begin analyzing the first batch of photographs while I was randomizing and renaming the second batch of photographs.

Once the photographs were randomized and renamed, I used Adobe Photoshop CS3 to measure tentacle color. Each photograph, or series of photographs (there were between one and three photographs for one anemone), was opened in Adobe Photoshop CS3; no adjustments to the color or white balance were made. I haphazardly selected ten tentacles for color analysis and sampled from the approximate midpoint of each tentacle. I considered two factors for sampling a specific tentacle: I did not sample a part of a tentacle if there were expelled algae growing on the glass beneath because this would affect the color reading of that tentacle; I also tried not to sample a tentacle that was

overlying another tentacle, although this wasn't always possible. Additionally, I was not always able to sample ten tentacles from each anemone—sometimes there weren't ten tentacles, or they were too tightly furled. I sampled the tentacles by using a circular selection tool. The diameter of the selection tool was between 15-20 pixels, depending on the thickness of the tentacles. The majority of the time, I kept the tool at a 20-pixel diameter, but because I wanted to be sure that I was selecting only the tentacle and not the background, if the anemones' tentacles were very thin, I changed the selection tool to a diameter of 15 pixels. Using the histogram function in Adobe Photoshop CS3, I collected six pieces of data per tentacle:

- The red mean and standard deviation,
- The green mean and standard deviation,
- The blue mean and standard deviation.

Tentacle Measurements

I decided to measure the mean tentacle width of anemones in the 23°C and 30°C treatments, to see if there was a significant change in width between the two temperatures over the course of the experiment. In order to make this determination, I measured the tentacles for the two extreme temperature treatments from the initial day of photographs and the final day of photographs, a total of 96 photographs. I did this because the results that I obtained for the temperature effect were the opposite of what I had been expecting—anemones at the highest temperature became darker. I hypothesized that this might have been due to host tissue contraction.

To test this hypothesis, used ImageJ 1.42q to measure the tentacle width of each anemone. Each photograph was opened in ImageJ 1.42q. To calibrate the measuring tool in ImageJ, I randomly selected photograph from the 23°C treatment, on the initial day, that had been taken with a ruler. I calibrated the measurement tool so that 10 mm equaled 390.36 pixels. I haphazardly selected ten tentacles for tentacle measurement and sampled from the approximate midpoint of each tentacle. Sometimes I was not able to measure ten tentacles from each photograph because the anemone didn't have ten tentacles. In these instances, I measured as many tentacles as possible.

Statistical analysis

Estimating color change

To statistically analyze the data collected from the photographs, I first calculated the slope of the least-squares regression line for the relationship between each color value (red, green, and blue) and time. I did this to create independent data; one datum describing the magnitude and direction of color change during the experiment for each anemone.

I then used R to analyze these data with a nested linear mixed-effects model. The response variable was the change in color value for a given color (i.e., the least-squares slope for the relationship between a particular color value and time), and the main fixed factors were temperature, a continuous variable, and predator, a categorical variable. Block was included as a random variable. To account for the split-plot design, I nested the block within temperature, and used this nested term as the error to calculate the effect

of temperature. I was unable to test for block interactions because temperature was not replicated in each block.

Tentacle measurements

I used R to analyze the tentacle measurement data with a nested linear mixed-effects model. The response variable was the final tentacle width measurement, and the main fixed effects were temperature, a continuous variable, and predator, a categorical variable. In addition, the initial tentacle width was included as a covariate.

Results

Color Data

Figures 2-4 illustrate how anemones change in color for the 11 days after exposed to predation and elevated temperatures. These figures plot the average color value for red, green, and blue for the temperature treatments (Figure 2), predation treatments (Figure 3), and the combination of temperature and predation treatments (Figure 4).

For each of the three colors, I observed no significant interactions between the main factors, temperature and predation, in any of the analyses (Table 2). I did, however, see significance in the main effects (Table 2). These effects differed according to color, however. For the analyses for blue and green, there was a significant temperature effect; anemones became darker with increasing temperature. The analyses for blue and green also showed a significant predator effect; anemones exposed to predation became darker than anemones that were not exposed to predation. There were no significant effects for red.

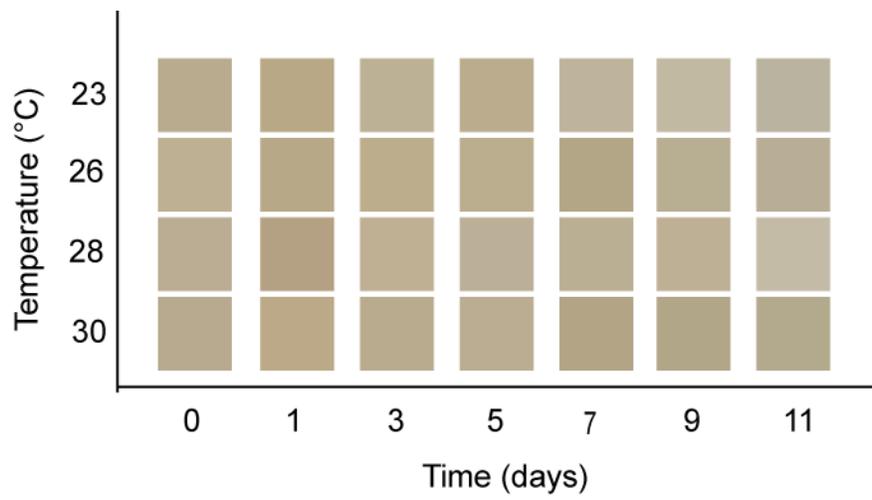


Figure 2. The color change of anemones for each temperature treatment (n = 12). The colors were determined from the means of red, green, and blue color values.

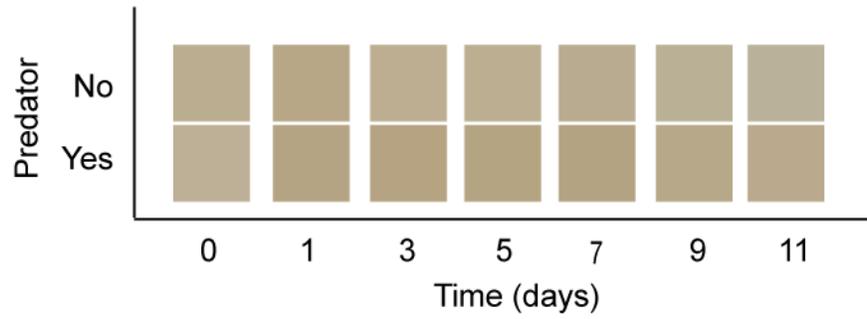


Figure 3. The color change of anemones for each predator treatment (n = 48). The colors were determined from the means of red, green, and blue color values.

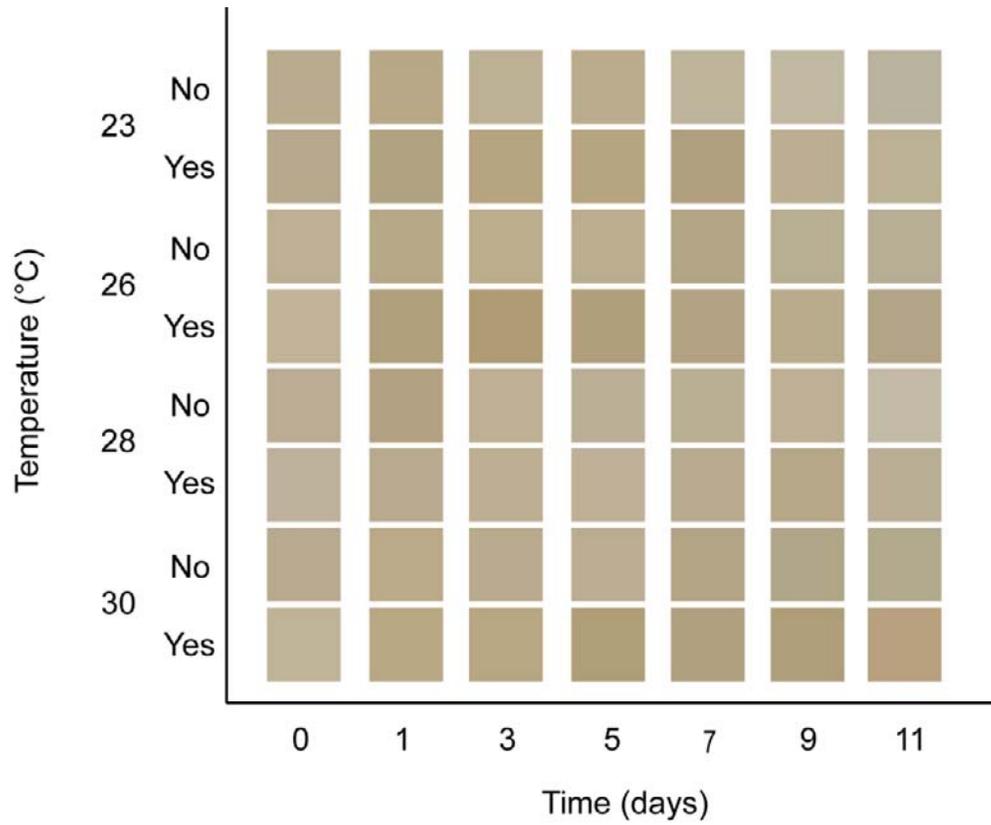


Figure 4. The color change of anemones for each of the eight possible combinations of temperature and predation treatments (n = 12). The colors were determined from the means of red, green, and blue color values.

Table 2. Results from the nested linear mixed-effects analysis of the split-plot design for blue, green, and red color-change values.

Color	Factor	df	F	p
Blue	Temperature	1, 8	9.715	0.014
	Predator	1, 82	10.41	0.002
	Temperature * Predator	1, 82	0.449	0.504
Green	Temperature	1, 8	5.907	0.041
	Predator	1, 82	8.905	0.004
	Temperature * Predator	1, 82	2.152	0.146
Red	Temperature	1, 8	4.197	0.075
	Predator	1, 82	0.719	0.399
	Temperature * Predator	1, 82	1.165	0.284

Temperature effect

For blue, there was a significant negative relationship between change in color intensity and temperature (change in blue color value = $16.97 \text{ intensity} - (0.608 \text{ intensity/day}) * x$; Table 2, Figure 5). Positive values for a change in color intensity indicate that individuals became lighter, and negative values indicate individuals became darker. Anemones in the 23°C control treatment had the largest (positive) change in color intensity values, and anemones in the 30°C treatment had the smallest (negative) change in color intensity values on average (Figure 5). The regression equation indicates that at 27.9°C the change in color intensity values was zero. In terms of change in color intensity values, the pattern was opposite to what I expected. Anemones became lighter after exposing them to temperature < 27.9°C and darker after exposing them to temperature > 27.9°C.

For green, there was a significant negative relationship between change in color intensity and temperature (change in green color value = $10.85 \text{ intensity} - (0.387 \text{ intensity/day}) * x$; Table 2, Figure 6). The analysis for green showed a similar trend to blue color values. Anemones in the 23°C treatment had the largest (positive) change in color intensity values, and anemones in the 30°C treatment had the smallest (negative) change in color intensity values on average (Figure 6). The regression equation indicates that at 27.34°C the change in color intensity values was zero. In terms of change in color intensity values, the pattern was opposite to what I expected. Anemones became lighter after exposing them to temperature < 27.34°C and darker after exposing them to temperature > 27.34°C.

For red, there was not a significant relationship between change in color intensity and temperature (change in red color value = $7.971 \text{ intensity} - (0.303 \text{ intensity/day}) * x$; Table 2, Figure 7). On average, the values for the intensity of red did not change after exposure to any temperature.

Predator effect

For blue, there was a significant difference in the change in color intensity values between the two predation treatments (Table 2, Figure 5). Anemones that were preyed on by shrimp had a negative average change in color intensity value, whereas anemones that were not preyed on by shrimp had a positive average change in color intensity value. The magnitude of mean color intensity change for the no predator treatment was three times the change for the anemones that were preyed on by shrimp. These values indicate that anemones in the no predator treatment became lighter and anemones in the predator treatment became darker on average.

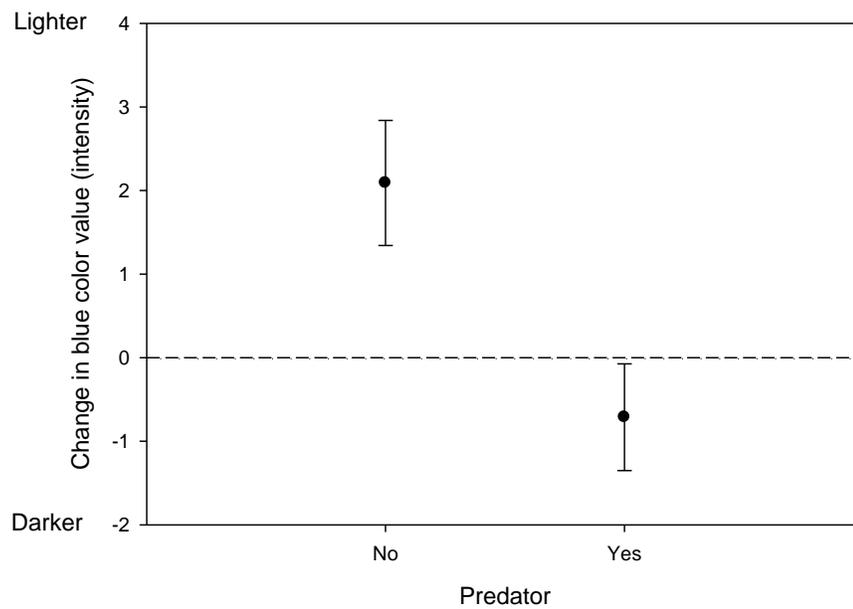
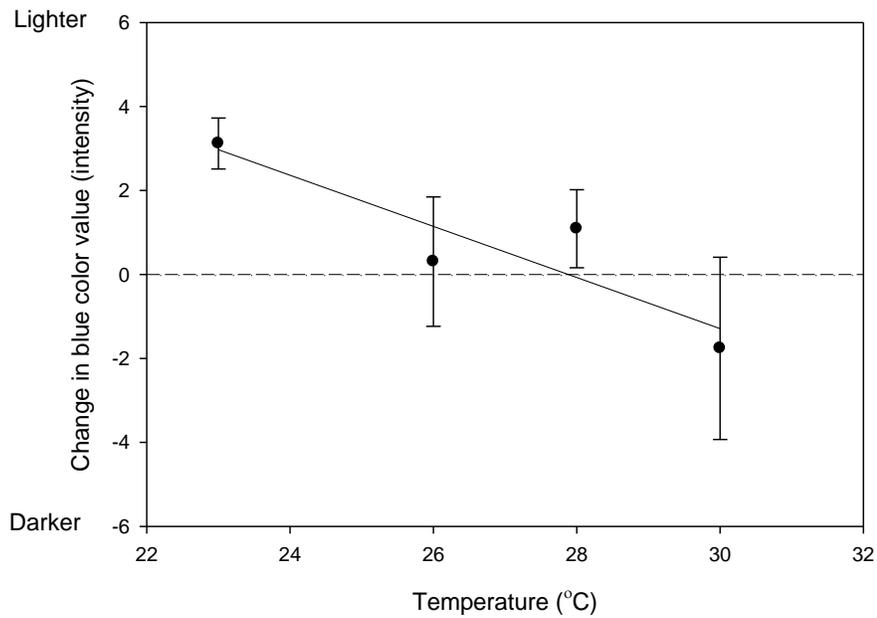


Figure 5. Comparisons of the mean change in blue color values over eleven days for each temperature and predator treatment. A positive color change value indicates a lighter color. For temperature, each circle represents the means from each incubator and block; the error bars represent standard error derived from these means ($n = 3$). For predator, each circle represents the mean for each treatment, and the error bars represent standard error of the mean ($n = 48$).

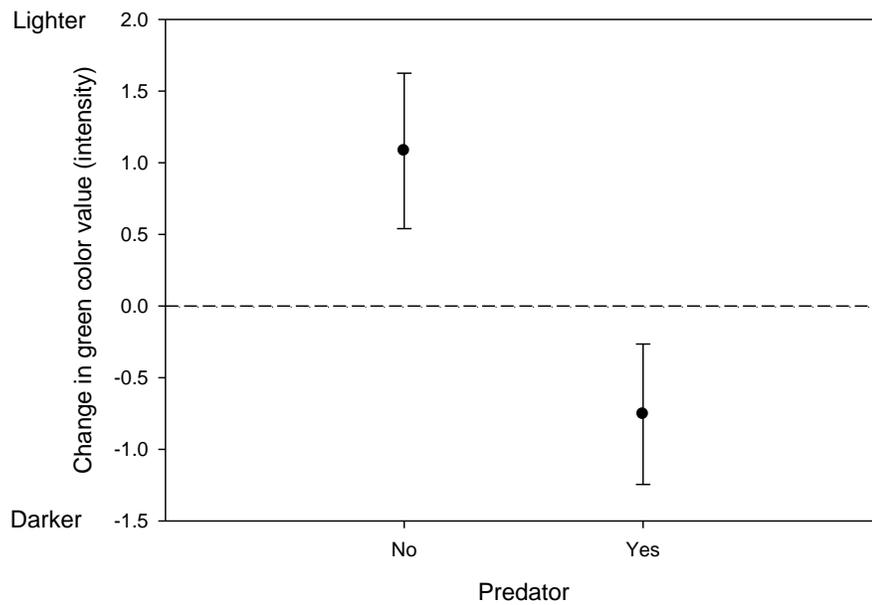
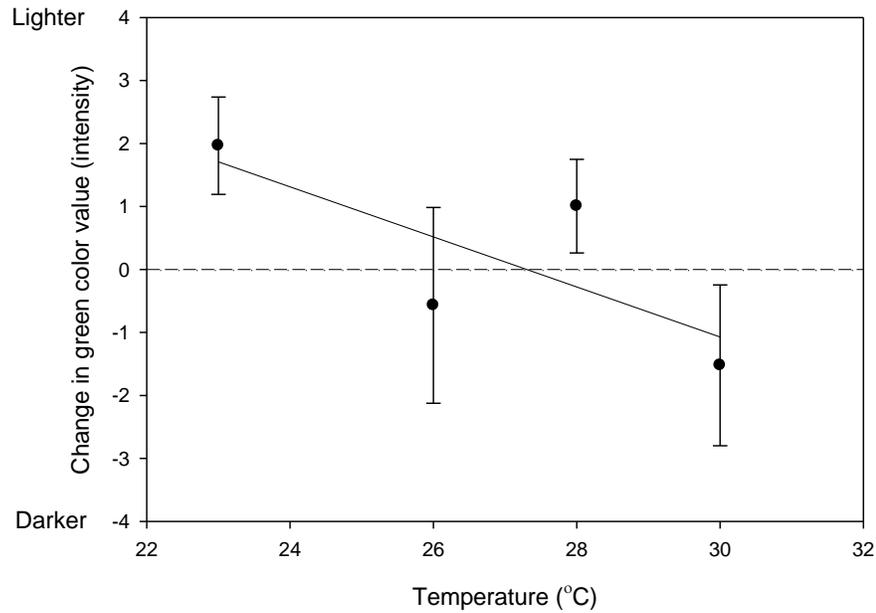


Figure 6. Comparisons of the mean change in green color values over eleven days for each temperature and predator treatment. A positive color change value indicates a lighter color. For temperature, each circle represents the means from each incubator and block; the error bars represent standard error derived from these means ($n = 3$). For predator, each circle represents the mean for each treatment, and the error bars represent standard error of the mean ($n = 48$).

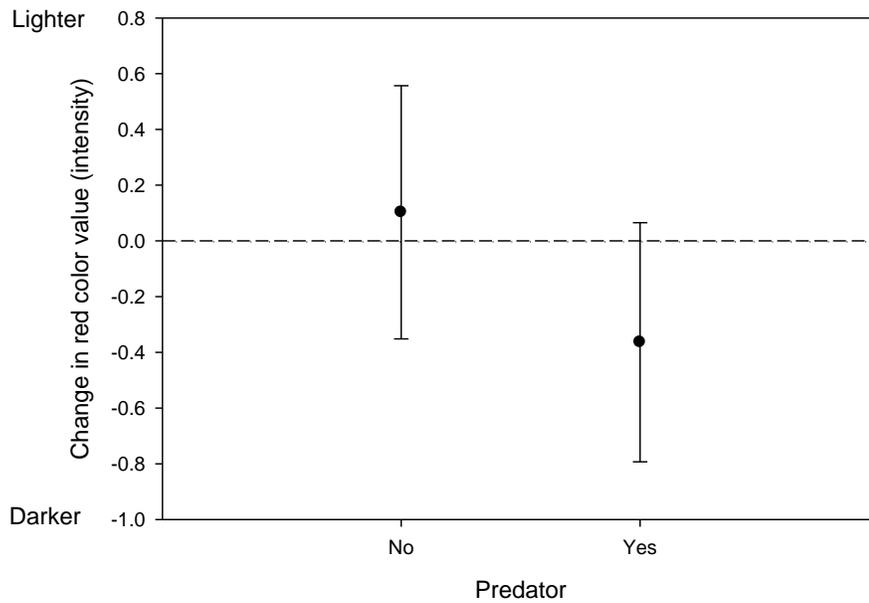
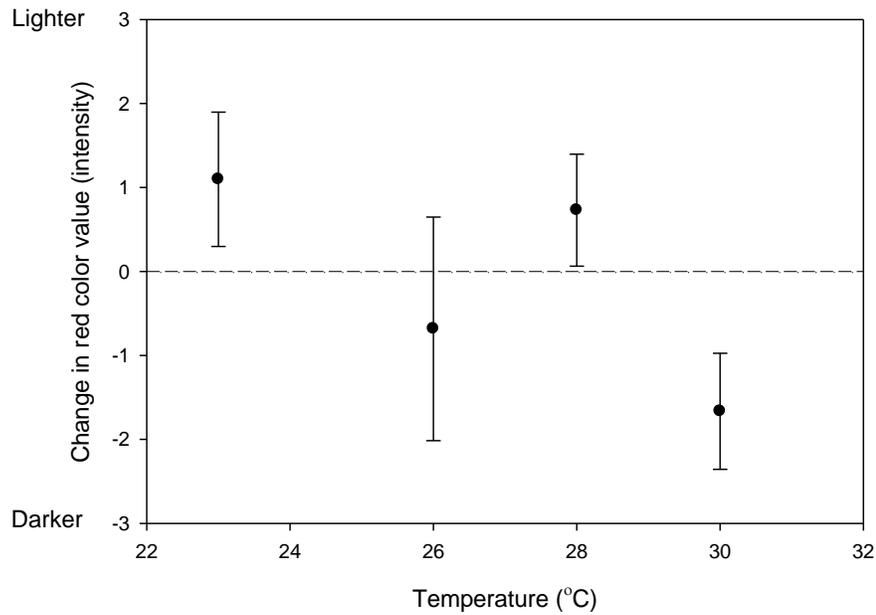


Figure 7. Comparisons of the mean change in red color values over eleven days for each temperature and predator treatment. A positive color change value indicates a lighter color. For temperature, each circle represents the means from each incubator and block; the error bars represent standard error derived from these means ($n = 3$). For predator, each circle represents the mean for each treatment, and the error bars represent standard error of the mean ($n = 48$).

Green had a similar pattern to blue—there was a significant difference in the change in color intensity values between the two predator treatments (Table 2, Figure 6). Anemones that were preyed on by shrimp had a negative average change in color intensity value, whereas anemones that were not preyed on by shrimp had a positive average change in color intensity value. The magnitude of mean change in color intensity for the no predator treatment was more than twice the change for the anemones that were preyed on by shrimp. These values indicate that anemones in the no predator treatment became lighter and anemones in the predator treatment became darker on average.

For red, there was not a significant difference in the change in color intensity values between predation treatments (Figure 7). On average, the values for the intensity of red did not change after exposure to either predator treatment.

Tentacle width

The analysis of the nested linear mixed-effects model for tentacle width indicated that only the covariate, initial width, was significant (Table 3). There was not a significant difference in the average tentacle width of anemones between the two extreme temperature treatments (23°C and 30°C) or predator treatments. For anemones in the 23°C treatment, the mean initial tentacle width was 0.68 mm, and the average width after 11 days at the end of the experiment was 0.70 mm. For anemones in the 30°C treatment, the mean initial tentacle width was 0.72 mm, and the average width after 11 days at the end of the experiment was 0.66 mm.

Table 3. Results from the nested linear mixed-effects analysis of the split-plot design for tentacle width.

Factor	df	F	p
Initial	1, 35	20.671	0.0001
Temperature	1, 2	1.373	0.362
Predator	1, 35	0.300	0.587
Initial * Temperature	1, 35	1.484	0.231
Initial * Predator	1, 35	0.001	0.971
Temperature * Predator	1, 35	0.902	0.349
Initial * Temperature * Predator	1, 35	0.189	0.667

Discussion

There were two primary findings in my research. First, I observed no synergy between the temperature and predator treatments. I had hypothesized that these two treatments would interact synergistically, with predation affecting the anemones' response to increased temperature, but this was not the case. Second, there was a significant predator effect. Predation caused the anemones to become darker.

Synergy

I had hypothesized that predation would have a synergistic interaction with thermal stress, potentially eliciting a greater bleaching response as a result of the combination of stressors. Conversely, there was the possibility that predation would invoke a stress response, making the individual more resilient to any stressors that immediately followed, such as thermal stress. There was also the possibility that predation would not have an effect on the organism's response to thermal stress—that any effects of thermal or predation stress would be independent of one another. This final hypothesis was supported by my results. There were no interactions between the main effects.

Research analyzing synergies and coral bleaching has so far focused on abiotic factors. For example, Coles and Jokiel (1978) looked at interactions between temperature, salinity, and ultraviolet radiation. They found synergistic effects between low salinity and high temperatures and radiation levels. More recently, several different studies have also

found synergistic effects between high temperatures and increased solar radiation (Fitt and Warner 1995, Glynn 1996, Lesser *et al.* 1990).

There has been little research into synergistic interactions that include biotic factors, particularly predation, in corals. Studies in amphibian biology have demonstrated the importance of synergistic effects between stressors that include predation or predator cues, though. Boone and Semlitsch (2001) found that carbaryl pesticide had a significant, synergistic effect on treefrog larval weight when coupled with exposure to predator cue. Relyea and Mills (2001) have found carbaryl to be 2-4 times more lethal in the presence of predator cue in amphibian larvae. Similar research has yet to be done with anthozoans. As Rotjan and Lewis (2008) remarked, “although coral stressors are becoming both more numerous and more severe, the interaction between corallivory and other stressors has not been well studied.”

With this study, I begin to address the paucity of studies into synergistic stressors in coral reefs. The results of this study suggest that the effects of predator and thermal stress are additive. Furthermore, the lack of a synergistic interaction between the two main factors is not because of a lack of significant main effects. I saw a significant effect for both main factors, temperature and predator.

My results suggest we can predict the combined effects of predation and thermal stress from experiments that manipulate only one factor. As mentioned above, a great deal of research has focused on heat stress as a primary cause of coral bleaching. My data suggest that an understanding of how predation stress relates to this previous research on heat stress can be done fairly easily. Because the effects of each variable appear additive,

we can predict the combined effects of both factors from different single-factor studies. Because there is so little work, though, much more needs to be done before we can confidently assume additivity in most cases. As global climate change continues to be a challenge, the more that is known about the ways that increasing ocean temperatures may interact with other stressors, the more equipped we are to make informed decisions.

Temperature

I hypothesized that as the temperature increased, the anemones would become lighter. This hypothesis is firmly supported by the literature—research has demonstrated the causative role that high temperatures have in coral bleaching (Brown 1997, Gates *et al.* 1992, Glynn 1984, Goreau 1964). However, this was not what I observed. In fact, I observed the exact opposite. Anemones in the control treatment bleached the most, while the anemones in the 30°C treatment became darker.

I proposed three biological hypotheses that could explain the temperature effect. One possible explanation for these results is that anemones contracted in response to heat stress making them appear darker. If the tentacles were contracting as part of a stress response, this would most likely skew my color data. Contracted tentacles should be shorter and thicker, which would not change the density of zooxanthellae within the tissues, but would increase the number of cells that light would need to pass through during the photographic measurements. This would very likely manifest as darker color values but not reflect a change in zooxanthellae density. A second possible explanation is that the anemones were not sufficiently heat stressed to cause bleaching. A third possible

explanation is that the color change reflects the response of the zooxanthellae to heat stress in darkness. The numbers of zooxanthellae within the host tissues fluctuate. The number of zooxanthellate cells within host coral cells may change by more than 10% within 24 hours (Cervino *et al.* 2003). My results might be illustrating this phenomenon.

I was able to test whether the host tissue contraction explained my results. I measured tentacle width for anemones in the 23 and 30°C treatments. A greater width in tentacles 30°C than 23°C would support the host contraction hypothesis. I observed over the course of the experiment that the anemones would contract in response to stimuli, similar to what previous researchers have observed (Hyman 1940). However, while there was a difference in tentacle width between the anemones exposed to 23°C or 30°C over the course of the experiment, it was not significant. The mean tentacle width for anemones in the 30°C treatment was 5.3% less—meaning they were narrower— than the mean tentacle width for anemones in the 23°C treatment. These results are inconsistent with the host tissue contraction hypothesis.

There is also further evidence that refutes the host tissue contraction affecting my results. The original protocol by Johnson and Goulet (2007) subjected anemones to a 32°C temperature treatment and had strong correlative relationships between color analysis and zooxanthellae densities ($r = -0.75$, $p < 0.0001$) and chlorophyll-*a* concentrations ($r = -0.70$, $p = 0.0001$). This provides further support that the assay I used to measure bleaching was appropriate; a small degree of host tissue contraction does not interfere with the accuracy of the color analysis.

There is little support for or against the remaining two hypotheses. It is possible that the temperature effects I saw were due to the fact that not all of the anemones were sufficiently stressed to bleach. There is no standardized protocol for using heat stress to cause bleaching, in either corals or anemones. Even when working on the same species, researchers use different temperatures and different exposure times. The temperatures that I used were similar to those used by Lesser (1996), who worked with *Aiptasia*, and Glynn and D’Croiz (1990), who worked with corals, but my exposure time was much shorter. I shortened the exposure time dramatically—maximum temperatures were reached in less than five minutes as opposed to the ten days that Lesser (1996) used—with the belief that this quick elevation time would still sufficiently stress the anemones, even with a shorter exposure period.

However, overall, I am unsure whether or not this was actually the case. The control anemones became lighter, which may have been the result of moderate cold stress. Previous research has defined the upper threshold for cold shock to be approximately 16°C, although this may increase to 20°C depending on exposure time and light levels (Muscatine *et al.* 1991, Saxby 2003). Coral reefs are areas with high light and mean temperatures of 27-28°C (Fautin and Buddemeier 2003). While *Aiptasia* would be unaffected by a lower temperature of 23°C, zooxanthellae have less resistance to fluctuations in temperature—either increasing or decreasing (Muscatine *et al.* 1991, Saxby *et al.* 2003). My control temperature, when coupled with darkness, might have been exceeding the thermal tolerance of the zooxanthellae (A. Banaszak, personal communication).

It is also possible that temperature effects I observed were the result of the zooxanthellae responding to the lack of light and also the temperature increase to 30°C. It has been shown that decreased light or shading will cause the zooxanthellae to increase the amount of photosynthetic pigments within their cells (J. Cervino, personal communication, August 25, 2009; Masuda 1993, Johnson and Goulet 2007, Muller-Parker and D'Elia 1997, Muscatine 1989). This might explain the effect that I saw, particularly if coupled with the increase in temperature. Moderately increased temperatures will frequently increase the metabolic rate of an individual; the metabolism of both corals and their symbionts are no exception to this (Muller-Parker 1997). The increased temperature may have simply increased the growth rate of the zooxanthellae. A temperature of 30° would be a reasonable and moderate increase from the mean temperature of most shallow water reefs. This is a temperature that would likely be experienced by a tropical anthozoan, and may have simply stimulated increased growth. This would have resulted in the darker color that I observed. It is difficult to know exactly what to expect when measuring zooxanthellae's response to different light and temperature levels, though. As Muller-Parker and D'Elia (1997) state, "...it is important to remember that each zooxanthella strain or species is likely to have different adaptive capabilities and tolerances to environmental extremes."

The results from my temperature treatments were not what I expected. Rather than becoming lighter with increasing temperature, my anemones became darker. There were several possible explanations for this, although I do not find any of them wholly satisfactory. Host tissue contraction was the most likely explanation, but the work of

Johnson and Goulet (2007), in addition to my simple tentacle width measurements did not bear this out. There is the possibility that the anemones were not sufficiently stressed to bleach, but it is difficult to make a judgment on this because there is no standardized laboratory protocol for using temperature to bleach anthozoans. I believe that the most likely explanation is one that requires more research. There is too much about zooxanthellae that we do not know for me to draw a reasonable conclusion about the temperature effect.

Predation

The other main factor besides temperature was predation, and this was of primary interest to me. I found that there was a significant predator effect; anemones exposed to predation were always darker than those that were not. This is in contrast to what I had hypothesized; I thought that predation would evoke a stress response that would increase the likelihood of bleaching. Bleaching is, after all, a generalized stress response (Baird *et al.* 2008, Buddemeier and Fautin 1993, Glynn 1991). However, these results suggest that while a stress response was being evoked, it perhaps provided physiological resilience, either to the host or the symbiont.

I initially had two possible explanations for this predator effect. The first possibility I considered was host tissue contraction, just as I hypothesized for the temperature effect. Because I observed similar effects—the anemones becoming darker from increased temperature and exposure to predation, this seemed a likely explanation. I also considered that the anemones might be creating heat shock proteins. Heat shock

proteins function on a cellular level to protect the organism against the negative effects of stress. Because this would bolster the organism's physiological functioning and maintain homeostasis, it might very well cause the anemones to become darker.

The first hypothesis, that the anemones appeared darker because of host tissue contraction, was ruled out for the same reasons I ruled it out as the cause of the temperature effect. There was no significant difference between tentacle width in the anemones in the 23°C and 30°C treatments. Again, Johnson and Goulet's work (2007) also supports this not being the reason for the darker appearance in the anemones.

I considered the creation of heat shock proteins as a possible cause for the predator effect. Heat shock proteins are created in response to many different stressors, including predation (Pauwels *et al.* 2005, Sorensen *et al.* 2003). The induction of heat shock proteins in response to predation maintains homeostasis and avoids potential reductions in fitness (Pauwels *et al.* 2005, Slos and Stoks 2008, Sorensen *et al.* 2003). However, if this had been the case, then I would have expected the anemones that were preyed on to have had protection against the increased temperatures to which they were later exposed. This would cause a synergy between predation and thermal stress, which I did not observe. Because there was no interaction between predation and temperature, it does not seem likely that production of heat shock proteins explains the predation effect.

Neither of my two hypotheses is well supported. There is the possibility that the zooxanthellae are increasing photosynthetic production in response to the host's increased energetic demands for tissue repair; this response could be mediated by either the host or the symbiont. However, the relationship between the two organisms is so

complex that a simple increase in production as a response to demand seems unlikely. There are many factors that affect the costs and benefits of the symbiosis; for instance, nutrient availability, toxic photosynthetic products (such as reactive oxygen species), and space limitations for zooxanthellae (Muller-Parker and D'Elia 1997, Weis 2008). There is also the possibility that the zooxanthellae are indirectly benefitting from the increased allocation of resources to the tentacles for tissue repair. This is possible, but I consider it to be unlikely, as previous research has demonstrated the major constraints the host places on the growth and production of its symbionts (Muller-Parker and D'Elia 1997). I think the best answer to this question lies in there being more research focusing on the effects of predation on individual organisms, in addition to studying the effects of corallivory on the reef as a whole.

There are two possible implications of this predator effect and bleaching. An immediate implication of this may be protection against further predation. According to my research, predation does have an effect on bleaching. After one episode of predation, the anemones actually became darker, either from an increase in the numbers of zooxanthellae, or an increase in pigment concentrations within the zooxanthellae. Either possibility points to an increase in the photosynthetic ability of the zooxanthellae. Previous research has determined that zooxanthellae translocate up to 95% of their photosynthetic products to their host (Muller-Parker and D'Elia 1997). This in itself may be a deterrent to further predation. Research has found that corallivorous fish, when given the choice, prefer bleached corals to unbleached corals (Cole *et al.* 2009, Slattery and Paul 2008). Slattery and Paul (2008) suggest that one reason for this dietary preference

may be the presence of defense metabolites within unbleached coral tissues. These metabolites are energetically costly and the host may very well be dependent on the translocated resources from zooxanthellae to produce them.

There are larger implications to these results, as well, as overfishing in coral reefs is becoming increasingly problematic. As Glynn (1988) discusses, the relationship between corallivores and coral reefs is a complex one. For instance, corallivorous fish are undoubtedly responsible for causing coral mortality, in addition to chronic stress within coral populations as a result of their feeding habits (Glynn 1988, Mumby 2009, Rotjan and Lewis 2005, Rotjan and Lewis 2006). However, many corallivorous fish are widely known to graze on algae, which, if left unchecked, would outcompete hermatypic corals for space (Glynn 1988, Mumby 2009, Rotjan and Lewis 2005, Rotjan and Lewis 2006).

My research suggests that the issue of overfishing is even more complex than frequently discussed. Certainly there are the direct effects to the fish populations themselves, but the indirect effects to the corals require consideration as well. Many corallivorous fish, especially parrotfish, are not so exclusively—they are also herbivores (Carpenter 1997, Glynn 1988, Mumby 2009, Rotjan and Lewis 2005, Rotjan and Lewis 2006). While it is accepted that this herbivory is beneficial, once the parrotfish begin grazing on corals, it is widely accepted that this is deleterious; indeed, as mentioned, parrotfish and other corallivores are undeniably responsible for a certain amount of coral mortality.

Integrating the results of my research into this model, though, would imply that even a certain degree of this coral grazing may be beneficial. Because the anemones in

my study became darker, it is reasonable to infer an increase in photosynthetic output by the zooxanthellae. An increase in the photosynthetic abilities of the zooxanthellae increases the available energy for growth, development, and reproduction for the corals. This growth and development may include the creation of toxic metabolites or more nematocysts, frequent antipredator responses in corals (Glynn 1988). These antipredator traits would definitely be favorable, even in areas subject to extreme overfishing; fish are not the only corallivores on the reef.

Furthermore, according to my results, if predators are removed from coral reefs, there is the likelihood of increased bleaching. If predation has a beneficial effect, then the removal of coral predators is going to increase the likelihood of bleaching. While we have become aware of the negative consequences to an ecosystem, including coral reefs, that result from overfishing (Jackson *et al.* 2001), it has not been widely explored that overfishing might indirectly contribute to coral bleaching. In addition, there is strong evidence that the removal of corallivorous fish increases the incidence of coral disease (Raymundo *et al.* 2009). Coral disease is also a factor contributing to coral bleaching (Brown 2000). It is crucial that we investigate predator effects on a single coral polyp, in addition to the widespread effects of corallivory on an entire reef, if we are to ameliorate coral bleaching.

Summary

I had two primary findings from my research: there was not a synergistic effect between predator and thermal stress, and there was a significant predator effect. No synergy between the two stressors means that we can make appropriate predictions about

the effects of both stressors from single-factor experiments. This is good news, as it suggests we can predict the combined effects of predation and thermal stress from experiments that manipulate only one factor.

I was also able to determine that there is a significant predator effect, as evidenced by all of the anemones that were preyed on by peppermint shrimp becoming darker. This has wide-ranging implications for both coral bleaching and overfishing. According to my results, overfishing might be having a negative impact on coral bleaching. Whether this impact is on the frequency or extent of coral bleaching requires more research, but this is a result that cannot be ignored as we consider the ramifications of global climate change and coral bleaching. Addressing the seemingly disparate issues of global climate change and overfishing needs to become a more cohesive discipline if we are to make appropriate decisions protecting coral reefs.

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