UNIVERSITY OF CALIFORNIA, SAN DIEGO

Coral Fluorescence and Symbiosis: Photoacclimation, Thermal Shock, Life History Changes, and Implications for Reef Monitoring

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Marine Biology

by

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2010

The Dissertation of Melissa Susan Roth is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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EPIGRAPH

You tickle my toes.
You embrace me whole.
You mesmerize my soul.
I could spend forever watching you.
You teach me everything.
You are calm, patient and forgiving.
You are powerful, challenging, and humbling.
You are constant, yet ever-changing.
You own the moment, the past and the future.
You take my worries and fears.
You give me hope, inspiration, and promise.
You are always with me.
Your voice sings in my ears,
Your colors live in my eyes,
Your waves dance with my soul.
You are my passion, my love, my life.
My ocean.

Melissa Roth
Written with my toes in the sand
Palmyra Atoll, 26 September 2004

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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MATERIAL SUBMITTED FOR PUBLICATION IN THE DISSERTATION

The following chapters are in preparation, have been submitted for publication or have been published.

Chapter II, in full, has been submitted for publication as: Roth, Melissa; Latz, Michael; Goericke, Ralf; Deheyn, Dimitri. "Green fluorescent protein regulation in the coral *Acropora yongei* during photoacclimation". The dissertation author was the primary investigator and author of this paper.

Chapter III is in preparation for publication as: Roth, Melissa; Goericke, Ralf; Deheyn, Dimitri. "Effects of cold and heat shock on the photophysiology of the coral *Acropora yongei*". The dissertation author was the primary investigator and author of this paper.

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ABSTRACT OF THE DISSERTATION

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Coral reefs ecosystems are diverse, productive and globally threatened. Corals have endosymbiotic dinoflagellates, which provide important nutrition. This symbiosis depends on a precarious balance of sunlight captured and processed through photochemistry that can be easily disrupted by environmental stressors including changes in irradiance and temperature. Photoprotective mechanisms are essential to maintaining the symbiosis equilibrium. Corals produce fluorescent proteins,

homologous to the popular green fluorescent protein (GFP), that have the potential to be used in photoprotection because of their inherent absorption and emission properties. This dissertation investigated the responses of corals and their dinoflagellate symbionts during photoacclimation, thermal shock and life history stages with particular focus on the effects on fluorescent proteins. In carefully controlled experiments on *Acropora yongei,* GFP abundance was positively correlated with light intensity and modulated the coral cell internal light environment. *A. yongei* thermal shock experiments caused the active degradation of GFP, possibly suggesting that GFP provided some role during stress. Cold shock caused more immediate pressure on the corals, but the heat shock was ultimately more deleterious. Life history stages of *Seriatopora hystrix* expressed different colors of fluorescent proteins, which shifted accordingly to changes in the light environment. Brooded larvae from *S. hystrix* exhibited a wide range of physiological characteristics, but there were subtle influences of parental environment on larval settlement. A novel methodology using fluorescence to enhance detection of coral recruits was developed and used to characterize the distribution, abundance and microhabitat of small juvenile corals (≤5 mm) on a healthy coral reef, providing a baseline for coral recruitment and re-evaluating the coral demographics of healthy coral reef. The responses of fluorescent proteins with photoacclimation and thermal shock and the life history patterns of fluorescence support a photoprotective function of fluorescent proteins in corals, which do not exclude other possible functions. This dissertation provides evidence that coral fluorescence could be used as an early indicator of coral stress and as a tool to

monitor coral recruits. Because multiple stressors threaten coral reefs, understanding the coral symbiosis physiology and having nondestructive tools to monitor coral health will be critical for the conservation and management of reefs.

CHAPTER I

Introduction to the dissertation

INTRODUCTION

Coral reefs flourish as one of the world's most diverse and productive ecosystems. Currently many coral reefs have become degraded and the remaining ones are globally threatened because of multiple stressors including those associated with global climate change (Hoegh-Guldberg, 1999; Hoegh-Guldberg et al., 2007; Baker et al., 2008; Riegl et al., 2009). Corals are sensitive to changes in their environment, and adult corals are sessile, which exacerbates their predicament. Coral physiology, and in particular how corals can respond to a changing environment, will be critical to their survival and future.

Coral-algal symbiosis. A key component of coral physiology is the coral-algal symbiosis. Coral cells contain endosymbiotic dinoflagellates of the genus *Symbiodinium*. Dinoflagellates convert sunlight and carbon dioxide into photosynthetically fixed carbon, most which is translocated to the coral host (Falkowski et al., 1984), and enhances coral calcification (Goreau, 1959). Reef-building corals live in oligotrophic tropical waters with high solar irradiance and warm seawater temperatures, which promote high rates of primary productivity (Muscatine and Porter, 1977; Muscatine, 1990). Moreover, the coral skeleton augments the amount of light within coral cells as compared to irradiance in the adjacent water column (Enriquez et al., 2005). Dinoflagellates, like all photosynthetic organisms, must maintain a delicate balance between the sunlight harvested and processed through photochemistry (Huner et al., 1998). Changes in light and temperature are among a suite of environmental variables that can create an imbalance and ultimately

lead to dissociation between the coral and the dinoflagellates that they require (Hoegh-Guldberg, 1999; Weis, 2008).

Photoacclimation. Because of the central role of irradiance in achieving high rates of productivity, the endosymbiotic dinoflagellates photoacclimate or adjust physiological parameters to the appropriate ambient light conditions. Corals photoacclimate to different depths (Falkowski and Dubinsky, 1981; Porter et al., 1984) and seasons (Warner et al., 2002), both of which influence the light environment. The cellular mechanisms of dinoflagellate photoacclimation has been well studied and includes changes in dinoflagellate density, photosynthetic pigment concentration, photosynthetic efficiency, mycosporine-like amino acids (MAAs), and antioxidants (Falkowski and Dubinsky, 1981; McCloskey and Muscatine, 1984; Porter et al., 1984; Dunlap and Chalker, 1986; Iglesias-Prieto and Trench, 1994; Shick et al., 1995; Brown et al., 1999; Warner et al., 2002; Shick, 2004; Ulstrup et al., 2008).

In contrast to their endosymbionts, the cellular and biochemical photoacclimation responses of the coral host are poorly understood. The described changes of corals under varying light regimes include extending/contracting of the polyp (Levy et al., 2003), varying antioxidant activity (Levy et al., 2006), and altering skeleton morphology (Falkowski and Dubinsky, 1981; Muko et al., 2000).

Photoprotection. While high intensities of solar irradiance are essential for high rates of productivity, excess light can be damaging for the photosynthetic system. Therefore, dinoflagellates and corals have a few photoprotective strategies to decrease pressure on the photosynthetic system and/or reduce the damage. The D1 protein, the

reaction center of photosystem II (PSII), is the site of primary damage and has a fast turnover rate in order to maintain high rates of photosynthesis (Adir et al., 1990; Warner et al., 1999). The xanthophyll cycle, another part of the photosynthetic system, safely dissipates excess energy as heat (Demmig-Adams and Adams, 1996). In dinoflagellates, energy is dissipated during the de-epoxidation of diadinoxanthin to diatoxanthin (Iglesias-Prieto and Trench, 1997), which can happen on the time scale of minutes. Dinoflagellates also synthesize MAAs, which absorb ultraviolet radiation, and transfer them to corals where they accumulate (Shick et al., 1996). The coral skeleton can also absorb ultraviolet radiation (Reef et al., 2009). Corals and dinoflagellates both have a variety of antioxidant defenses that can be used to neutralize reactive oxygen species, the sources of oxidative stress (Lesser, 2006).

Fluorescent proteins. Corals have another potential mechanism of photoprotection, fluorescent proteins. Corals produce fluorescent proteins that are homologues to the green fluorescent protein (GFP) originally isolated from jellyfish, which is now such an important tool in biomedical research (Tsien, 1998) that those who discovered and developed the protein were awarded the 2008 Nobel Prize in Chemistry. Fluorescent proteins have the potential to be photoprotective because they absorb potentially harmful high-energy photons of light and emit lower energy photons of light. Fluorescent proteins contribute to the diversity of coral color (Dove et al., 2001; Labas et al., 2002; Oswald et al., 2007); a rainbow of fluorescent proteins have been found in corals (Alieva et al., 2008), including ones that only absorb light but do not fluoresce, also called pocilloporins or GFP-like homologues (Dove et al.,

1995; Dove et al., 2001; Alieva et al., 2008). All fluorescent proteins identified to date have the same configuration of an 11-stranded β–barrel fold and a central α–helix, which contains the three amino acid chromophore (Figure 1-1; Shanar, 2006). This structure makes *in vitro* fluorescent proteins stable and resistant to changes in temperature and pH (Tsien, 1998). The protein is formed by a three-step process of cyclization, dehydration, and oxidation (Cubitt et al., 1995). Fluorescent protein monomers are usually about 28 kDa in size, but in anthozoans FPs are often found as tetramers (Leutenegger et al., 2007) and occasionally dimers and trimers (Dove et al., 1995; Dove et al., 2001). Fluorescent proteins are a superfamily because of the diversity in color while remaining very similar on a structural level (Shagin et al., 2004). Fluorescent proteins are universal in reef-building shallow corals (Salih et al., 2000; Alieva et al., 2008; Gruber et al., 2008) and can often make up a significant portion of the total soluble protein (Leutenegger et al., 2007).

Despite the prevalence of these proteins in corals, the function of fluorescent proteins remains ambiguous and controversial. It is probable that different fluorescent proteins will have different functions. Early hypotheses include photoprotection and photosynthesis enhancement (Kawaguti, 1944; Kawaguti, 1969). While there has been some further support for a photoprotective role such as more fluorescence in higher light corals and reduced photoinhibition with higher fluorescence intensity (Salih et al., 2000; Brown et al., 2002), the photoprotection hypothesis is weakened by a lack of correlation between fluorescent proteins and depth (Takabayashi and Hoegh-Guldberg, 1995; Vermeij et al., 2002; Mazel et al., 2003). The role of fluorescent

proteins as photosynthesis enhancers appears unlikely because of inefficient transfer of fluorescence emission to endosymbiotic dinoflagellates (Gilmore et al., 2003). A host membrane called the symbiosome surrounds the dinoflagellates, which creates too much distance for energy transfer to occur. Many additional hypotheses have been proposed including antioxidant capacity (Bou-Abdallah et al., 2006; Palmer et al., 2009a), regulation of symbiotic dinoflagellates (Field et al., 2006; Dove et al., 2008), coral innate immune response (Palmer et al., 2009b), and camouflage (Matz et al., 2006). The evolution of GFP-like homologues may suggest that the absorption properties of fluorescent proteins are more important to their function or that the different types of fluorescent proteins have different functions.

More recently, blue-light was shown to regulate fluorescent protein concentration in corals (D'Angelo et al., 2008), which may suggest a physiological connection with the high-energy portion of the light spectrum. Bay and colleagues (2009) found a decrease in GFP-like homologue expression but not abundance during a translocation from the reef (high light) to the laboratory (low light). Experimental heat stress caused a reduction in GFP-like homologues abundance and gene expression in adult corals (Dove et al., 2006; Smith-Keune and Dove, 2008) and GFP-like homologue abundance in larvae (Rodriguez-Lanetty et al., 2009). The physiological function of fluorescent proteins in corals, as well as other organisms, remains unknown and controversial, but fluorescent proteins may play an important role and particularly with regard to light in corals.

GFP is the most common fluorescent protein in corals and is thought to be the ancestral protein (Shagin et al., 2004; Alieva et al., 2008). Additionally, GFP has been found in copepods and amphioxus (Shagin et al., 2004; Deheyn et al., 2007). GFP absorbs high-energy blue light and emits lower energy green light. Blue light transmits well in oligotrophic waters (Falkowski et al., 1990), and has many influences on corals. Corals have cryptochromes, ancient-circadian clock proteins that are sensitive to blue light (Levy et al., 2007). Blue light also increases coral bleaching during thermal stress (Fitt and Warner, 1995), increases antioxidant activity (Levy et al., 2006), increases coral growth and chlorophyll *a* (Kinzie et al., 1984), regulates FPs (D'Angelo et al., 2008), and is required for the regeneration of coral growth tips (Kaniewska et al., 2009). In the photosynthetic system of cyanobacteria, blue light in addition to ultraviolet radiation damages photosystem II directly, and inhibits the repair of photosystem II (Nishiyama et al., 2006). Therefore, blue light is important to the physiology of the coral holobiont.

Coral-algal symbiosis breakdown. Because dinoflagellates have high rates of photosynthesis within coral cells, a large amount of dissolved oxygen is produced creating the potential for the formation of reactive oxygen species (ROS) (Lesser, 2006). There can be more than twice the amount of oxygen within coral tissue than in the surrounding water during daylight (Kuhl et al., 1995). ROS can cause significant cellular damage to DNA, proteins and membranes and lead to programmed cell death, apoptosis (Lesser and Farrell, 2004; Lesser, 2006). Under conditions of elevated seawater temperature and high irradiance, the breakdown of the coral-dinoflagellate

symbiosis is caused by oxidative stress damage to the photosynthetic apparatus: the reaction center (D1 protein) of photosystem II (PSII), the Calvin cycle, and/or the thylakoid membranes, which generates large amounts of ROS (Figure 1-2) (Lesser, 1996; Jones et al., 1998; Hoegh-Guldberg, 1999; Warner et al., 1999; Takahashi et al., 2004; Tchernov et al., 2004). Corals and dinoflagellates contain a number of antioxidant defenses including superoxide dismutase, catalase and ascorbate peroxidase (Lesser, 2006). This disruption of the symbiosis can lead to the dissociation of the coral and the dinoflagellate through a number of pathways including host cell apoptosis or exocytosis (Gates et al., 1992; Lesser, 1997; Franklin et al., 2004; Lesser and Farrell, 2004). Corals can become bleached, or visually appear white because their tissue becomes transparent and the coral skeleton becomes visible. Corals can bleach from either a decrease in dinoflagellate density and/or a reduction in photosynthetic pigment concentration (Coles and Jokiel, 1978; Hoegh-Guldberg and Smith, 1989; Warner et al., 1996). Many environmental factors in addition to temperature and irradiance influence the photochemistry balance including turbidity, flow and salinity (Brown and Suharsono, 1990; Glynn, 1996; Lesser, 1996; Lesser and Farrell, 2004; Nakamura et al., 2005).

Coral life history. Corals, like many other benthic marine invertebrates, have a pelagic larval stage. Adult corals either release gametes (broadcast spawners) or larvae (brooders) during particular lunar phases (Richmond and Hunter, 1990; Fan et al., 2002). Some larvae are competent to settle shortly after release, while others may remain in the water column for long periods of time (competent for ≥100 d;

Richmond, 1987; Harii et al., 2002). There are some anecdotal observations of fluorescence in coral eggs (Hirose et al., 2000; Leutenegger et al., 2007; Roth et al., 2007), embryos in *Montastrea cavernosa* (Leutenegger et al., 2007), larvae in *Stylophora pistillata* (Rinkevich and Loya, 1979), first polyps in *Acropora millepora* (D'Angelo et al., 2008), and in many families and genera of recent recruits (Roth and Knowlton, 2009). As a result of the difficulty in surveying small coral recruits, the ecology and post-settlement dynamics of recruits are poorly understood, but will have important consequences on future populations of corals.

The need for coral reef monitoring tools: Current state and future of coral reefs. Currently, nearly 20% of coral reefs worldwide have been destroyed, and another 35% are at risk without even considering global climate change (Wilkinson, 2008). Local threats to coral reefs include direct and indirect effects of overfishing and pollution (Hughes et al., 2003). However, with global climate change, changes in seawater temperature and ocean acidification will have profound effects on the future of coral reefs worldwide (Hoegh-Guldberg, 1999; Hoegh-Guldberg et al., 2007; Baker et al., 2008; Riegl et al., 2009). Coral bleaching events have been increasing since the 1980's and are mostly correlated with warm-seawater temperatures (Hoegh-Guldberg, 1999; Baker et al., 2008). In addition to elevated seawater bleaching events, cold-water bleaching events can have great consequences for corals reefs (Roberts et al., 1982; Coles and Fadlallah, 1991; Hoegh-Guldberg and Fine, 2004; Hoegh-Guldberg et al., 2005). Thermal anomalies such as those that occur during the El Nino-Southern Oscillation (ENSO), Pacific Decadal Oscillation, and Indian Ocean Dipole are often

associated with large-scale bleaching events (Brown and Suharsono, 1990; Glynn, 1990; Glynn, 1996; Hoegh-Guldberg, 1999; Jokiel and Brown, 2004; McClanahan et al., 2007). Global climate change is expected to change the frequency and intensity of thermal anomalies (Urban et al., 2000), and it is likely this will have grave consequences on the future of coral reefs. Because of the unprecedented changes in the future of coral reefs, a greater physiological and ecological understanding is needed for the protection and conservation of coral reefs. Additionally, nondestructive tools that can help monitor the health of the coral reef as well as the health of corals will be crucial to guide managers on the best course of action to take for reef preservation.

SCOPE OF THE DISSERTATION

This dissertation explores the physiology and ecology of the coral and its dinoflagellate symbionts and can be divided in two main parts: 1) investigations of the dynamics of fluorescent proteins and dinoflagellates in corals, and 2) applications of coral fluorescence as a monitoring tool. Chapters II-IV explore the photophysiology of Scleractinian corals and their endosymbiotic dinoflagellates during photoacclimation, shock, and life history stages. This dissertation focuses on the role of a common yet poorly understood protein produced by the coral, the fluorescent protein. Chapter V explores utilizing the inherent fluorescent nature of corals to monitor coral reef health nondestructively to enable studying the ecology of coral recruits. The small size of coral recruits makes them difficult to detect on a reef and therefore this life history

stages lacks basic ecological data. Understanding the changes in physiology of corals and ecology of coral reefs is an important component of the conservation and management of coral reef ecosystems.

Chapter II investigates the photoacclimation of the branching coral *Acropora yongei.* Photoacclimation or the ability for an organism to adapt to its local light environment will be an important determinant for whether a photosynthetic organism will be able to survive and thrive. Primary productivity rates are directly related to the light environment. Because reef-building corals rely on their symbionts for their energetic needs (Muscatine, 1990), the light environment is of critical importance for the coral and its symbiont. In this chapter, I conducted carefully controlled experiments testing the photoacclimation capacity of both the coral and its dinoflagellate. I have observed a single GFP in *A. yongei*, which makes it a good model system to test the responses of GFP. These experiments showed that corals use GFP to photoacclimate to both increases and decreases in light, and modulate the internal light environment mitigating the response of the dinoflagellates.

Chapter III investigates the response of corals and their dinoflagellates to thermal shock. Changes in temperature can upset the balance of capturing and processing light through photochemistry, which can lead to oxidative stress (Huner et al., 1998). In corals, oxidative stress can damage protein and DNA and leads to dissociation of the coral-algal symbiosis (Hoegh-Guldberg, 1999; Lesser, 2006). In this chapter, I conducted both a heat and cold shock on *Acropora yongei*, and examined the coral and the symbionts response to thermal stress. These experiments

elucidated different photophysiological responses on different time scales between cold and heat shock. However, both treatments induced a decrease in GFP abundance. Thermal anomalies are likely to increase in frequency and intensity with global climate change, and thus it is essential to understand the effects of thermal stress on coral physiology. These experiments suggest that corals will be more sensitive to changes in elevated seawater temperature than cold temperature changes.

Chapter IV describes the patterns of fluorescence during coral life history stages and explores how the parental environment influences coral larvae physiology and settlement. Corals have a pelagic larval dispersal stage where they are likely to encounter a different light regime compared to their benthic parents. In this chapter, I examined fluorescence patterns during life history stages in the brooding coral *Seriatopora hystrix* in Taiwan and found that different life history stages expressed different fluorescent proteins. In this chapter, I also conducted an experiment testing how the parental light environment affected the larvae physiology and settlement. In general, adults produce larvae with a wide range of physiological characteristics, but parental environment had subtle effects on larval settlement.

Chapter V develops a new nondestructive methodology utilizing the inherent fluorescent nature of juvenile corals to detect them reliably. This technique was used to characterize the distribution, abundance and microhabitat of small coral recruits (≤5 mm) on a healthy coral reef, Palmyra Atoll in the central Pacific. Coral demography and in particular juvenile coral populations can inform managers about the health and future prospects of a coral reef. Chapter V offers the first ecological characterization

of an important life history stage of corals, which may give a baseline for recruit populations on a healthy coral reef.

Together, these investigations of corals and their symbionts during photoacclimation, thermal shock and life history stages provide a greater understanding of the coral photophysiology and gives new insights into the function of fluorescent proteins in corals. The dissertation implications on the function of fluorescent proteins, for using coral fluorescence as an indicator of coral physiology, and for using coral fluorescence to monitor coral reef health are discussed in a cross-chapter synthesis with conclusions of the dissertation in **Chapter VI**.

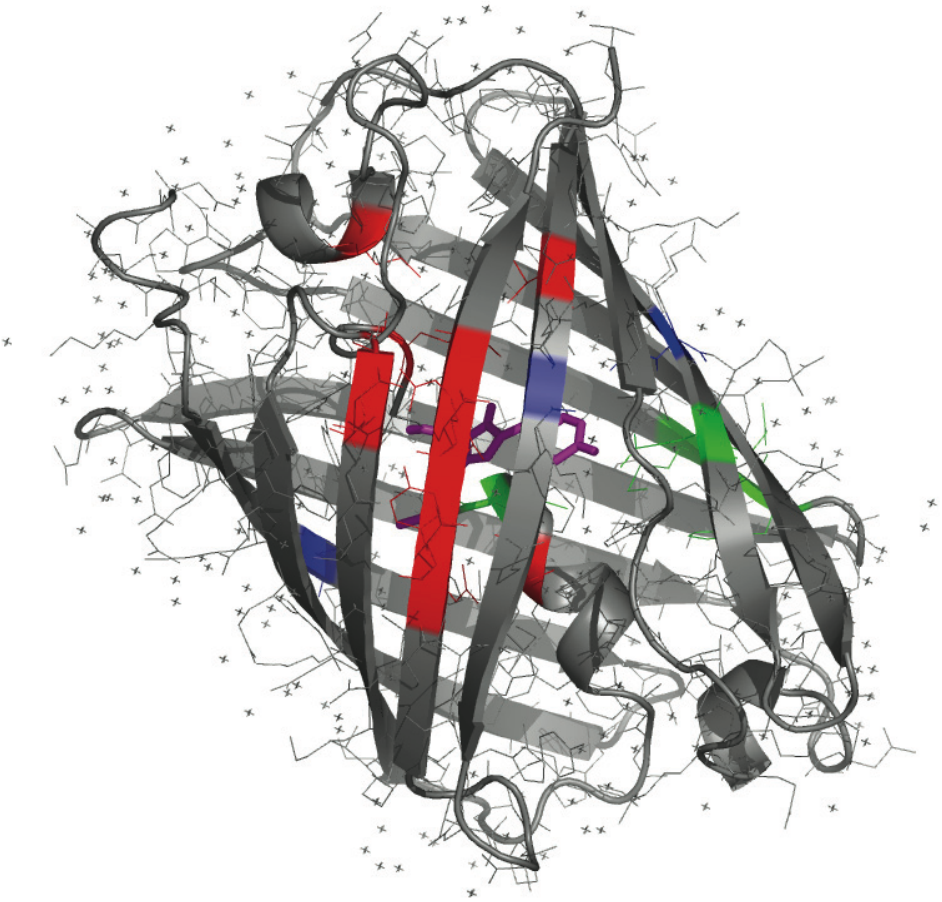

Figure 1-1. Ribbon representation of the fluorescent protein mCherry with the chromophore shown in magenta. Figure from Shanar (2006).

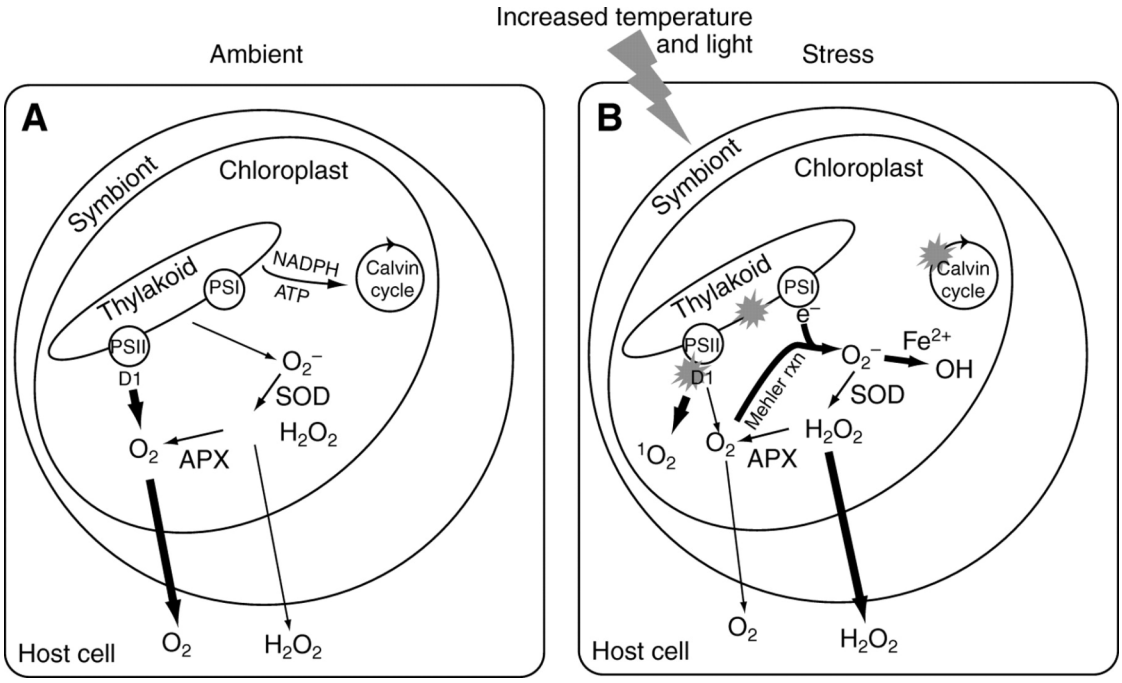

Figure 1-2. Schematic representation of the oxygen-handling pathways in *Symbiodinium* resident in host cells under ambient (A) and elevated temperature and light (B) conditions. Under ambient conditions, the photosynthetic apparatus, consisting of photosystem II (PSII) and photosystem (I) on the thylakoid, operates normally and produces large quantities of oxygen that diffuse into the host. ROS that are produced are converted back to oxygen with superoxide dismutase (Daily et al.) and ascorbate peroxidase (APX). Under stress conditions, damage to the photosynthetic apparatus occurs in at least three places (depicted as 'flashes' in the figure); the D1 protein in PSII; in the Calvin cycle; and on the thylakoid membranes. This damage acts to generate large amounts of ROS in the form of singlet oxygen ($^1O_2$) and superoxide (O_2^-) that overwhelm the oxygen-handling pathways. O_2^- is converted to both the most highly reactive hydroxyl radical ($^{\cdot}OH$) and the more stable and highly diffusible hydrogen peroxide (H_2O_2), which can move into host tissues. Figure from Weis (2008).

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CHAPTER II

Green fluorescent protein regulation in the coral *Acropora yongei* during photoacclimation

ABSTRACT

Reef-building corals inhabit high light environments and are dependent on photosynthetic endosymbiotic dinoflagellates for nutrition. While photoacclimaton responses of the dinoflagellates to changes in illumination are well understood, host photoacclimation strategies are poorly known. This study investigated fluorescent protein expression in the shallow-water coral *Acropora yongei* during a 30 d laboratory photoacclimation experiment in the context of its dinoflagellate symbionts. Green fluorescent protein (GFP) concentration measured by Western blotting changed reversibly with light intensity. The first 15 d of the photoacclimation experiment led to a ~1.6x increase in GFP concentration for high light corals (900 µmol quanta m^{-2} s^{-1}) and a ~4x decrease in GFP concentration for low light corals (30 µmol quanta m^{-2} s^{-1}) compared to medium light corals (300 µmol quanta m^{-2} s^{-1}). Green fluorescence increased ~1.9x in high light corals and decreased ~1.9x in low light corals compared to medium light corals. GFP concentration and green fluorescence intensity were significantly correlated. In comparison, smaller changes in the dinoflagellate density, photosynthetic pigment concentration, and photosynthetic efficiency were observed. Although fluorescent proteins are ubiquitous and abundant in scleractinian corals, their functions remain ambiguous. These results suggest that scleractinian corals regulate GFP to modulate the internal light environment of the endosymbiotic dinoflagellates, and support the hypothesis that GFP plays a photoprotective role. The success of photoprotection and photoacclimation strategies, in addition to stress responses, will

be critical to the fate of scleractinian corals exposed to climate change and other stressors.

INTRODUCTION

Scleractinian corals are the foundation of one of the world's most diverse and productive ecosystems—coral reefs—because of a critical endosymbiotic relationship with dinoflagellates. This symbiosis provides the corals with significant nutrition (Muscatine, 1990) and enhances coral skeleton calcification (Goreau, 1959). Reef-building corals live in oligotrophic tropical waters, characterized by bright light and warm temperatures, which allow their symbionts to maintain high rates of photosynthesis (Muscatine and Porter, 1977; Muscatine, 1990). While solar radiation is required for high productivity, excessive levels can be deleterious to corals and/ or their symbionts. This can happen either directly, through inactivation of photosystem II (PSII) or, indirectly, through an imbalance of light energy absorbed and processed through photochemistry that can trigger damaging oxidative stress (Lesser and Shick, 1989; Lesser et al., 1990; Lesser, 1996; Lesser, 1997; Lesser, 2006). High irradiance, often accompanied by elevated seawater temperatures, can lead to coral bleaching, the breakdown of the coral-dinoflagellate symbiosis (Lesser, 1997; Hoegh-Guldberg, 1999; Weis, 2008) that can cause a decline in coral health and even mortality (Brown and Suharsono, 1990; Hoegh-Guldberg, 1999). Comprehensive understanding of both stress responses and acclimation to light and temperature are important to the conservation of scleractinian corals.

Photoacclimation, the physiological response to changes in the ambient light environment, involves a variety of processes acting on different time scales. The cellular mechanisms of photoacclimation to decreased light intensity by the

endosymbiotic dinoflagellates are well known and include: increases in photosynthetic pigment concentrations; increases in the photochemical efficiency of photosystem II (PSII); increases in dinoflagellate density, except in cases of extreme low light, which decreases density (Falkowski and Dubinsky, 1981; McCloskey and Muscatine, 1984; Porter et al., 1984; Shick et al., 1995; Brown et al., 1999; Warner et al., 2002; Ulstrup et al., 2008); increases in photosynthetic unit size and number (Iglesias-Prieto and Trench, 1994); decreases in levels of ultraviolet radiation (UVR) absorbing mycosporine-like amino acids (MAAs) (Dunlap and Chalker, 1986; Shick et al., 1995; Shick, 2004); and decreases in levels of antioxidants (Shick et al., 1995).

Photoacclimation in corals has been primarily studied in terms of behavioral and morphological changes. On short time scales (minutes), corals expand and contract polyps in response to light (Levy et al., 2003); on daily cycles corals regulate their antioxidant activity (Levy et al., 2006b); and on long time scales (months to years) corals change their morphology (Falkowski and Dubinsky, 1981; Muko et al., 2000).

In contrast to their endosymbiotic dinoflagellates, cellular and biochemical photoacclimation strategies of the coral itself remain poorly understood. Corals produce fluorescent proteins (FPs) that are homologous to the green fluorescent protein (GFP), originally isolated from jellyfish and now a widely used tool in cellular and molecular biology (Tsien, 1998). FPs inherently affect the internal light microenvironment of the coral by absorbing high-energy light and emitting lower-energy light. FPs are ubiquitous in scleractinian corals (Salih et al., 2000; Alieva et al.,

2008; Gruber et al., 2008) and can constitute a significant portion of the total protein content (up to 14%) (Leutenegger et al., 2007).

The functions of FPs in corals remain ambiguous and controversial. Hypothesized roles for FPs include photoprotection (Kawaguti, 1944; Salih et al., 2000), photosynthesis enhancement (Kawaguti, 1969), camouflage (Matz et al., 2006), antioxidant capacity (Bou-Abdallah et al., 2006; Palmer et al., 2009b), regulation of symbiotic dinoflagellates (Field et al., 2006; Dove et al., 2008), and being part of the coral immune response (Palmer et al., 2009a). Corals produce a number of FPs with different spectral properties (Alieva et al., 2008), including FPs that do not emit visible fluorescence, which are often called pocilloporins or GFP-like proteins (Dove et al., 1995; Dove et al., 2001). FPs contribute to the diversity of coral coloration (Dove et al., 2001; Labas et al., 2002; Oswald et al., 2007), and it is possible that dissimilar FPs will have different functions while the same FP could have multiple functions. The principal hypothesis, photoprotection, is weakened by a lack of correlation between FPs and depth (Vermeij et al., 2002; Mazel et al., 2003; Dove, 2004). Recently, variation of exposure to blue light was shown to regulate FP concentration (D'Angelo et al., 2008), suggesting a physiological connection between FPs and the high-energy portion of the light spectrum. In addition, corals with and without the GFP-like proteins can have different ecological and physiological characteristics (Takabayashi and Hoegh-Guldberg, 1995).

The objective of this study was to investigate the dynamics of GFP concentration in a fluorescent corals in response to changes in light intensity, and to

simultaneously monitor the population of symbiotic dinoflagellates. A laboratory controlled photoacclimation experiment using the reef-building coral *Acropora yongei* clearly revealed that corals reversibly increase GFP concentration under increased light intensity, and decrease GFP concentration under reduced light intensity. As compared to the magnitude of the corals response, the endosymbiotic dinoflagellates responded with relatively minor changes in cell density, photosynthetic pigment concentrations, and photochemical efficiency, suggesting that GFP may modulate the cellular light environment of the endosymbiotic dinoflagellates.

MATERIALS AND METHODS

Photoacclimation experiment design

Specimens of *Acropora yongei* Veron and Wallace 1984 were obtained from the Birch Aquarium at the Scripps Institution of Oceanography in San Diego, CA, USA. *A. yongei*, a common shallow-water branching coral endemic to the Indo-West Pacific, is cream to brown colored under white light illumination, and shows intense green fluorescence under blue light excitation (Figure 2-1A,B). The excitation spectrum peak was 470 nm, and the fluorescence emission spectrum peak was 516 nm, with a full width at half maximum (FWHM) of 28 nm when excited with blue (470 nm) light (Figure 2-1C). These spectral characteristics were obtained using a SpectraMax M2 fluorescence reader (Molecular Devices, Sunnyvale, CA, USA). The original wild-type GFP isolated from the jellyfish *Aequorea victoria* has an *in vitro* blue excitation peak of 476 nm and emission peak of 503 nm (Heim et al., 1994).

For the experiment, corals were cut into ~5 cm long fragments with all tips and side branches removed to obtain uniform experimental replicates, and were attached to terracotta tiles with cyanoacrylic adhesive and placed in an individual 1 L glass aquarium. Coral fragments are individual ramets and could possibly be derived from only one genet (corals were not genotyped), as is possible when corals originate from a single location of collection. This is especially true for *Acropora* whose colonies distribute around locally by spreading broken branches upon high physical disturbances. The limited genetic variability of replicate ramets was a strength of the study, allowing for more carefully controlled experimental approach in that responses would not be due to genetic variability. Another strength of the experimental design was that each coral ramet was individually maintained in its own aquarium without mixing of seawater with any other conspecific and the possibility of chemical interactions. Each aquarium had two seawater inlet hoses (inner diameter 0.32 cm) with a combined flow rate of ~0.7 L min^{-1}. Corals were maintained under a photoperiod of 12:12 h light:dark at a seawater temperature of 26.5 ± 1ºC. One or two herbivorous snails per aquarium were used to control algal growth. Light intensity was manipulated by using neutral density shade cloth (Easy Gardener Products, Ltd, Waco, TX, USA) and changing the distance of the coral from the light fixture (T5 Teklight with two Midday and two Aqua Blue+ 54W Powerchrome fluorescent lamps, Sunlight Supply, Vancouver, WA, USA). Light intensity of photosynthetically active radiation (PAR) was measured using a 4-channel cosine radiometer (BIC, Biospherical Instruments, San Diego, CA, USA).

The light environment during the 2-4 week steady-state phase, starting immediately after fragmentation, was 300 µmol quanta m^{-2} s^{-1}. A two-phase photoacclimation experiment was then conducted (Figure 2-2). At the beginning phase I, corals were placed in three light treatments for 15 d (0-15 d): low light (LL: 30 µmol quanta m^{-2} s^{-1}), medium control light (ML: 300 µmol quanta m^{-2} s^{-1}), and high light (HL: 900 µmol quanta m^{-2} s^{-1}). At the beginning of phase II, corals were returned to ML for 15 d (15-30 d). Every five days, corals (0 d: *N*=40, 5-15 d: *N*=11-12 per treatment; 20-30 d: *N*=5-6 per treatment) were non-destructively measured for fluorescence emission, green fluorescence intensity, frequency distribution of green fluorescence, maximum quantum yield of photosystem II (PSII), and growth. A subset of coral samples (*N*=5-6) was collected at 0, 15 and 30 d for the following three destructive analyses: quantitative Western Blots, dinoflagellate abundance, and chlorophyll and carotenoid concentrations. Corals were collected just before dawn, frozen in liquid nitrogen, and stored at -80ºC until further analyses.

Quantitative Western Blots (immunoblot)

Custom GFP antibodies were designed based on the GenBank published peptide sequences for GFP in *Acropora aculeus* (AAU06845), *Acropora millepora* (AAU06846) and *Acropora nobilis* (AAU06847). The peptide sequence used was DMPDYFKQAFPDGMSYER (aa 80-97) and the result was a specific antibody that cross-reacted with a ~27 kDa protein (Figure 2-A1A), which corresponds to the expected size of GFP (Prasher et al., 1992).

A 16 mm long piece from each coral was ground to a fine powder with a liquid nitrogen chilled mortar and pestle. The piece was cut 8 mm below the tip of the coral to avoid growing tip regions, which may have a different physiology, and was the same portion of the coral that was measured for optical fluorescence. Samples were boiled and the protein extracted in the denaturing buffer (50 mM Tris-HCl (pH 6.8), 2% w/v SDS, 25 mM dithiothreitol, 10 mM EDTA, 4% w/v polyvinylpolypyrrolidone, 1% DMSO) and protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA). The homogenate was vortexed and incubated twice at 90ºC for 3 min, centrifuged at 13,000*g* for 15 min, and the middle phase containing the extracted protein was removed. Protein extract concentration was determined using the RC/DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Using tissue from extra corals, a large amount of protein was extracted, homogenized and used as a coral standard, to compare results across gels and create a standard curve from each gel, which was used to convert optical density into relative protein concentration (Figure 2-A1B). Seven concentrations of the coral standard and 8 mg of protein from each sample with loading buffer were run on 6% 96-well gels (Invitrogen, Carlsbad, CA, USA). All samples and standards were run in triplicate. Samples were then transferred to PVDF and immunoblotted using the custom made polyclonal GFP antibody (described above) or polyclonal conjugated ubiquitin antibody (Stressgen, Ann Arbor MI, USA). Conjugated ubiquitin, a marker of protein degradation, was used as a proxy to assess coral general health. The proteins were then visualized using secondary antibodies with a peroxidase label (Assay Designs, Ann Arbor, MI, USA) and a

chemiluminescence kit (Pierce Biotechnology, Rockford, IL, USA) before being scanned on a Typhoon 9410 Imaging Workstation (Amersham Biosciences, Piscataway, NJ). The optical densities for each sample were measured and were subtracted by an adjacent background value of the same size using image analysis software (ImageJ, NIH, Bethesda, MD, USA). The optical densities were then transformed by the standard curve for that particular gel. Thus, these data always represented the relative concentrations of the specific protein of interest.

Green fluorescence intensity

Green fluorescence of a 16 mm long section of the coral fragment 8 mm from the tip was measured prior to its use in the immunochemistry. Every five days during the experiment, the coral was imaged with an epifluorescence stereoscope (Nikon SMZ1500 with 100 W mercury lamp and filter cube with excitation 450-490 nm and longpass emission barrier >500 nm, Melville, NY, USA) coupled to a Retiga 2000R color digital camera (Figure 2-1; QImaging, Surrey, Canada). Images were taken under the same settings, which included an exposure of 0.048 s. Additionally, images were taken of the same field under white light to use in image processing.

The images were processed in MATLAB 7.5 (Mathworks, Natick, MA, USA). The average green fluorescence intensity of the coral was obtained by first taking the white light image and determining the area of coral tissue. Second, using the green channel of the fluorescence image the background value was determined by averaging the pixel intensity of the area outside of the coral tissue. Third, the green pixel

intensity of the coral area, minus the average background, was summed and divided by the coral area to obtain the average green fluorescence intensity of the coral section. Fluorescence intensity had an 8-bit resolution scaling from 0-255. To examine whether changes in green fluorescence were the result of a particular region or the entire area of coral changing, the frequency distribution of green fluorescence intensity was analyzed. The image from each coral was normalized to the highest pixel intensity of each image.

Fluorescence emission spectrum

The fluorescence emission spectrum was measured from the same 16 mm long section of the live coral fragment, 8 mm from the tip that was used in immunochemistry and green fluorescence measurements. Every five days during the experiment, the spectrum was measured with a low-light Echelle SE200 Digital Spectrograph (Catalina Scientific, Tucson, AZ, USA). The measurement probe, which consisted of a single optical fiber, was manipulated at a fixed angle with a micromanipulator (M-3333, Narishige, Tokyo, Japan) so that the tip of the probe was placed 2 mm from the coral using a plastic spacer. Emission spectra were measured with blue (450-490 nm) and cyan (426-446 nm) excitation light. Cyan emission spectra did not differ from blue emission spectra and thus these data are not presented. GFP emission peaks were characterized by the wavelength of the peak and the FWHM from smoothed curves (KestrelSpec, Catalina Scientific, Tucson, AZ, USA). At two occasions of emission curves, the shoulder of the peak extended into the excitation

light so these spectra were excluded from analyses because the FWHM could not be determined. The spectrograph was calibrated each time using a mercury lamp and tungsten lamp (Ocean Optics, Dunedin, FL, USA) to ensure its resolution of 1 nm.

Dinoflagellate density and photosynthetic pigment concentrations

A 10 mm long piece of the coral fragment (24 mm from the tip) was used to determine the density of the endosymbiotic dinoflagellates and the concentration of photosynthetic pigments. The coral tissue was removed by an artist's airbrush and filtered seawater. Samples were maintained on ice and under low light conditions to prevent degradation. The coral slurry was centrifuged at 1,500 r.p.m at 4ºC for 10 min. The supernatant was removed and the dinoflagellate pellet resuspended in filtered seawater. The surface area of the underlying skeleton was determined by measuring its height and diameter and calculated using simple cylinder geometry. Any branches that grew longer than 5 mm from the coral piece were also measured and the surface area was included in the calculation.

Dinoflagellate density was determined from three aliquots of the resuspension. Aliquots (10 µl) were added to 20 mL filtered seawater so that dinoflagellate cells could be counted with the Elzone II 5390 particle counter (Micromeritics, Norcross, GA, USA). The volume of ~2,000 particles between 7-12 µm diameter was determined. The dinoflagellate concentration was normalized by the coral piece surface area, and the average of the three replicates was calculated. To verify the use of a particle counter as an adequate method, the dinoflagellate density was also

determined using a Neubauer ruled hemacytometer for a subset of corals (*N*=10). The two methods were highly correlated with an average 5% higher count for the particle counter.

Concentrations of chlorophyll *a* (chl *a*), chlorophyll c_2 (chl c_2), peridinin (per), carotene and the xanthophylls diadinoxanthin (Dd) and diatoxanthin (Dt) were determined using high-pressure liquid chromatography (HPLC). An aliquot of the resuspension was centrifuged at 1,500 r.p.m at 4ºC for 10 min. The supernatant was discarded and samples were extracted for 18 h at 4ºC in 1.6 ml acetone that had been spiked with an internal standard (canthaxanthin). The samples were analyzed on an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA, USA) on a Waters Symmetry C8 column (3.5 μm particle size, 4.6 x 150 mm, silica, reverse-phase; Waters, Milford, MA, USA). Pigments were eluted using a gradient method, employing two solvents: (A) a mixture of methanol, acetonitrile and an aqueous pyridine solution (0.25 M, pH=5) (50:25:25 v:v:v); and (B) a mixture of methanol, acetonitrile, and acetone (20:60:20 v:v:v) and the following gradient (time, %A, %B): (0, 100, 0), (12, 60, 40), (36, 0, 100), (38, 0, 100), (40, 100, 0). Data were normalized to dinoflagellate density and reported in pg $cell^{-1}$.

Photochemical efficiency of photosystem II

To assess the photoacclimation and physiological status of the dinoflagellates, the photochemical efficiency of PSII was measured using a diving pulse amplitude modulated (PAM) fluorometer (Walz Inc., Effeltrich, Germany). Dark-acclimated

maximum quantum yield of PSII (F_v/F_m; F_v, variable fluorescence; F_m maximum fluorescence) was measured pre-dawn similar to previously described methods (Warner et al., 1996; Rodrigues et al., 2008). The measurement was taken at a standardized distance from the coral using a 1 cm piece of surgical tubing on the fiber optic cable that was placed ~1 cm below the tip of the coral on the same side of the coral where the spectroscopy and optical fluorescence measurements were taken. Measurements were taken every five days during phases I and II of the experiment.

Linear extension growth

The growth rates of the corals were determined by linear extension from the tip of the coral. Digital images were captured through the stereoscope (Nikon SMZ1500) under white light every five days. The coral was positioned in the same way in each image. The linear extension was then measured from a landmark with image analysis software (ImageJ). The difference between two time points was taken and divided by the number of days between the two images to obtain the average daily growth rate.

Statistical analyses

Statistical analyses were conducted using JMP version 7.0 (SAS, Cary, NC, USA) and R software version 2.2.1 (Table 2-1). A one-way nested analysis of variance (ANOVA) compared the effects of light treatments on GFP, conjugated ubiquitin immunoblots, and dinoflagellate densities. A repeated-measures multivariate analysis of variance (MANOVA) was used to compare the effects of time and light treatments

on green fluorescence intensity. Because this test violated the sphericity assumption ($p < 0.001$) it was not possible to do further univariate analyses. One-way ANOVAs were conducted for phases I and II to test the effect light treatments on green fluorescence intensity, frequency distribution of green fluorescence intensity peak, emission characteristics, growth rate, maximum quantum yield of PSII, and concentrations of chlorophyll and carotenoids. For all significant ANOVAs, post-hoc Tukey-Kramer HSD pairwise comparisons were used to test which groups were significantly different. Simple correlation analyses were also used to test relationships between GFP concentration and green fluorescence, and growth with each of those parameters. Averages represent arithmetic means ± standard deviations. Statistical differences were significant at the $\alpha=0.05$ level.

RESULTS

Green fluorescent protein response during photoacclimation

GFP expression was dynamic and reversible under different treatments of the photoacclimation experiment. Phase I of the experiment led to significantly different GFP concentrations among corals of the LL, ML, and HL conditions with each treatment being significantly different from each other (Table 2-1; Figure 2-3A; Figure 2-A2). The GFP concentration of the HL corals increased ~1.6 times compared to the ML control corals, while that of the LL corals decreased ~4.0 times compared to the ML corals. At the end of phase II, GFP concentrations of HL and LL returned to similar levels as ML corals and these differences were no longer significant.

Green fluorescence was dynamic and reversible under different treatments of the experiment. There was a significant treatment effect during phase I (MANOVA, $F_{6,58}$=36.1, P<0.0001). Phase I of the experiment led to significantly different green fluorescence pixel intensity among treatments with each treatment being significantly different from each other (Table 2-1; Figure 2-3B; Figure 2-A2). The green fluorescence of HL corals increased ~1.9 times compared to ML corals, while that of LL corals decreased ~1.9 times compared to ML corals. At the end of phase II, the green fluorescence pixel intensity in phase I LL and ML corals were similar while the HL corals remained significantly different and higher.

Green fluorescence intensity at the end of phase I was significantly correlated with GFP concentration (Figure 2-3C), based on the least-squares linear regression between protein abundance and green fluorescence ($F_{1,16}$=23.4, P<0.001, R^2=0.59).

The shifts in frequency distributions of green pixel intensity indicated that all parts of the coral changed in GFP concentration as opposed to only a small part of the coral changing GFP concentration (Figure 2-4). The frequency distributions showed unimodal distributions. There was a shift in the peaks of the frequency distributions of green fluorescence at the end of phase I, and HL corals had a broader distribution of pixel intensity and LL corals had a narrower distribution compared to the ML corals (Figure 2-4B). The peak pixel intensities among the treatments were significantly different from each other (Table 2-1). The HL corals peaked at higher pixel intensity and the LL corals peaked at lower pixel intensity than the ML corals. After phase II, the peak pixel intensity was similar among treatments, but the HL corals were still

significantly different (Figure 2-4C). It is noteworthy that all frequency distributions of green fluorescence displayed jagged curves. To ensure this was not an instrumentation artifact, different color channels of the same images and the white light images of the corals were examined; none of the frequency distributions displayed the jagged pattern (M. S. Roth, unpublished). Moreover, additional coral species with varying skeletal morphology and polyp structure were examined and the same jagged pattern was observed: *Galaxea fasicularis* Linnaeus 1767 and *Pavona* cf. *decussata* Dana 1846, both of which displayed green fluorescence and had jagged green fluorescence distribution curves, and the red fluorescing *Montipora capricornis* Veron 1985, which displayed jagged red fluorescence distribution curves but not in curves of the green channel (M. S. Roth, unpublished). Therefore, the jagged pattern in the curves is not likely to be caused by the coral anatomy, but rather is a property of FPs in corals, perhaps from protein arrangement or position in the coral.

The fluorescence emission spectrum (Figure 2-1C) did not change during the photoacclimation experiment. Two spectral characteristics, the emission peak maximum and FWHM, did not vary in any treatment during phases I or II. The average emission peak was 516.2 ± 0.8 nm (N=90). Additionally, the average FWHM was 29.0 ± 1.5 nm (N=88), and there were no significant differences among treatments (Table 2-1).

Dinoflagellate response during photoacclimation

Maximum quantum yield of PSII, a measure of photochemical efficiency, was 0.63 at the end of the steady-state phase. At the end of phase I, maximum quantum yield increased 3% in LL corals and decreased 4% in HL corals compared to ML corals (Figure 2-5); each treatment was significantly different from the other (Table 2-1). At the end of phase II, treatments had more similar yields, although the ML and HL corals were still significantly different from each other.

Dinoflagellate density also changed during the experiment (Table 2-2). At the end of phase I, there was a significant difference in dinoflagellate density among treatments (Table 2-1); the density in LL corals was 36% lower and significantly different than ML corals, while the HL density was not significantly different from ML corals. At the end of phase II, the dinoflagellate densities of both the LL and HL corals were similar to the ML control corals.

The concentrations of chlorophyll *a*, chlorophyll c_2, and peridinin were significantly different among light treatments at the end of phase I, and these differences were primarily based on changes for the HL corals, which had lower concentrations of the pigments (Tables 2-1, 2-2). There was a trend of higher ratios of chl c_2 : chl *a* and per : chl *a* in the HL treatment, but the differences were not significant. At the end of phase II, none of the observed differences were significant. The concentrations of the photoprotective pigments produced by the symbiotic dinoflagellates, which include carotene and diadinoxanthin + diatoxanthin, did not vary among treatments after phases I or II (Tables 2-1, 2-2). There was little

diatoxanthin observed, which was expected because samples were collected pre-dawn and diatoxanthin disappears rapidly under low light (Goericke and Welschmeyer, 1992; Levy et al., 2006a). Because of the changes in chl *a*, the ratios of carotene : chl *a* and Dd + Dt : chl *a* were significantly different at the end of phase I, where the corals showed slightly greater relative concentrations of photoprotective pigments under HL. At the end of phase II of the light treatment, these ratios were back to their initial values and showed no significant difference among treatment groups.

Coral condition during photoacclimation

All corals survived and grew measurably during phases I and II regardless of treatment. From 10-15 d at the end of phase I, the linear extension rates were 0.094 ± 0.088 mm day^{-1} for LL corals, 0.215 ± 0.090 mm day^{-1} for ML corals, and 0.312 ± 0.115 mm day^{-1} for HL corals. There were significant differences among treatments because the growth rate of LL corals was different and lower from that of ML and HL corals (Table 2-1). At the end of phase II (25-30 d), there were no significant differences among the linear extension rates; the average linear extension rate pooled among treatments was 0.251 ± 0.131 mm day^{-1}.

Any treatment causing a stress response would likely result in an increase in conjugated ubiquitin levels; however, conjugated ubiquitin concentration was similar among treatments (Table 2-1). At the end of phase I, the relative concentration of conjugated ubiquitin was 4.8 ± 9.6 for LL corals, 5.7 ± 14.0 for ML corals, and 6.9 ± 14.6 for HL corals, and the differences were not significant among treatments. At the

end of phase II, the relative concentration of conjugated ubiquitin was 8.7 ± 6.6 for LL corals, 10.6 ± 7.2 for ML corals, and 8.5 ± 6.6 for HL corals, and the differences were not significant among treatments.

Because significant differences in growth at the end of phase I were observed, the correlation between GFP and growth was examined (Figure 2-6). Both the correlations between growth rate and relative GFP concentration ($F_{1,16}$=11.2, P<0.01, R^2=0.41) and growth rate and green fluorescence ($F_{1,32}$=20.3, P<0.001, R^2=0.39) were significant.

DISCUSSION

GFP in *A. yongei* maximally absorbs blue light, which has significant physiological effects on corals and their symbionts. Corals are sensitive to blue light (Gorbunov and Falkowski, 2002) and have cryptochromes, blue light photoreceptors that are thought to play a role in synchronizing coral spawning (Levy et al., 2007). Blue light amplifies synergistic bleaching between elevated seawater temperatures and ambient light (Fitt and Warner, 1995), increases antioxidant activity (Levy et al., 2006b), increases coral growth and chlorophyll *a* (Kinzie et al., 1984), regulates FPs (D'Angelo et al., 2008), and is required for the regeneration of coral growth tips (Kaniewska et al., 2009). In cyanobacteria, blue light in addition to UVR primarily damages PSII directly, and secondarily inhibits the repair of PSII through production of reactive oxygen species (Nishiyama et al., 2006). Because shallow-water corals receive a considerable amount of solar radiation and blue wavelengths transmit well

through oligotrophic ocean water (Falkowski et al., 1990), mechanisms to dissipate high-energy blue light may be important to optimize photosynthesis and provide photoprotection to both the coral and its symbionts. This study provides evidence that suggest GFP may be important to modulate photosynthesis and support the hypothesis that GFP may play a photoprotective role.

Regulation of GFP as a coral photoacclimation strategy

There was a positive correlation between light intensity and GFP concentration during the photoacclimation experiment of the shallow-water coral *Acropora yongei*. Both an increase and decrease in light intensity led to changes in GFP concentration and these changes were reversible when original light conditions were restored. These results show that GFP has a strong photoacclimation response and is consistent with the hypothesis that FPs participate in photoacclimation and may modulate the internal light environment, which could influence the physiology of both coral and dinoflagellate cells. These results are consistent with lower green fluorescence in corals under low light (80-100 µmol quanta m^{-2} s^{-1}) compared to higher light conditions (400 µmol quanta m^{-2} s^{-1}; D'Angelo et al., 2008). There was no difference in green fluorescence at a light intensity > 400 µmol quanta m^{-2} s^{-1}, whereas this study found a ~1.9x increase in green fluorescence between ML and HL corals. A 10 d translocation experiment from the field to the laboratory (corresponding to a higher to lower light treatment) triggers a down-regulation of fluorescent protein genes including GFP, without being associated with a decrease of fluorescent protein levels

(Bay et al., 2009), thus indicating that the pool of fluorescent proteins is kept constant in the cells despite an increased turnover induced by environmental change. This is in contrast with prior results (D'Angelo et al. 2008) showing that the level of fluorescent proteins is modulated by exposure to variable blue light intensity. In addition, such environmental modulation of the levels of fluorescent proteins in cells was also shown to occur with GFP-like proteins have a positive correlation with light intensity over time scales of months (Takabayashi and Hoegh-Guldberg, 1995; D'Angelo et al., 2008) to years (Dove et al., 2008). The fact that both fluorescent proteins and GFP-like proteins show a correlation with ambient light characteristics may suggest that the light absorption properties of the fluorescent proteins may be the critical component.

The increase in green fluorescence occurred in <5 d (within the 5 d resolution of the experiment) after an increase in light intensity, while decreases in green fluorescence were observed 10-15 d after the reduction in light intensity for the LL corals and 5-10 d after the reduction in light intensity for the HL corals (phase II). The delayed reduction in green fluorescence suggests GFP has a slow turnover and long lifetime. This property of the *A. yongei* GFP is consistent with irreversible green-to-red photoconvertible FPs, which have slow decay rates and protein half-lives of ~20 days (Leutenegger et al., 2007).

The ability of corals to rapidly respond to increases in light would be important protection against solar radiation causing direct and indirect damage of their symbionts' photosynthetic apparatus. Corals are susceptible to oxidative stress because of elevated concentrations of oxygen within coral cells (Kuhl et al., 1995),

and enhanced irradiance within coral cells (Enriquez et al., 2005). As a result, there is higher antioxidant activity in corals at shallow depths (Shick et al., 1995) and during the daytime (Levy et al., 2006b). Ambient light levels (e.g., <700 μmol quanta m^{-2} s^{-1} in Fitt and Warner, 1995) can cause coral bleaching when temperatures are elevated. The photoacclimation experiment in this study showed that the corals regulated GFP expression in the absence of other indicators of stress. The increase in GFP concentration under higher light conditions may be a pre-emptive strategy of photoprotection because the coral would be more susceptible to a temperature stress with higher light (Lesser and Farrell, 2004).

High light corals increased green fluorescence (~1.9x) and GFP concentration (~1.6x) in nearly a 1:1 relationship. However, there was a discrepancy between the ~1.9x decrease in green fluorescence and ~4.0x decrease in GFP concentration in the LL corals. It is possible that the decrease in green fluorescence in LL corals was offset by an increase in fluorescence due to: (1) strong scattering of the coral skeleton (Enriquez et al., 2005), and (2) the reduction in dinoflagellate density that would result in more transparent coral tissue. Thus, the net decrease in green fluorescence would be less than the actual decrease in GFP concentration. Alternatively, this discrepancy could be related to changes in other GFP homologs that are not fluorescent, but still immunoreactive. If the GFP homologs have the same molecular weight, such as observed following genetic manipulations of GFP *in vitro* (Bulina et al., 2002), then they would be impossible to detect separately on immunoblots.

While the expression of GFP varied with light level, the fluorescence emission properties of the protein were unaffected by the treatments. Corals that have multiple FPs can have FPs with differing responses where green, red, and non-fluorescent FPs increase in concentration while cyan FPs decrease under increasing light intensity (D'Angelo et al., 2008). The response of GFP to light intensity was similar in this study where GFP was observed in isolation compared to when GFP was observed concurrently with other colors of FPs (D'Angelo et al., 2008).

During the photoacclimation experiment, there was no evidence that the treatments caused any physiological stress. During both phases of the experiment, all of the corals appeared visibly healthy, grew in length, and showed no difference among treatments for the general marker of physiological health. No differences among treatments in absolute values of carotene and total xanthophylls, but small increases in the ratios of carotene and total xanthophyll pool to chlorophyll *a* slightly at the end of phase I, suggest that there was an unchanging capacity for photoprotection even though light harvesting capacity changed. Additionally, the ratios of peridinin and chlorophyll c_2 to chlorophyll *a* did not change, which suggest the proportions of antennae and cores of the photosynthetic apparatus remain unchanged. Moreover, the recovery of the maximum quantum yield after changing light conditions showed that the treatments did not induce any long-term damage in the symbionts. The light levels used in this experiment are well below maximum PAR irradiance measured on coral shallow coral reefs (Lesser et al., 1990; Lesser et al., 2000). Therefore, this studies shows that GFP concentrations change in the absence of

damaging light on the photosynthetic apparatus and reinforce the notion that GFP concentrations can be regulated as a photoacclimation strategy, and not a stress response.

Photoacclimation response by endosymbiotic dinoflagellates

As opposed to most previous coral symbiont photoacclimation studies, which have examined corals from a natural light gradient due to depth of occurrence, field transplant studies, seasonality, and outside tank experiments (Falkowski and Dubinsky, 1981; McCloskey and Muscatine, 1984; Titlyanov et al., 2001; Warner et al., 2002; Ulstrup et al., 2008), this study involved a highly controlled laboratory experiment with careful manipulations of light intensity. In contrast to the large magnitude of the changes in coral GFP concentration during the photoacclimation experiment, endosymbiotic dinoflagellates displayed relatively smaller photoacclimation responses despite a 30 fold difference in light intensity between HL and LL treatments. *A. yongei* is a shallow-water coral so both the coral and its symbionts would be exposed to high light conditions in its natural environment.

The largest photoacclimation response by the endosymbiotic dinoflagellates was in symbiont density, which decreased in LL, but did not change in the HL treatment as compared to the ML corals. The decrease in dinoflagellate density in the LL treatment led to extremely low symbiont numbers for a coral that is found in very high-light environments; this finding is consistent with previous studies (Titlyanov et al., 2001). A decrease in density may reduce self-shading and/or be a result of a

reduction in tissue biomass per unit surface area of the coral (Yellowlees and Warner, 2003). In the field, higher dinoflagellate density has been described on the upward facing regions of branches of corals as opposed to the downward facing portions (Dubinsky and Jokiel, 1994). In the present study under these laboratory conditions, dinoflagellate densities returned to level present in ML control corals during the 15 d of phase II of the experiment showing rapid photoacclimation. Significant changes in symbiont density can occur in 8 days, but may take up to 40 days to reach acclimation to a light decrease (Titlyanov et al., 2001).

Although the changes in light intensity between treatments were considerable, the photoacclimation response of the photosynthetic efficiency of PSII was small. Maximum quantum yield increased in low light corals and decreased in high light corals during phase I. Immediately after returning corals to original light conditions in phase II, the maximum quantum yield converged. The rapid photoacclimation of the HL corals during phase II provides evidence that the light treatment did not cause long-term photodamage. These results are consistent with seasonal and depth patterns of photochemical efficiency where corals under lower light levels, such as at greater depths and in the winter, have higher efficiency (Warner et al., 2002).

The observed changes in maximum quantum yield coincided with changes in photosynthetic pigments. An increase in photosynthetic pigments in cells under low light conditions, also consistent with previous field studies (Falkowski and Dubinsky, 1981; McCloskey and Muscatine, 1984; Porter et al., 1984), can result from either an

increase in size of the light harvesting antenna in the photosynthetic unit and/or the number of photosynthetic units (Chang et al., 1983; Iglesias-Prieto and Trench, 1994).

Intriguingly, the photoacclimatory responses of the endosymbiotic dinoflagellates *in vivo*, i.e., in the coral, were relatively minor compared to the photoacclimatory responses of endosymbiotic dinoflagellates *in vitro*, i.e., in culture. In this study, endosymbiotic dinoflagellates in the HL corals had 22% less chlorophyll *a* per cell than in the LL corals at the end of phase I. In contrast, various strains of cultured endosymbiotic dinoflagellates have 25-83% (depending on clade) less chlorophyll *a* per cell in HL than LL treatments (Iglesias-Prieto and Trench, 1994; Robison and Warner, 2006; Hennige et al., 2009). Additionally, those experiments had a smaller difference between their light treatments (6x) than what was used in this study (30x). In the present study, endosymbiotic dinoflagellates in the HL corals had 39% less chlorophyll c_2 per cell and 31% less peridinin per cell than in the LL corals, while dinoflagellates in culture have 51% less chlorophyll c_2 per cell and 52% less peridinin per cell than the LL culture (Iglesias-Prieto and Trench, 1994). Furthermore, in the present study the HL corals had 7% less maximum photochemical efficiency than the LL corals at the end of phase I. Dinoflagellates in culture have 22% (average of four clades) less maximum photochemical efficiency (Robison and Warner, 2006). The light environment within the coral host is most likely different than the environment in culture, yet experiments in culture may provide some valuable insights on the capacity the dinoflagellate photosynthetic system can develop in response to changes in ambient light. Here, we showed that the endosymbiotic dinoflagellates in

corals displayed the classic down-regulation of PSII as evidenced by the reduction in photochemical efficiency, similar to that documented in previous photoacclimation studies in *Symbiodinium* (Warner et al., 2002; Hennige et al., 2009). Responses of the photosynthetic pigment and photochemical efficiency observed in our study were reduced compared to those described from the literature in culture (Iglesias-Prieto and Trench, 1994; Robison and Warner, 2006; Hennige et al., 2009), which may be some indication that the coral host may actually provide a buffer from the otherwise dramatic changes in light levels experienced by the dinoflagellates. Our study thus supports the hypothesis that coral GFP may modify the internal light environment through influencing not only the intensity of light, but also the light spectrum surrounding the dinoflagellate.

The concentration of coral blue and orange absorbing GFP-like proteins, similar to GFP, are positively correlated with light intensity (Dove et al., 2008). During the onset of thermal bleaching, orange absorbing GFP-like protein levels are correlated with maximum photosynthesis of endosymbiotic dinoflagellates (Dove et al., 2008). To investigate further the possibility of the coral host and GFP affecting photosynthesis in this study, the ratio of photosynthetic pigments were examined in comparison to endosymbiotic dinoflagellate experiment in culture. Chlorophyll *a*, chlorophyll c_2 and peridinin are important photosynthetic pigments, which have distinct absorption properties. GFP absorbs blue light, and consequently has the potential to play a photoprotective role for pigments that absorb the same light wavelengths. While all three pigments absorb blue light, chlorophyll *a*, and to a lesser

extent chlorophyll c_2, also absorb red light (Falkowski et al., 1990). Surprisingly, there were no differences in the ratios between chl c_2 : chl *a* and per : *chl a* among the treatments at the end of phase I. In contrast, endosymbiotic dinoflagellates in culture decrease the relative amount of peridinin and chlorophyll c_2 to chlorophyll *a* (Iglesias-Prieto and Trench, 1994; Hennige et al., 2009). If endosymbiotic dinoflagellates maintain relatively more peridinin and chlorophyll c_2 to chlorophyll *a* under HL than dinoflagellates in culture, then there is a greater opportunity for GFP to play a photoprotective role. However, changes in photosynthetic pigments with irradiance are nonlinear (Sosik et al., 1989), and therefore further examination of this aspect is warranted. Overall, these scenarios suggest that the coral host fluorescent proteins are regulated to influence symbiont photosynthesis.

Evaluation of GFP as a monitoring tool for coral health

Reef-building corals are under immediate threat from global climate change, which can cause the breakdown of the coral-algal symbiosis and lead to coral bleaching (Hoegh-Guldberg et al., 2007; Anthony et al., 2008). Scientists and managers alike are searching for new non-invasive tools that can detect coral stress. Because of the inherent visual nature of some FPs, there was an early interest in using them as a non-invasive indicator of coral health (Mazel, 1995; Myers et al., 1999). This study demonstrates that green fluorescence was correlated with GFP concentration in the coral and that GFP is dynamic (6-fold changes) within a ”normal” range in healthy corals. The high natural variability of fluorescence is however

correlated with environmental factors, which add to the complexity of using fluorescence as a coral health indicator at this stage, but warrants further characterization of such possibility.

As a start for such characterization, fluorescence change in corals has first to be associated with a measure of coral health under laboratory controlled stress and non-stressful conditions, in order to establish a reference scale for a biological or ecological interpretation in changes of fluorescence patterns. In the present photoacclimation study (representing controlled non-stressful conditions), there were no differences in the amount of conjugated ubiquitin amongst light treatments validating that the corals were not stressed, but also indicating that the observed changes in GFP were not reflective of changes in this stress protein. These changes in GFP could actually be linked to a more general metabolic balance because growth rates in corals correlated with GFP concentration as well as green fluorescence intensity. This experiment was conducted on a relatively short time scale, yet on longer time scales such as months to years, corals with lower growth rates may have reduced roles in reproduction or reef accretion. Thus, monitoring changes of coral fluorescence over time might provide good estimates of corals global health through large and long scale environmental changes. In conclusion, this study described the possible range of variation of GFP for a model coral under “normal” non-stressful condition. It also highlighted that there is a complex and intricate relationship between ambient light, GFP, and physiological and/or metabolic processes, requiring additional experimental investigation to exploring the use of fluorescence as a tool for

monitoring corals health. In particular, studies on the response of coral fluorescence to different environmental stressors will be instrumental in deciding if, how, and for which corals species, fluorescence could be used as indicator of coral health. Previous studies have shown that expression of GFP homologs is down-regulated with heat stress (Smith-Keune and Dove, 2008), which also decreases green fluorescence (Zawada and Jaffe, 2003). The results of laboratory studies can then be combined with measurements made directly in the field for further validation (or not) of fluorescence as a coral health indicator. However, in order to take into consideration GFP-like proteins and other coral/symbiont pigments that do not necessarily fluoresce but that provide color to the coral, development of an underwater hyperspectral imaging system, used for both fluorescence and white light imaging, would provide more comprehensive and integrated optical measurements, possibly for better assessment on coral health.

Implications for the function of GFP

The present study suggests that GFP can function to change coral host physiology. Because of the rapid changes of GFP concentration in response to increases and decreases in light level, the coral could be regulating GFP to modulate the internal light environment. Both fluorescent proteins and their non-fluorescing homologs are correlated with light intensity (this study, Takabayashi and Hoegh-Guldberg, 1995; D'Angelo et al., 2008; Dove et al., 2008), suggesting that light absorption is a critical aspect of the GFP function in the corals. The dissipation of

high-energy light would be beneficial for both the coral host and the endosymbiotic dinoflagellates. The changes of GFP were observed on a <5 day time scale; in comparison with changes of dinoflagellate photosynthetic pigment photoacclimation that occur in 2-4 days and changes in symbiont densities that occur in 8-40 days (Titlyanov et al., 2001). The dinoflagellate photoacclimation response was relatively minor in comparison to the coral photoacclimation response, suggesting that the coral GFP may buffer the dinoflagellates from external light variations. This finding is consistent with the use of GFP-like proteins in some corals to regulate photosynthesis of dinoflagellates at the onset of thermal bleaching (Dove et al., 2008). Coral photoacclimation involves a hierarchy of responses, where the primary acclimation response is by the host coral and does not involve changes in photoprotective pigments of the endosymbiotic dinoflagellates. Under stressful conditions, outside this range, the dinoflagellate responses also become significant. Additionally, short time scale dinoflagellate responses are important, such as photoprotective xanthophyll cycling, which operates in minutes. GFP absorbs high-energy blue light, which can damage PSII directly and inhibit the repair of PSII through the production of reactive oxygen species (Nishiyama et al., 2006). Oxidative stress is particularly damaging for the coral-dinoflagellate symbiosis and in extreme cases can result in coral bleaching. FPs, like antioxidants, are regulated by blue light (D'Angelo et al., 2008). This study supports a model whereby GFP levels in shallow-water corals are regulated to buffer dinoflagellates from light variation creating an optimal environment for photosynthesis, which in turns leads to a fixed carbon source for the coral. Such

photoacclimation may be an important mechanism for corals to reduce oxidative stress and to withstand the stressors of climate change. Advances in methodologies that would allow molecular and/or genetic manipulations of corals, such as FP gene knockdown and small interfering or silencing of RNA from specific FPs, considered in combination with acclimation and stress experiments, may help elucidate the functions of fluorescent proteins in corals.

LIST OF ABBREVIATIONS

chl *a*	chlorophyll *a*
chl c_2	chlorophyll c_2
FP	fluorescent protein
F_v/F_m	variable fluorescence / maximum fluorescence
FWHM	full width at half maximum
GFP	green fluorescent protein
HL	high light treatment
ML	medium light treatment
LL	low light treatment
PAM	pulse amplitude modulated
per	peridinin
PSII	photosystem II

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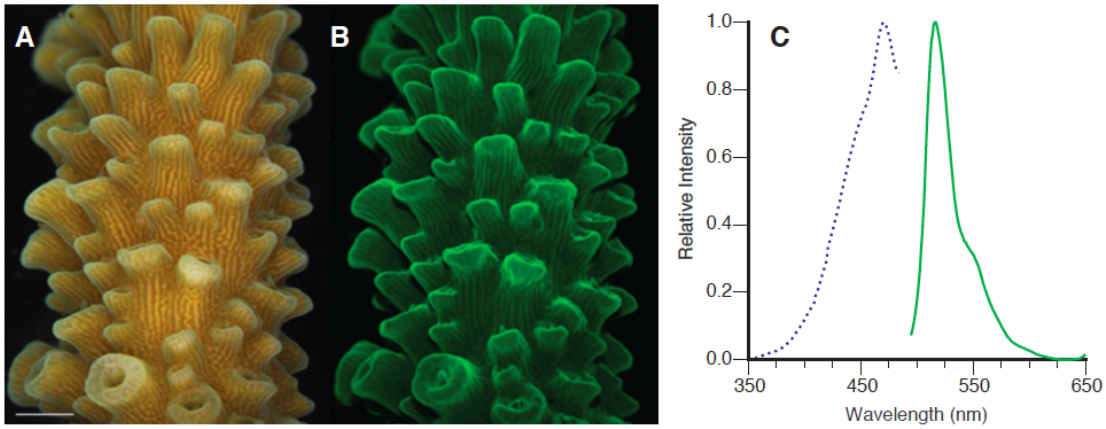


Figure 2-1. Optical properties of *Acropora yongei*. Images with (A) white light illumination and (B) blue light excitation (470 nm) with a longpass filter (transmission > 500 nm). Scale bar represents 2 mm. (C) Spectral characteristics showing the excitation spectrum (dotted line) of emission at 517 nm, and emission spectrum (solid line) with 470 nm excitation. The excitation peak is 470 nm and the emission peak is 517 nm with a FWHM of 26 nm.

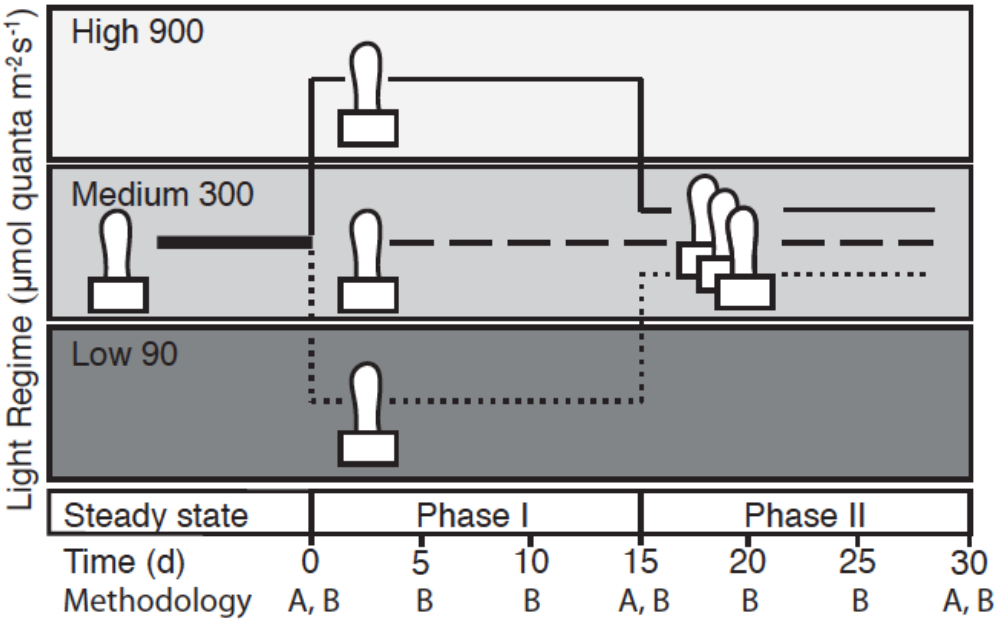
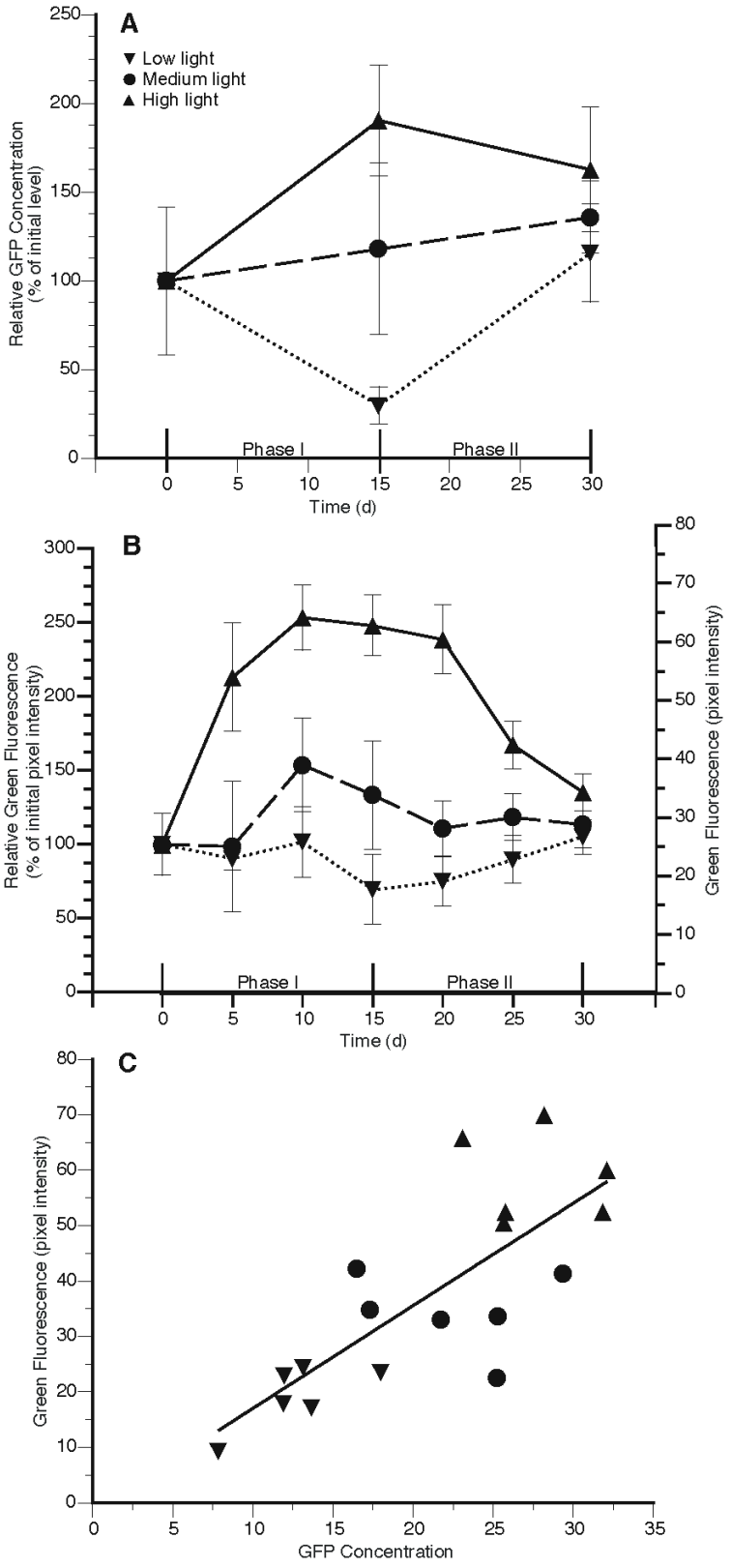


Figure 2-2. Schematic of the photoacclimation experiment design. At the beginning of the steady-state phase, corals were fragmented and placed under medium light for 2-4 weeks. At the beginning of phase I, corals were divided into three light intensity treatments for 15 d. At the beginning of phase II, corals were returned to medium light for 15 d. Methodologies included in group A are quantitative Western Blots, dinoflagellate density, and photosynthetic pigment concentration. Methodologies included in group B are fluorescence emission, green fluorescence intensity, frequency distribution of green fluorescence, maximum quantum yield of PSII, and growth rate.

Figure 2-3. Photoacclimation of the green fluorescent protein of *Acropora yongei*. (A) GFP concentration represented as treatment means ± SD (*N*=5-6) as relative to initial level at 0 d. (B) Green fluorescence pixel intensity represented as treatment means ± SD (*N*=40 for 0 d, *N*=11-12 for 5-15 d, *N*=5-6 for 20-30 d). (C) Relationship between GFP concentration (refer to methodology) and green fluorescence. The line represents the least-squares linear regression of fluorescence intensity vs. GFP concentration.


A
Low light
Medium light
High light
Relative GFP Concentration (% of initial level)
Phase I
Phase II
Time (d)
B
Relative Green Fluorescence (% of inittal pixel intensity)
Green Fluorescence (pixel intensity)
Phase I
Phase II
Time (d)
C
Green Fluorescence (pixel intensity)
GFP Concentration

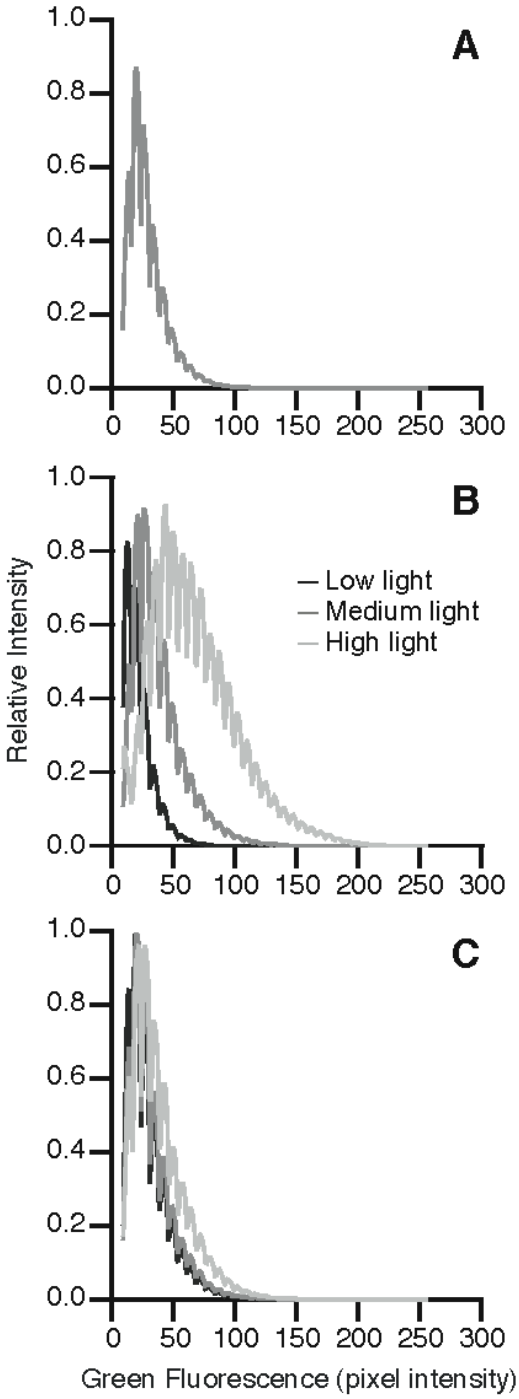


Figure 2-4. Frequency distribution of green fluorescence intensity of *Acropora yongei*. Average pixel intensity frequency distribution from images taken of corals at (A) the end of steady-state phase (0 d, *N*=40), (B) end of phase I (15 d, *N*=11-12), and (C) end of phase II (30 d, *N*=5-6).

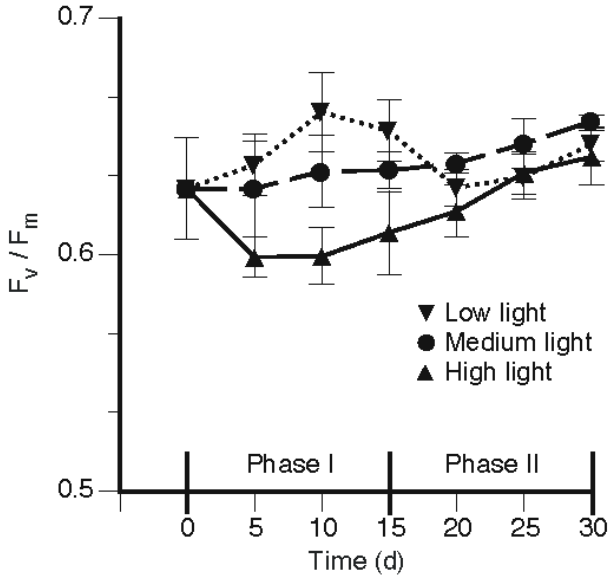


Figure 2-5. Photoacclimation of the photochemical efficiency of PSII of *Acropora yongei*, measured as pre-dawn maximum quantum yield (F_v/F_m). Values are means ± SD (*N*=38 for 0 d, *N*=11-12 for 5-15 d, *N*=5-6 for 20-30d).

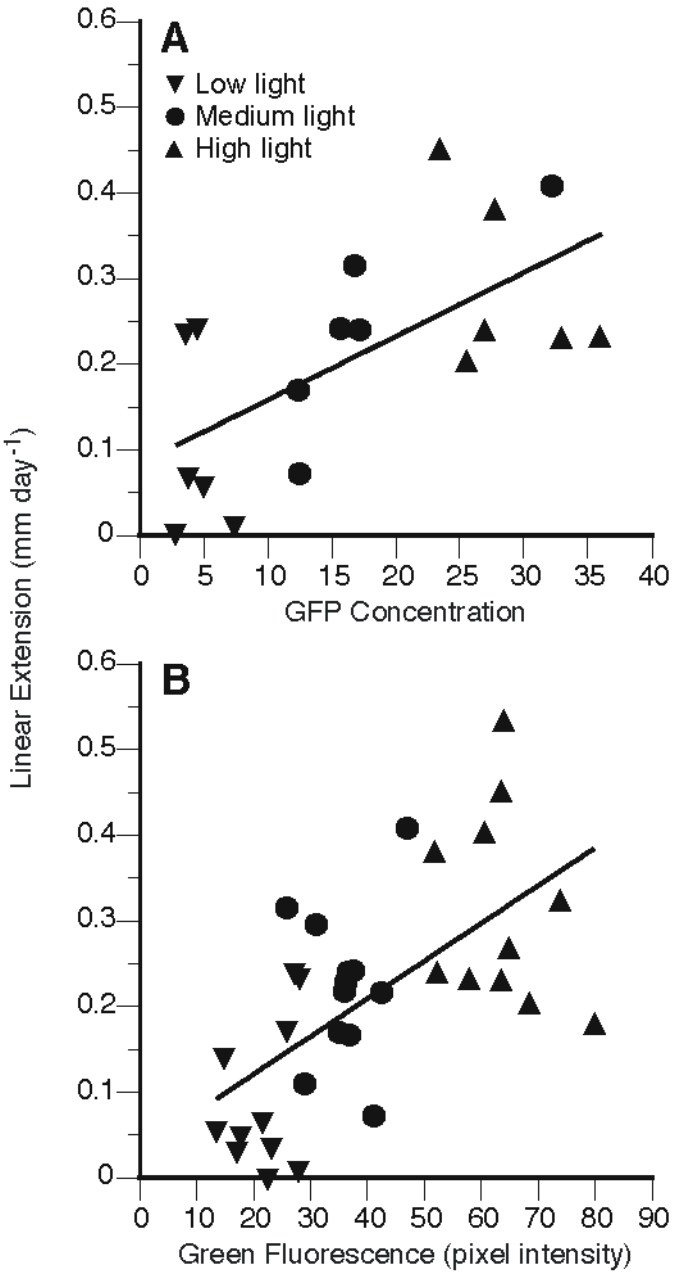


Figure 2-6. Positive correlations between green fluorescent protein and coral growth rate. Lines represent simple correlations between linear extension (from 10-15 d) and (A) relative GFP concentration at 15 d, and (B) green fluorescence at 15 d.

Table 2-1. Summary of analysis of variance (ANOVA) statistics to test differences among treatment groups at the end of phase I (15 d) and phase II (30 d). Post-hoc pairwise groupings represent the results from Tukey pairwise comparisons with differences $P<0.05$.

Parameter	Phase	Day	F statistic	Degrees of freedom	*P*	Post-hoc pairwise grouping		
						LL	ML	HL
Coral								
GFP concentration	I	15	219.3	2,15	***	A	B	C
GFP concentration	II	30	17.6	2,13	0.06			
Green fluorescence pixel intensity	I	15	117.7	2,31	***	A	B	C
Green fluorescence pixel intensity	II	30	6.9	2,13	**	A	A	B
Frequency distribution of green fluorescence intensity peak	I	15	73.2	2,31	***	A	B	C
Frequency distribution of green fluorescence intensity peak	II	30	6.6	2,13	*	A	A	B
FWHM	I	15	0.1	2,29	0.94			
FWHM	II	30	0.6	2,13	0.57			
Growth rate	I	15	14.2	2,31	***	A	B	B
Growth rate	II	30	0.6	2,13	0.66			
Conjugated ubiquitin concentration	I	15	0.3	2,15	0.95			
Conjugated ubiquitin concentration	II	30	1.3	2,13	0.80			
Dinoflagellate								
Maximum quantum yield of PS II	I	15	30.0	2,31	***	A	B	C
Maximum quantum yield of PS II	II	30	5.4	2,13	*	A,B	A	B
Dinoflagellate density	I	15	106.8	2,15	***	A	B	B
Dinoflagellate density	II	30	120.7	2,13	**	A	A,B	B
Chl *a*	I	15	4.8	2,15	*	A	A	B
Chl *a*	II	30	1.3	2,13	0.30			
Chl c_2	I	15	6.2	2,15	*	A	A,B	B
Chl c_2	II	30	2.1	2,13	0.16			
Chl c_2 : chl *a*	I	15	2.2	2,15	0.14			
Chl c_2 : chl *a*	II	30	1.5	2,13	0.27			
Peridinin	I	15	5.8	2,15	*	A	A	B
Peridinin	II	30	1.1	2,13	0.35			
Peridinin : chl *a*	I	15	2.1	2,15	0.15			
Peridinin : chl *a*	II	30	0.3	2,13	0.77			
Carotene	I	15	1.4	2,15	0.27			
Carotene	II	30	1.5	2,13	0.27			
Carotene : chl *a*	I	15	13.2	2,15	***	A	A	B
Carotene : chl *a*	II	30	0.2	2,13	0.83			
Dd + Dt	I	15	0.2	2,15	0.80			
Dd + Dt	II	30	1.7	2,13	0.23			
Dd + Dt : chl *a*	I	15	13.6	2,15	***	A	A	B
Dd + Dt : chl *a*	II	30	1.0	2,13	0.40			

* $P<0.05$

** $P<0.01$

*** $P<0.001$

Table 2-2. Dinoflagellate density and photosynthetic pigment composition from *Acropora yongei* during photoacclimation experiment. Values are treatment means (s.d.) (*N*=5-6).

	Pre-experiment (0 d)	Phase I (15 d)			Phase II (30 d)		
Light Regime	ML	LL	ML	HL	LL	ML	HL
Density ($x10^6$ cells cm^{-2})	2.76 (0.56)	1.95 (0.47)	3.04 (0.43)	2.89 (0.39)	2.49 (0.42)	3.32 (0.75)	3.61 (0.68)
Chl *a* (pg $cell^{-1}$)	2.51 (0.36)	2.54 (0.26)	2.55 (0.42)	1.98 (0.39)	2.35 (0.43)	2.06 (0.37)	2.03 (0.18)
Chl c_2 (pg $cell^{-1}$)	0.59 (0.11)	0.74 (0.18)	0.67 (0.16)	0.45 (0.09)	0.67 (0.15)	0.56 (0.12)	0.53 (0.07)
Chl c_2: chl *a*	0.24 (0.05)	0.29 (0.06)	0.26 (0.03)	0.23 (0.05)	0.28 (0.02)	0.26 (0.02)	0.26 (0.04)
Per (pg $cell^{-1}$)	1.45 (0.22)	1.69 (0.38)	1.67 (0.25)	1.17 (0.23)	1.52 (0.28)	1.35 (0.24)	1.32 (0.13)
Per : chl *a*	0.58 (0.01)	0.66 (0.11)	0.66 (0.03)	0.59 (0.01)	0.65 (0.02)	0.66 (0.01)	0.65 (0.01)
Carotene (pg $cell^{-1}$)	0.08 (0.01)	0.08 (0.01)	0.07 (0.01)	0.07 (0.01)	0.07 (0.01)	0.07 (0.01)	0.07 (0.00)
Carotene : chl *a* $x10^{-2}$	3.27 (0.11)	3.01 (0.28)	2.76 (0.19)	3.36 (0.09)	3.01 (0.28)	3.25 (0.15)	3.24 (0.07)
Dd + Dt (pg $cell^{-1}$)	0.44 (0.06)	0.42 (0.08)	0.43 (0.07)	0.40 (0.06)	0.40 (0.07)	0.34 (0.06)	0.34 (0.03)
Dd + Dt : chl *a*	0.18 (0.00)	0.17 (0.02)	0.17 (0.00)	0.20 (0.01)	0.17 (0.00)	0.17 (0.00)	0.17 (0.02)
Dt / (Dd + Dt) $x10^{-2}$	1.53 (0.19)	1.56 (0.25)	1.05 (0.17)	1.66 (0.39)	1.07 (0.27)	1.05 (0.12)	1.04 (0.13)

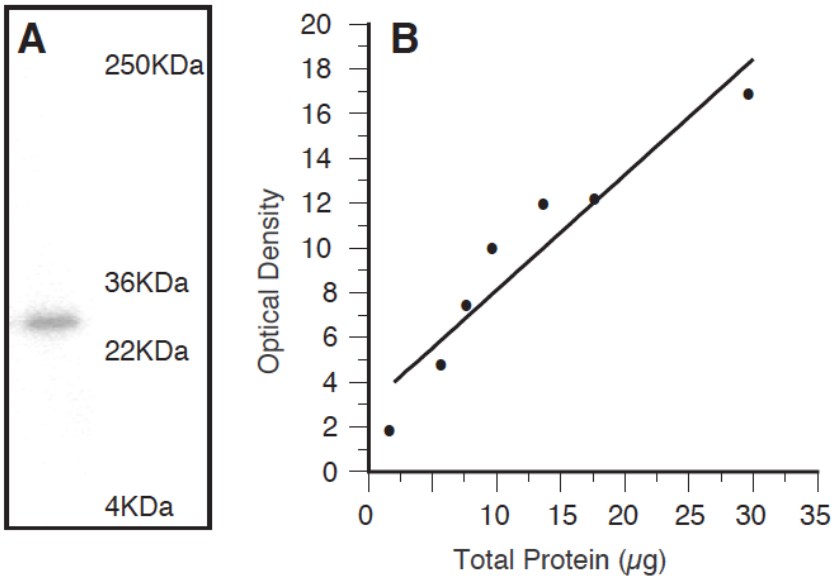


Figure 2-A1. Quantitative Western Blot methodology**.** (A) Immunodetection of the GFP custom polyclonal antibody showing a high specificity for the ~27 kDa protein. (B) Representative standard curve from a GFP Western Blot ($F_{1,5}$=44.6, P<0.01, R^2=0.90). The equation of the line, y = 2.97 + 0.52x, was then used to determine the relative GFP concentration for the samples on the Western Blot.

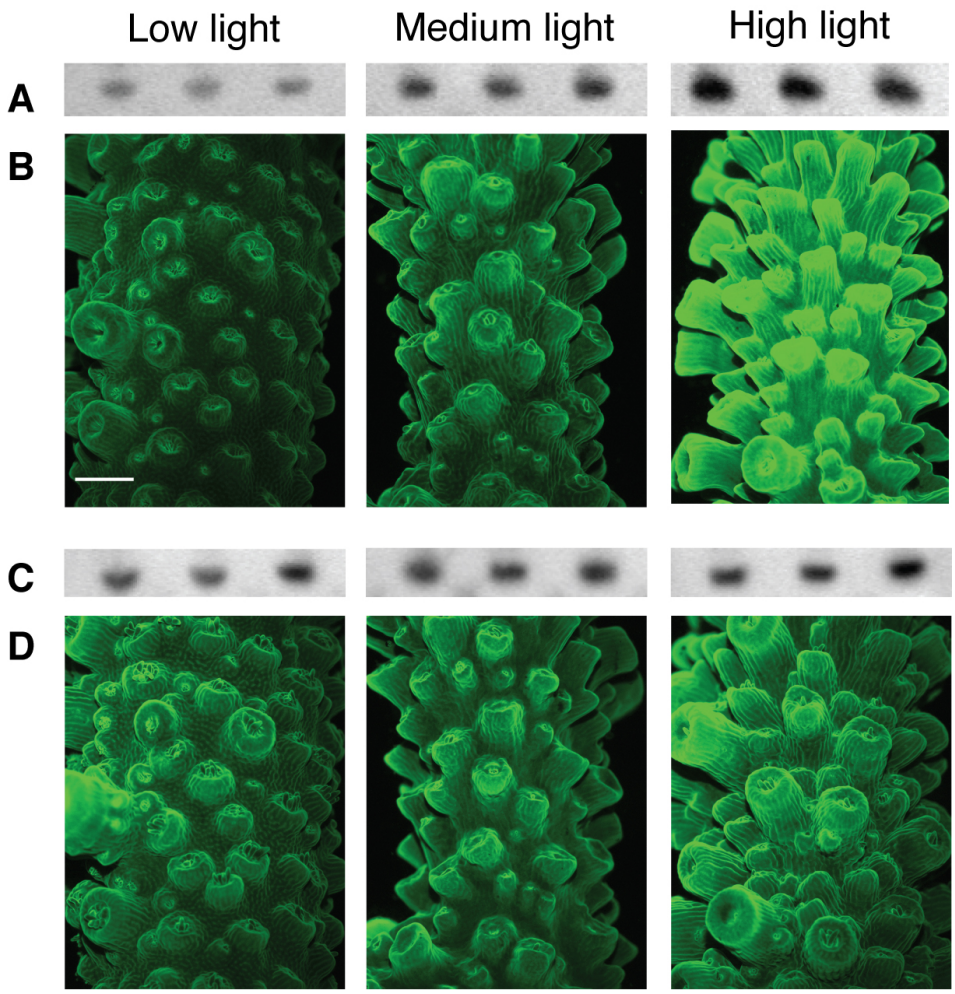


Figure 2-A2. Representative GFP expression and fluorescence in *Acropora yongei*. (A,C) GFP Western Blots and (B,D) fluorescence images from (A,B) the end of phase I (15 d), and (C,D) end of phase II (30 d). Images were taken under identical settings. Scale bar represents 2 mm.

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CHAPTER III

Effects of cold and heat shock on the photophysiology of the coral *Acropora yongei*

ABSTRACT

Global climate change and in particular thermal anomalies cause profound effects on coral physiology and often lead to coral mortality. The effects of cold and heat shock were investigated on a reef-building coral and its endosymbiotic dinoflagellate. A 20 d thermal shock (±5ºC) experiment was conducted on the branching coral *Acropora yongei*. Initially the cold treatment corals showed a stronger response with larger reductions in coral growth, green fluorescent protein (GFP) abundance, and electron transport rates, and increased pressure to photosystem II and xanthophyll cycling compared to heat treatment corals. However, the changes by the cold shock corals stabilized and even reversed by the end of the experiment. In contrast, the health of the heat treatment corals, after a short delay, steadily declined throughout the experiment and eventually bleached. Heat treatment corals had continual decreases in growth, dinoflagellate density, electron transport rates, and increases pressure to photosystem II and xanthophyll cycling. Coral bleaching in the heat treatment and paling in the cold treatment resulted from a decrease in dinoflagellate density rather than a loss of photosynthetic pigments. Rapid decreases in GFP abundance in both cold and heat treatment corals suggest that the protein was expended or actively degraded, and GFP abundances recovered by the end of the experiment. Large decreases in dinoflagellate density caused an apparent increase in green fluorescence intensity, which did not correspond to an increase GFP abundance. This study demonstrated that both cold and heat shock reduce the coral growth rate, and that ultimately the heat shock was more deleterious for the coral holobiont.

Because of global climate change, thermal anomalies may become more frequent and intense having severe repercussions on the immediate coral physiology and the long-term stability of coral reefs.

INTRODUCTION

Global climate change has profound effects on coral reef ecosystems (Hoegh-Guldberg, 1999; Hoegh-Guldberg et al., 2007; Baker et al., 2008; Riegl et al., 2009). Changes in sea surface temperature and ocean pH have the potential to be principal drivers of continued coral reef degradation (Hoegh-Guldberg, 1999; Hoegh-Guldberg et al., 2007; Baker et al., 2008; Riegl et al., 2009). Scleractinian corals are sensitive to environmental changes and in particular to increases and decreases in seawater temperature (Brown and Suharsono, 1990; Coles and Fadlallah, 1991). The coral-algal symbiosis is a key component to the survival and success of reef-building corals, but is disrupted by changes in temperature that can lead to the disassociation of the relationship between the coral and endosymbiotic dinoflagellates (Muscatine and Porter, 1977; Gates et al., 1992; Hoegh-Guldberg, 1999). Because the dinoflagellates provide significant amounts of energy to its host and enhance coral calcification (Goreau, 1959; Muscatine, 1990), the separation can cause declines in coral health and even coral mortality (Brown and Suharsono, 1990; Hoegh-Guldberg, 1999). In addition to the changes in temperature, coral bleaching can be influenced by solar irradiance, turbidity, flow and salinity (Brown and Suharsono, 1990; Glynn, 1996; Lesser, 1996; Lesser and Farrell, 2004; Nakamura et al., 2005).

The number of coral bleaching events has been increasing since the 1980's and the frequency and intensity is expected to increase (Hoegh-Guldberg, 1999; Baker et al., 2008). Bleaching events are well correlated with warm seawater temperatures, and specifically thermal anomalies such as those that occur as part of the El Nino-Southern

Oscillation (ENSO), Pacific Decadal Oscillation, and Indian Ocean Dipole (Brown and Suharsono, 1990; Glynn, 1990; Glynn, 1996; Hoegh-Guldberg, 1999; Jokiel and Brown, 2004; McClanahan et al., 2007). Laboratory bleaching experiments due to increasing seawater temperature as well as bleaching events in the field have shown that coral bleaching can result from a decline in dinoflagellate density, in photosynthetic pigments, or a combination of these factors (Coles and Jokiel, 1978; Hoegh-Guldberg and Smith, 1989; Warner et al., 1996). Under conditions of elevated seawater temperature and high irradiance, the breakdown of the coral-dinoflagllate symbiosis is caused by oxidative stress damage to the photosynthetic apparatus: the reaction center (D1 protein) of photosystem II (PSII), the Calvin cycle, and/or the thylakoid membranes, which generates large amounts of reactive oxygen species (Lesser, 1996; Jones et al., 1998; Hoegh-Guldberg, 1999; Warner et al., 1999; Takahashi et al., 2004; Tchernov et al., 2004). This oxidative stress leads to host cell apoptosis or exocytosis (Gates et al., 1992; Lesser, 1997; Franklin et al., 2004; Lesser and Farrell, 2004).

Corals elicit a number of physiological responses under heat stress. With elevated seawater temperatures, corals increase biochemical markers such as heat shock proteins, antioxidants, and protein-denaturing proteins (Downs et al., 2000; Lesser and Farrell, 2004; Fitt et al., 2009), decrease tissue thickness (Ainsworth et al., 2008), and decrease mucus production (Fitt et al., 2009). Under heat stress, coral DNA can become damaged and the expression of genes that modulate damage increase (Lesser and Farrell, 2004). Overall physiological process also decline including

growth, calcification, and reproduction (Jokiel and Coles, 1977; Jokiel and Guinther, 1978; Goreau and Macfarlane, 1990; Meesters and Bak, 1993). The responses however can be highly variable amongst corals since some species and color morphs of corals are more sensitive to increased seawater temperatures, as are some clades and sub-clades of symbiotic dinoflagellates (Jokiel and Coles, 1977; Goreau and Macfarlane, 1990; Dove et al., 2006; Venn et al., 2006; Abrego et al., 2008; Fitt et al., 2009).

In general, symbiotic dinoflagellates are more susceptible to heat stress than their coral hosts (Strychar and Sammarco, 2009), and therefore often become a source of oxidative stress for its host (Yakovleva et al., 2009). Responses of dinoflagellates to increased seawater temperature include reductions in density, photosynthetic pigment concentration, maximum photosynthesis, photosynthetic efficiency, and D1 concentration (Hoegh-Guldberg and Smith, 1989; Warner et al., 1996; Warner et al., 1999; Lesser and Farrell, 2004; Rowan, 2004; Venn et al., 2006; Fitt et al., 2009); and increases in photoprotective xanthophyll cycling (Dove et al., 2006; Venn et al., 2006). Moreover, the repair processes of PSII are inhibited by elevated temperatures under light (Takahashi et al., 2004).

In contrast to the widespread and numerous observations of warm water bleaching events (Baker et al. 2004), many fewer cold-water bleaching events have been observed. The reefs of Florida, Bahamas, Persian Gulf and more recently Australia have all had large-scale bleaching from decreased seawater temperatures, which often caused massive coral mortality (Roberts et al., 1982; Coles and Fadlallah,

1991; Hoegh-Guldberg and Fine, 2004; Hoegh-Guldberg et al., 2005). Global climate change affects the frequency and intensity of thermal anomalies (Urban et al., 2000), which may generate more cold-water events. There have only been a few cold shock experiments on reef-building corals. Jokiel and Coles (1977) observed decreased growth and calcification rates with lower seawater temperatures. Muscatine and co-authors (1991) were able to induce bleaching in many species of corals hours after subjecting them to a cold shock. Corals can bleach due to cold shock by the same mechanisms as by heat shock including detachment of host cells containing dinoflagellates (Gates et al., 1992). A bleaching event during cold weather in Australia showed a decrease in the effective quantum yield (EQY) of bleached compared to healthy coral tissue, but no difference in maximum quantum yield (MQY) (Hoegh-Guldberg et al., 2005). Endosymbiotic dinoflagellate responses to decreased temperatures include decreases in dinoflagellate density and photosynthetic efficiency, and changes photosynthetic pigment concentrations (Muscatine et al., 1991; Saxby et al., 2003); like responses to heat stress, the responses to cold stress are dependant on irradiance levels (Saxby et al., 2003).

Because of the role of light in thermal stress, photoprotective mechanisms of both the coral and dinoflagellate can have important functions in reducing the potential for oxidative stress. The highly reflective coral skeleton enhances light levels within coral cells (Enriquez et al., 2005), helping to maintain high rates of photosynthesis but also exacerbating the effects of thermal stress and the reduction of dinoflagellates and/or photosynthetic pigments. Dinoflagellates have photoprotective

pigments as part of their photosynthetic system including carotenoids and xanthophylls, which dissipate excess light energy (Demmig-Adams, 1990; Ambarsari et al., 1997). Additionally, dinoflagellates have mycosporine-like amino acids (MAAs) that absorb ultraviolet radiation and can be transferred to the coral (Dunlap and Chalker, 1986; Shick et al., 1995; Demmig-Adams and Adams, 1996; Shick, 2004). Furthermore, both corals and dinoflagellates have antioxidant defenses to counteract the reactive oxygen species generated during oxidative stress (Lesser, 2006).

Corals make fluorescent proteins (FPs), which have the potential to be photoprotective because they absorb high energy photosynthetically active radiation (PAR) and emit lower-energy wavelengths of light (Kawaguti, 1944; Salih et al., 2000). Coral FPs are homologues to the green fluorescent protein (GFP), originally isolated from jellyfish and currently pervasive tool in biomedical research (Tsien, 1998). Corals produce a variety of FPs with different spectral characteristics (Alieva et al., 2008), which factor into the color pigmentation of corals (Dove et al., 1995; Dove et al., 2001; Labas et al., 2002; Oswald et al., 2007). Corals can have FPs that do not emit light, called pocilloporins or GFP-like homologues, which often causes dark pigmentation (Dove et al., 1995; Dove et al., 2001).

The presence of FPs in corals is ubiquitous throughout shallow-water reef building corals (Salih et al., 2000), and FPs can be present at high concentrations within corals cells (Leutenegger et al., 2007b). However, the functions of FPs are unknown and controversial. The principal hypotheses of FPs include photoprotection, antioxidant capacity, modulating the light environment, symbiont regulation and

innate immune response (Kawaguti, 1944; Salih et al., 2000; Dove, 2004; D'Angelo et al., 2008; Palmer et al., 2009b; Palmer et al., 2009a; Roth et al., in review). FPs are not energetically costly to produce (Leutenegger et al., 2007b) and mature FPs are stable at high temperatures *in vitro* (Tsien, 1998). During photoacclimation, corals increase FP concentration in response to increased light levels, suggesting a photoprotective function (Brown et al., 2002; D'Angelo et al., 2008; Dove et al., 2008; Roth et al., in review). Salih and colleagues (2000) observed a positive correlation between dinoflagellate density and fluorescence intensity in corals during a severe bleaching event, and they attributed the FPs to bleaching resistance during heat stress. However, in sea anemones, abundance of GFP has no effect on bleaching (Leutenegger et al., 2007a). Experimental heat shock in corals causes a decrease in GFP-like homologues abundance and gene expression (Dove et al., 2006; Smith-Keune and Dove, 2008), and a switch from primarily green to primarily orange fluorescence as corals near mortality (Zawada and Jaffe, 2003); however, the source of the orange fluorescence was not determined in that particular study and it is possible that it could have been phycoerythrin from endosymbiotic cyanobacteria instead of a FP (Lesser et al., 2004).

This study investigated the responses of a 20 d thermal shock, including both cold and heat treatments, in the coral *Acropora yongei*. A comprehensive suite of physiological parameters of both the coral and the dinoflagellate were explored. Coral measurements included growth, GFP abundance and GFP fluorescence. Dinoflagellate measurements included density, photosynthetic efficiency, rapid light curves, photosynthetic pigment concentrations, and neutral lipid concentrations. Both

treatments had severe effects on the coral physiology; the cold shock caused more immediate pressure on the coral holobiont, but the heat shock was ultimately more deleterious. Both increased and decreased seawater temperatures are expected in the near future due to changes associated with global climate change, and this study provided opportunities to compare the effects of heat and cold shock on the coral and its dinoflagellates, and specifically on the photosynthetic apparatus.

MATERIALS AND METHODS

Thermal shock experiment design

The common shallow-water branching coral *Acropora yongei* Veron and Wallace 1984, obtained from the Birch Aquarium at the Scripps Institution of Oceanography in San Diego, CA, USA, was used in this study. The photoacclimation of *A. yongei* and its endosymbiotic dinoflagellates has been well studied and gives a context for the changes observed in this experiment (Roth et al. in review). Moreover, *A. yongei* is an appropriate model organism for investigating fluorescence because it has intense green fluorescence with blue light excitation due to a GFP with an excitation peak of 470 nm and an emission peak of 516 nm and a full width at half maximum (FWHM) of 28 nm (Figure 2-1, Roth et al. in review).

The experimental design of this experiment was similar to the photoacclimation experiment described in Chapter II. Corals were cut into ~5 cm long fragments with all tips and side branches removed to obtain uniform experimental replicates, and were attached to terracotta tiles with cyanoacrylic adhesive. Each coral

was placed in an individual 1 L glass aquarium with two seawater inlet hoses with a combined flow rate of ~0.7 L min^{-1}. Corals were maintained under a 12:12 h light:dark photoperiod and an intensity of 300 µmol quanta m^{-2} s^{-1} (T5 Teklight with two Midday and two Aqua Blue+ 54 W Powerchrome fluorescent lamps, Sunlight Supply).

Prior to temperature shock, corals were fragmented and maintained in steady-state at 26.1 ± 0.4ºC for 16 d in their individual aquaria. There was no coral mortality during this period and tissue grew completely over the cut region within days. A 20 d thermal shock followed the steady-state phase. The temperature was manipulated to achieve a 5.2 ± 0.5ºC decrease in the cold shock treatment and a 5.3 ± 0.8ºC increase in the heat shock treatment. The temperature changes were introduced incrementally over a 5 h period starting 0 d at sunrise (when the lights turned on). During the experiment, non-destructive coral measurements including green fluorescence intensity, fluorescence emission, maximum quantum yield of PSII, effective quantum yield of PSII, and growth were conducted on 0 d, 5 d, 9 d, 12 d, 16 d, and 20 d. Sample sizes were N=15 (0-5 d), N=10 (9 d), N=5 (12-20 d) for the cold and control treatments and N=19 (0-5 d), N=14 (9 d), N=9 (12 d), N=4 (16-20 d) for the heat treatment. Due to time constraints, rapid light curves were conducted on a subset of corals (N=3-5) on 0 d, 5 d, 9 d, 12 d, and 20 d. A subset of samples (N=4-5) each time point was used for destructive analysis including GFP abundance, dinoflagellate density, photosynthetic pigment concentrations, and dinoflagellate lipid concentrations and sampled on 0 d, 5 d, 9 d, 12 d (heat treatment only), and 20 d. For destructive sets

of samples, corals were collected just before the end of the light phase, frozen in liquid nitrogen, and stored at -80ºC until further analyses.

Quantitative Western Blots (immunoblot)

Quantitative western blots were used to determine the protein abundance of GFP in the coral during the experiment. A 16 mm long piece of each experimental coral was used. The piece was 8 mm below the tip of the coral to avoid the growth region of the coral, which may have different physiology, and was the same portion of the coral used to obtain the green fluorescence optical measurement (see below). The protein extraction and quantitative western blot methodology were conducted as described in Chapter II. In brief, protein was extracted in a denaturing buffer and 10 mg of protein from each sample were run in triplicate on 96-well gels. Custom GFP primary antibodies produced based on coral peptide sequences were used (Roth et al. in review). Immunoblots were visualized with secondary antibodies with a peroxidase label (Assay Designs) and a chemiluminescence kit (Pierce Biotechnology), and scanned on a high performance blot imager (Typhoon 9410 Imaging Workstation, Amersham Biosciences). The optical density for each sample was measured and the adjacent background subtracted in image analyses software (ImageJ, NIH). The optical densities were then transformed into relative GFP concentration using a coral standard, and the data were log transformed for statistical analyses (Roth et al. in review, Chapter II).

GFP fluorescence intensity

Green fluorescence intensity was determined on live coral on a 16 mm long piece, 8 mm from the tip, prior to its use in immunochemistry. The coral was imaged with an epifluorescence stereoscope (Nikon SMZ1500 with light X-Cite Series 120 EXFO and filter cube with excitation 450-490 nm and longpass emission barrier >500 nm) coupled to a color digital camera (Retiga 2000R), as described in Chapter II. An exposure of 0.20 s was used for fluorescence images. Additionally images of the same field were taken under white light to use in image processing.

Images were processed in MATLAB 7.5 (Mathworks) as described in Chapter II. In brief, the average green fluorescence intensity was determined by taking the average pixel intensity of the coral (as determined by the white light image) in the green channel of the fluorescence image subtracted by the average background. The study in Chapter II showed that GFP abundance and GFP fluorescence were positively correlated (Figure 2-3).

Fluorescence emission spectrum

The fluorescence emission spectrum was measured on the live coral fragment using the same 16 mm long section, 8 mm from the tip, as used in the green fluorescence measurement and immunochemistry. The fluorescence emission spectrum was measured with a low-light Echelle Spectrograph (SE200 Digital Spectrograph, Catalina Scientific) and a fiber optic probe as described in Chapter II. The spectrograph was calibrated prior to each day of measurements using a mercury

and tungsten lamp (Ocean Optics) to ensure its resolution of 1 nm. The probe was placed a few millimeters from the coral under ultraviolet (379-401 nm), blue (450-490 nm), and cyan (426-446 nm) excitation light. The only fluorescent protein observed was GFP, and therefore the emission spectra captured under blue light excitation was used for further analyses. GFP emission peaks were characterized by the peak wavelength and the FWHM from the smoothed curves (KestrelSpec, Catalina Scientific). On a few occasions, the FWHM could not be clearly determined in the emission spectrum captured from blue light and therefore the cyan emission spectrum was used instead.

Dinoflagellate density, photosynthetic pigment and lipid concentrations

A 15 mm long section of the coral (24 mm from the tip) was used for dinoflagellate analyses. The coral tissue was removed with an artist's airbrush and filtered seawater. Samples were maintained on ice and under low light conditions to prevent degradation. The coral slurry was centrifuged at 1,500 r.p.m. at 4ºC for 10 min, the supernatant was removed, the dinoflagellate pellet was resuspended in filtered seawater and the process was repeated.

The dinoflagellate density was determined using a Neubauer ruled hemacytometer, and samples were run in triplicate. Samples were normalized to the surface area of the coral. The surface area of the underlying skeleton was determined by measuring the height and diameter with calipers and calculated using simple cylinder geometry. Any branch that grew ≥5 mm from the coral fragment was also

measured and the surface area added in the calculation.

Concentrations of photosynthetic pigments of the dinoflagellates were determined using high-pressure liquid chromatography (HPLC). The photosynthetic pigments of interest included chlorophyll a (chl *a*), chlorophyll c_2 (chl c_2), peridinin (per), carotene, and the xanthophylls diadinoxanthin (Dd) and diatoxanthin (Dt). Acetone extracted pigments were run on an Agilent 1100 series HPLC system (Agilent Technologies) as described in Chapter II. Data were normalized to dinoflagellate density and reported in pg $cell^{-1}$. Because of the large decline in the dinoflagellate density in the heat treatment, photosynthetic pigment results could not be obtained for the heat treatment at 12 d and 20 d.

Quantitative cytoplasmic neutral lipid droplet measurements were determined using the fluorescent dye nile red (NR), 9-diethylamino-5H-benzo[α]phenoxazine-5-one, which stains these types of lipids (Greenspan and Fowler, 1985; Greenspan et al., 1985; Cooksey et al., 1987; Elsey et al., 2007). Dinoflagellate cells were incubated with NR in acetone (final concentration 1.56 μg ml^{-1}) for 30 min and 576 nm fluorescence was measured with an excitation of 485 nm (Greenspan et al., 1985; Cooksey et al., 1987; Elsey et al., 2007). The linear range of cell concentration was determined prior to running the samples and all samples were run in this range. Quantitative neutral lipid assays were conducted in 96-well microplates and samples were run in triplicate, except for 10 heat shock samples, which were concentrated to meet in the linear range requirement of the assay, and therefore there was only enough sample for a single replicate.

Pulse amplitude-modulated fluorescence (PAM)

The effects of thermal shock on the dinoflagellate photosynthetic system were measured using a pulse amplitude-modulated (PAM) fluorometer (Walz Inc.). Effective quantum yield (EQY), maximum quantum yield (MQY), pressure over photosystem II (Q_m), and relative photosynthetic electron rates (RETR), calculated from chlorophyll fluorescence parameters, were determined.

The dark-acclimated MQY of PSII (F_v/F_m; F_v, variable fluorescence; F_m, maximum fluorescence) were measured pre-dawn as described in Chapter II. The measurement was taken ~1 cm below the tip of the coral on the same side of the coral as the spectroscopy and optical fluorescence measurements. The light-acclimated EQY of PSII ($\Delta F/F_m'$) were measured at the corals' midday in the same place as the MQY measurement. The pressure over PSII was determined as: $Q_m = 1 - [(\Delta F/F_m'$ at noon) / (F_v/F_m at pre-dawn)] (Iglesias-Prieto et al., 2004).

Rapid light curves were obtained on a subset of corals from each treatment (N=5). Rapid light curves (RETR vs. actinic light) were used to estimate various fluorescence parameters: relative maximum electron transport rate ($RETR_{max}$), alpha (slope of initial rise of the rapid light curve), and saturating irradiance (I_k). Rapid light curves were fit to a non-linear two-parameter model to obtain the parameter estimates (Frenette et al., 1993). In all samples, the model fit the data well with significant R^2 values (R^2>0.95, P<0.0001, data not shown). PAM PAR values were calibrated with a cosine radiometer (BIC, Biospherical Instruments Inc.).

Growth rate

Growth rates of the corals during the experiment were determined by linear extension of the tip of the coral. Digital images were captured with the stereoscope (Nikon SMZ1500) under white light with the coral positioned the same way in each image. The linear extension was measured from a landmark with image analyses software (ImageJ). The difference between two time points was obtained and divided by the number of days between the two images to calculate the average daily growth rate.

Statistical analyses

Statistical analyses were conducted using JMP version 8.0 (SAS Institute, Inc.). Data were tested for assumptions of normality and homoscedasticity, and data were transformed accordingly prior to analyses. Two-way analyses of variance (ANOVA) tests were used to test the effects of the thermal shock and time on GFP abundance, dinoflagellate density, dinoflagellate pigment concentrations, $RETR_{max}$, alpha, I_k, and dinoflagellate lipids (Table 3-1). Two-way nested ANOVA tests were used to test the effects of thermal shock and time with the coral nested for GFP fluorescence, growth rate, EQY, MQY, and Q_m (Table 3-1). In two-way ANOVA tests, the R^2 value indicates the percentage of variance that is accounted for by each predictor variable, as well as the interaction term. R^2 were calculated by dividing the sum of squares of the factor of consideration by the total of all sum of squares and the

residuals; therefore nested ANOVA R^2 values included the nested factor. All nested factors were not significant (data not shown). For all significant factors in the two-way ANOVA tests, post-hoc Tukey-Kramer HSD pairwise comparisons were used to test which groups were significantly different. Because of the significant decline in dinoflagellate density in the heat treatment at 20 d, neither the PAM measurements nor photosynthetic pigment concentrations were below detection limit of our instrumentation. Therefore, *t*-tests were used to test if the difference between control and cold treatment corals at 20 d was significant (Table 3-2). Equal variance was verified on each dataset, and if the variance was unequal then *t*-tests assuming unequal variance were used. Data represent arithmetic means ± standard errors. Statistical differences were significant at the α=0.05 level.

RESULTS

Coral response to thermal shock

The 20 d thermal shock treatment had a dramatic effect on the appearance of corals in both cold and heat treatments (Figure 3-1); however, no mortality or tissue loss in any treatment was observed. By the end of the experiment, corals in the cold treatment had paled slightly and heat treatment corals had bleached completely.

Thermal shock rapidly decreased the growth rates of corals in cold and heat treatments, which were significantly different than the control corals for 0-5 d; and the growth rates in treatments remained lower and significantly different than the control corals throughout the entire experiment (Figure 3-2). Upon closer examination of the

growth rates, there were some notable differences between the cold and heat treatments. Immediately after the shock was initiated, the growth was lower in the cold treatment than in the heat treatment. In cold treatment corals, the growth rate remained low until 16 d when the growth rate increased substantially and they had their fastest growth of the experiment from 16-20 d. In contrast, the growth rate of corals in the heat shock treatment gradually decreased throughout the experiment, and the growth at the end of the experiment was almost zero. The growth rates of the control corals ranged from 0.40-0.46 mm^{-1} day and therefore grew substantially throughout the 20 d experiment (Figure 3-2). The two-way ANOVA showed that both treatment and time were significant main effects, and that the interaction of treatment x time was significant (Table 3-1). However, treatment was the largest contributor (55%) to the variance. Post-hoc analyses showed that the response of the control treatment was significantly different than the response of the cold and heat treatments, but that the cold and heat treatments were not significantly different from each other.

A decrease in GFP abundance was observed in both cold and heat treatments, while an increase in GFP abundance was observed in control corals (Figure 3-3). At 5 d, GFP abundance was 35% less in the heat treatment and 65% less in the cold treatment as compared to the controls. For both thermal treatments a minimum GFP abundance was reached during 9-12 d, which resulted in a 91% decrease in the cold treatment as compared to the controls. The GFP abundance in both cold and heat treatments recovered slightly by the end of the experiment, yet levels were still lower than at 0 d. The overall pattern of decrease and recovery in GFP abundance was

similar between cold and heat treatments. The two-way ANOVA showed that both the treatment and time were significant main effects, and that there was a significant interaction between treatment x time (Table 3-1). Most of the variability in the experiment was explained by the experimental treatments (55%). Post-hoc analyses showed that the response for each treatment was significantly different and that 5 d and 9 d grouped together and 5 d and 20 d grouped together.

Green fluorescence of the corals initially showed a similar pattern of change to GFP abundance (Figure 3-1, Figure 3-4). GFP fluorescence was less in both thermal treatments at 5 d as compared to the controls, although in contrast to GFP abundance the cold treatment decreased more dramatically than the heat treatment. At 9 d, the cold treatment corals had a 79% decrease in green fluorescence as compared to the controls, and after the green fluorescence in the cold treatments began to recover. The GFP fluorescence in the heat treatment dramatically rose after 9 d, surpassing the control corals by 12 d. The GFP fluorescence in the control corals steadily rose throughout the experiment. The two-way ANOVA showed that both treatment and time were significant main effects, and that there was a significant interaction between treatment x time (Table 3-1). All three components accounted for the variability in the experiment, although treatment was the largest contributor (33%). Post-hoc analyses showed that the response for each treatment was significantly different and that 5 d and 9 d grouped together, 10 d and 12 d grouped together, while 16 d and 20 d were significantly different.

Despite the changes in abundance of fluorescent proteins, green fluorescence

was the only color of fluorescence observed in all treatments throughout the experiment. The average treatment emission peak ranged between 516 and 517 nm throughout the experiment, which is well within the resolution of the instrument. The average treatment FWHM ranged from 27-31 nm throughout the experiment, often depending on the height of the peak. These spectral characteristics match the GFP peak previously described in *A. yongei* (Chapter II, Figure 2-1).

Endosymbiotic dinoflagellate response to thermal shock

The endosymbiotic dinoflagellates, similar to the coral host, had a strong response to the thermal shock experiment and the dinoflagellates in the cold and heat treatments responded differently. While the dinoflagellate density of both thermal treatments decreased, the heat shock treatment corals bleached by 12 d (Figure 3-1, Figure 3-5). At 5 d of thermal shock, the heat and cold treatments exhibited the same 27% decrease in dinoflagellate density as compared to the controls. The heat shock treatment caused a dramatic decline (98%) from 5-12 d, and the low density leveled off from 12-20 d. The cold shock treatment corals decreased gradually throughout the experiment ultimately resulting in a 45% decrease at 20 d compared to control corals. The two-way ANOVA showed that both treatment and time were significant main effects, and there was a significant interaction of treatment x time (Table 3-1). All three components accounted for most of the variability in the experiment (98%), although the treatment factor was the largest contributor (40%). Post-hoc analyses showed that the response of each treatment was significantly different as well as each

time point.

The quantum yield of PSII showed interesting differences between the effects of thermal shock. The EQY, which represents the more immediate pressure on the system, showed an immediate decline and then leveling off in the cold treatment and a delayed but dramatic decline in the heat treatment (Figure 3-6a). By 5 d, the EQY decreased nearly 20% in the cold shock treatment and only 2% in the heat shock treatment. However, the cold shock leveled off with total 27% decrease by 20 d while the heat shock declined rapidly with a 85% reduction by 12 d (the last day measurement was possible in heat shock corals because of the declining signal as a result from the low dinoflagellate density, see above paragraph). The EQY in the control corals remained constant through the experiment. The two-way ANOVA showed that both treatment and time were significant main effects; the interaction between treatment x time was also significant (Table 3-1). All three components accounted for the variability in the experiment, although treatment x time was the largest contributor (30%). Post-hoc analyses showed that each treatment and each day was significantly different. At 20 d, the cold and control treatments were significantly different than each other (Table 3-2).

The response of MQY with thermal shock showed a similar pattern as the EQY (Figure 3-6b). At 5 d, there were slight decreases in the MQY in both cold and heat shock treatments as compared to the controls. The cold treatment corals continued to decrease, leveling off at 20 d with a 10% reduction in MQY. The heat treatment corals dramatically declined after 5 d, and reached a 47% decrease by 12 d, the last

measurable day. The MQY of the control corals remained constant throughout the experiment. The two-way ANOVA showed that both treatment and time were significant main effects, and there was a significant interaction between treatment x time (Table 3-1). All three variables contributed to the variability measured in the experiment (treatment: 33%, time: 23%, treatment x time: 32%). Post-hoc analyses showed that each treatment is significantly different, and that each time point was significantly different except for 0 d and 5 d, which grouped together. At 20 d, the MQY of cold shock and control treatments were significantly different (Table 3-2).

The pressure over PSII, Q_m, showed clearly the differences on the photosynthetic system between the two thermal treatments (Figure 3-6c). In the cold shock treatment, the pressure over PSII increased >2 fold from 0 d to 5 d, and then leveled off. In contrast, the heat shock treatment did not increase until after 5 d, where it increased dramatically until 12 d (the last day where measurement was possible), overall a 6.8 fold increase. The pressure over PSII of the control corals remained low throughout the experiment. The two-way ANOVA showed that both treatment and time were significant main effects, and the interaction between treatment x time was significant (Table 3-1). All three variables accounted for variability in the experiment, with the treatment x time interaction as the largest contributor (32%). Post-hoc analyses showed that each treatment and time point was significantly different. At 20 d, the cold shock and control treatments were significantly different (Table 3-2).

Accordingly, the rapid light curves also showed the effects of thermal shock and the differences between the cold and heat shock treatments (Figure 3-7). The

control treatment corals showed similar rapid light curves through time. The cold shock treatment showed a changed in rapid light curve at 5 d, with only subtle changes through time. The $RETR_{max}$ of cold treatment corals decreased immediately by 5 d, continued to decline to a minimum at 9 d, and then increased slightly by 12 d and 20 d (Figure 3-8a). The saturating irradiance (I_k) and alpha (slope of initial rise) showed similar patterns (Figure 3-8b,c). In contrast, the heat shock treatment showed a more complex pattern. At 5 d, the $RETR_{max}$ increased to 22% higher than the control corals, after which it rapidly declined to levels near the cold shock treatment at 9 d, and the below the cold shock treatment at 12 d (measurements at 20 d were not possible). I_k increased to 27% greater than the control corals at 5 d, similar to the $RETR_{max}$, then dropped substantially but remained above cold treatments I_k. Alpha decreased slightly at 5 d, and then decreased dramatically. In the control corals, $RETR_{max}$ and I_k showed small increases during the experiment, while alpha stayed relatively constant through time. The two-way ANOVAs of the $RETR_{max}$, I_k, and alpha showed that both treatment and time were significant main effects, as well was the interaction terms treatment x time were significant (Table 3-1). For all three ANOVAs, the treatment was the largest contributor to the variability of the experiment, followed by the treatment x time variable. Post-hoc analyses for all three ANOVAs showed that each treatment was significantly different and that 5 d was significantly different 9 d and 12 d. Additionally, the 20 d cold shock and control treatments were significantly different for $RETR_{max}$ (Table 3-2).

The thermal shock caused major changes in the dinoflagellate photoprotective

photosynthetic pigments and minor changes in the other photosynthetic pigments (Figure 3-9). In the cold and heat treatments, the total xanthophyll pool (Dd + Dt) increased ~20% in each as compared to the controls, and the ratio of total xanthophylls to chlorophyll *a* increased dramatically, more than 4x in the heat treatment (9 d). In the heat treatment, the relative component of the xanthophyll Dt increased throughout the entire experiment, while in the cold treatment it increased until 9 d and then decreased by 20 d. These changes showed significant effects of treatment and time and the interaction of treatment x time (Table 3-1). There were no significant differences between treatments in the abundance of chl *a*, chl c_2, per and carotene per cell. However, there were small but significant changes in the relative ratio of these pigments and chl *a*. There were no clear trends in ratios of chl c_2 : chl *a*; the ratios of per : chl *a* were higher in thermal shock treatments compared to controls at the end of the experiments; and the ratios of carotene : chl *a* were higher in the thermal shock treatments until 20 d when the ratio in the cold treatment decreased and it was no longer different than the control group (Table 3-2).

The neutral lipid concentration in dinoflagellates showed a response different from that of the other physiological parameters (Figure 3-10). The cold shock and control treatments did not change the amount of lipids per cell through time. The heat shock treatment increased the amount of neutral lipids in cell, particularly after 9 d. The two-way ANOVA showed that both treatment and time were significant main effects; additionally, the interaction between treatment x time was significant (Table 3-1). The treatment was the largest contributor to the variability of the experiment

(27%). Post-hoc analyses show that the heat shock treatment was significantly different than the control and cold shock treatments, and that 20 d was significantly different than 5 d and 9 d.

DISCUSSION

This study included simultaneous cold and heat shock experiments, which provided opportunities to compare the effects of cold and heat stress on corals and their symbionts. Corals were subjected to a significant change in temperature (±5ºC) for an extended period of time (20 d), but no corals experienced tissue loss or mortality. It is possible that the low level of light used in this experiment (300 μmol quanta m^{-2} s^{-1}) weakened the effects of the temperature shock, which were considerable but not lethal. Nevertheless, cold and heat treatments had clearly different effects on the coral and the symbiont and at different time scales. By the end of the experiment, the heat treatments corals had bleached, whereas the cold shock corals paled slightly. In both treatments, the bleaching primarily resulted from a decline of dinoflagellate density rather than a reduction in photosynthetic pigment concentration. Occasionally corals under cold shock (6 out of 15) secreted a white substance (Figure 3-1i), which was never observed in the heat treatment nor the control corals.

The coral growth rate is an overall indicator of the physiology of the holobiont. Thermal shock reduced the coral growth rate, but cold and heat treatments affected the corals differently. Corals in the cold treatment responded immediately to the decrease

in temperature; and the growth rate of the cold treatment was significantly smaller than the control group as well as the heat treatment during 0-5 d. The cold treatment reached its lowest growth rate during 5-9 d, but was able to recover by the end of the experiment, suggesting that the corals were able to somewhat acclimate to the temperature change. In contrast, the heat shock corals showed a steady decline in growth rate throughout the experiment suggesting that it is likely they would have died if the treatment continued. These experiments may suggest that the heat treatment was more deleterious than the cold treatment. Thermal shock of Hawaiian corals cause significant declines in growth and mortality (Jokiel and Coles, 1977). However, the cold shock was more harmful than heat shock, and moreover, mortality occurred more rapidly in heat shock corals than cold shock corals (Jokiel and Coles, 1977). Different species were used in their study than in this study and it is possible that some corals are living closer to their upper thermal limits while other corals are living closer to their lower thermal limits. Temperature has been shown to have different effects on photosynthesis and respiration in the corals of the same species from different locations (Coles and Jokiel, 1977), thus possibly explaining the fact that our results are different from the one similar study on this topic available in the literature.

The simultaneous cold and heat shock experiments combined with data collected throughout the 20 d helped time course elucidate the effects of heat and cold shock on corals. In the cold treatment the pressure over PSII increased directly and by 5 d had reached its maximum. As a result, the photosynthetic rates declined in the cold treatment and the corals were much closer to their saturating irradiance. The continued

cold treatment did not cause further pressure over PSII throughout the rest of the experiment and the electron transport rates were able to stabilize, perhaps because the irradiance in this experiment was lower than the saturating irradiance as determined by rapid light curves. The irradiance during short-term (30 h) cold shock has a large effect on the decreases in photosynthetic efficiency, but not on the dinoflagellate density (Saxby et al., 2003). In this study, the dinoflagellate density also declined gradually resulting in a 37% reduction at the end of the experiment, but this variation is with the seasonal range on a coral reef (Warner et al., 2002). A 4 h extreme cold shock (12-18ºC) causes dinoflagellate expulsion in 12 h (Muscatine et al., 1991), and in this experiment the greatest change in dinoflagellate density occurred during 0-5 d in cold shock. In this study, the dinoflagellates were perhaps able to compensate perhaps by changing the concentration of proteins or enzymes. The xanthophyll cycle works to protect PSII from excess excitation by the de-epoxidation of diadinoxanthin (Dd) to diatoxathin (Dt). The thermal shock caused an increase in the total xanthophyll pool, which increased the capacity for photoprotection, and the higher relative Dt component of the xanthophyll pool signified more xanthophyll cycling. By 20 d, the xanthophyll cycling decreased suggesting that the system was able to compensate and the pressure had reduced. If the light levels were any higher (>400 μmol quanta m^{-2} s^{-1}), the cold treatment corals may not have been able to acclimate because of incurring too much photodamage.

The heat treatment caused a very different photophysiological response than the cold response. Similar to many heat stresses a decrease in photosynthetic

efficiency and dinoflagellate density were observed (Hoegh-Guldberg and Smith, 1989; Warner et al., 1996; Warner et al., 1999; Fitt et al., 2009); however, the time course of this study provided many insights into the effects of heat on the coral and dinoflagellate. The pressure over PSII did not start to increase until after 5 d. At 5 d, the heat treatment corals actually showed increased photosynthetic rates and saturating irradiances, possibly a result of temperature on enzyme activity rates (Somero, 1995). However, perhaps damage was accumulating and the system was overwhelmed because after 5 d the pressure over PSII begin to rise steadily, and the photosynthetic rates and saturating irradiance begin to drop. The xanthophyll cycle responded to the heat stress, but not as quickly or as great of a changes as in the cold shock. The high xanthophyll cycling at 9 d confirmed the significant excitation pressure on the photosynthetic system. By 9 d, the electron transport rates dropped to the same levels at the cold treatment, by 12 d the rates have dropped to below the cold treatment, and by 20 d there was not a strong enough of a signal to get a measurement. The dinoflagellate density declined slightly by 5 d, and then dramatically until 12 d, resulting in a very low remaining dinoflagellate population. The visual appearance of the corals began to pale after 5 d, were bleached by 9 d and completely stark white 12-20 d. These results suggest that the symbionts under the heat treatment were under significant photostress and the coral-algal symbiosis was disrupted, most likely because of the dinoflagellates became a source of oxidative stress (Lesser, 1996; Lesser, 1997). Experiments in coral larvae under heat stress show that larvae with symbionts have higher antioxidant activities than those without dinoflagellates

(Yakovleva et al., 2009), suggesting that it may be less damaging for the coral without its symbionts during heat stress.

In contrast to the response of the dinoflagellates, the effect of thermal shock on GFP concentration in corals was similar between heat and cold treatments, and on similar time scales. In this study, heat and cold shock decreased GFP concentration in corals, which is consistent to what has been observed with the abundance and gene expression of GFP-like homologues under heat stress (Dove et al., 2006; Smith-Keune and Dove, 2008; Rodriguez-Lanetty et al., 2009). Here, the cold treatment reduced GFP concentration more than the heat treatment. The time scale of responses was similar between the two treatments; GFP concentration declined until ~9 d, and after which GFP concentration increased. The decline in GFP concentration under shock was faster than the decay rate of the FPs (half-life ~20 d, Leutenegger et al., 2007b) and more rapid than decreases in GFP concentration observed with decreases in light during acclimation (Roth et al., in review, Chapter II). The rapid reduction in GFP may suggest that the protein was actively degraded and/or depleted, possibly from antioxidant activities, as opposed to resulting from a stop in production. The similar pattern between heat and cold treatments could suggest that the host response to thermal shock was independent of the symbiont response, which differed in the cold and heat treatments, and possibly that the effects of thermal shock were more harmful for the dinoflagellate than the coral. In agreement, heat stress has been shown to affect the dinoflagellates before the coral host (Strychar and Sammarco, 2009).

The GFP fluorescence response to thermal shock was distinct from that of the

GFP concentration. GFP fluorescence in the cold treatment matched the GFP concentration response, showing a decrease in fluorescence that reached a low at 9 d and then increasing until the end of the experiment. In the heat treatment, however, GFP fluorescence declined until 9 d, at which point the fluorescence increased and surpassing the control treatment by 12 d and at the end of the experiment was more than 1.5 fold above the control. The cause of the apparent increase in GFP fluorescence was likely the bleaching of the coral, in which the dinoflagellate density decreased dramatically. Large decreases in dinoflagellate density may allow for more excitation light to reach GFP and/or the fluorescence emission of GFP to be less obstructed. The reduction in dinoflagellate density may cause more reflection and scattering by the coral skeleton and amplify the fluorescence. Therefore, these data suggest that the optical fluorescence of GFP can be affected by the presence versus absence of symbiotic dinoflagellates and their pigments.

In a heat stress experiment of *Montastrea faveolata,* the ratio of green (515 nm) to orange (575 nm) fluorescence switched when corals bleached and died (Zawada and Jaffe, 2003). Unfortunately it was not determined if the source of the orange fluorescence was from coral pigments or phycoerythrin from endosymbiotic cyanobacteria. In this study, fluorescence was only observed from GFP of corals and chlorophyll of the dinoflagellates. GFP clearly declined with thermal shock, however, other types of FPs may have different responses to thermal stress as well as different functions.

The GFP abundance and fluorescence in the control corals increased

throughout this experiment. This increase may be accounted for by the high growth rate (>0.4 mm day^{-1}), which over the course of the 20 d experiment means a minimum of ~8 mm growth in length. Because the measurements were always taken 8 mm from the tip of the coral, the measurements were shifted at each time point and often including an area of recent growth (Figure 3-1). However, these data support that the control corals very healthy throughout the experiment.

Because of global climate change, corals will experience more thermal anomalies that will cause physiological stress, have long-term repercussions on growth and reproduction, and increase coral mortality. This experiment suggests that for some corals, elevated seawater increases will be more deleterious than cold temperatures and that the dinoflagellate physiology is more sensitive to thermal shock than its host. At some cold temperatures, corals and their symbionts may be able to acclimate to changed environment allowing them to survive. Significant changes in coral fluorescence were observed prior to coral bleaching, suggesting that changes in coral fluorescence may be used as an early signal of coral stress.

LIST OF ABBREVIATIONS

chl *a*	chlorophyll *a*
chl c_2	chlorophyll c_2
Dd	diadinoxanthin
Dt	diatoxanthin

FP	fluorescent protein
Fv/Fm	variable fluorescence / maximum fluorescence
FWHM	full width at half maximum
GFP	green fluorescent protein
PAM	pulse amplitude modulated
PAR	photosynthetically active radiation
per	peridinin
PSII	photosystem II

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Acropora yongei". The dissertation author was the primary investigator and author of this paper.

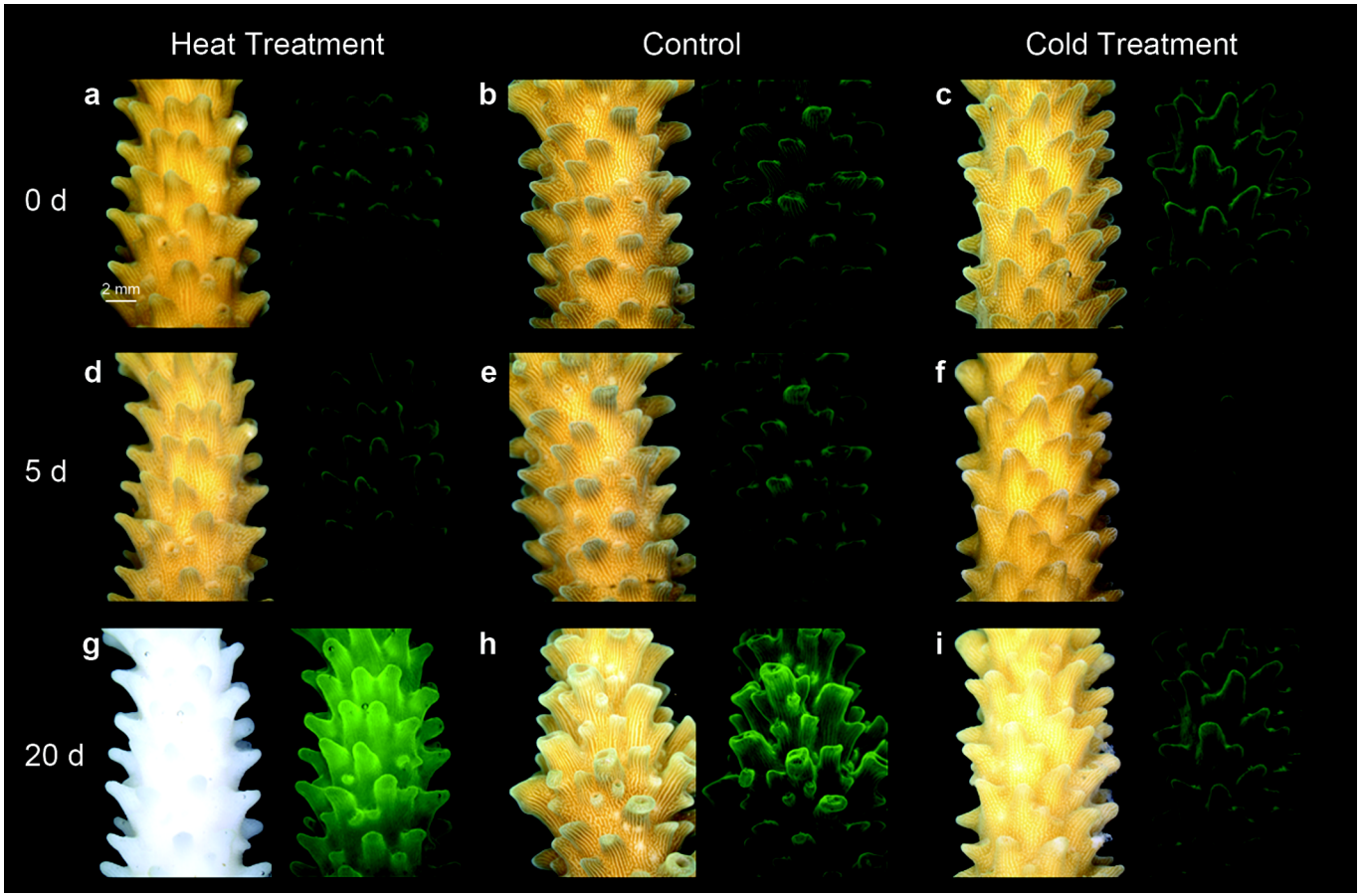


Figure 3-1. Representative *Acropora yongei* samples from the different treatments and time points during the thermal shock experiment. Each sample includes an image under white light (left panel) and blue light (excitation 470 ± 40 nm and longpass emission filter ≥500 nm; right panel); the same coral colony is shown through time. The treatments include heat shock (**a**, **d**, **g**), control (**b**, **e**, **h**), and cold shock (**c**, **f**, **i**) at 0 d (**a**, **b**, **c**), 5 d (**d**, **e**, **f**), and 20 d (**g**, **h**, **i**).

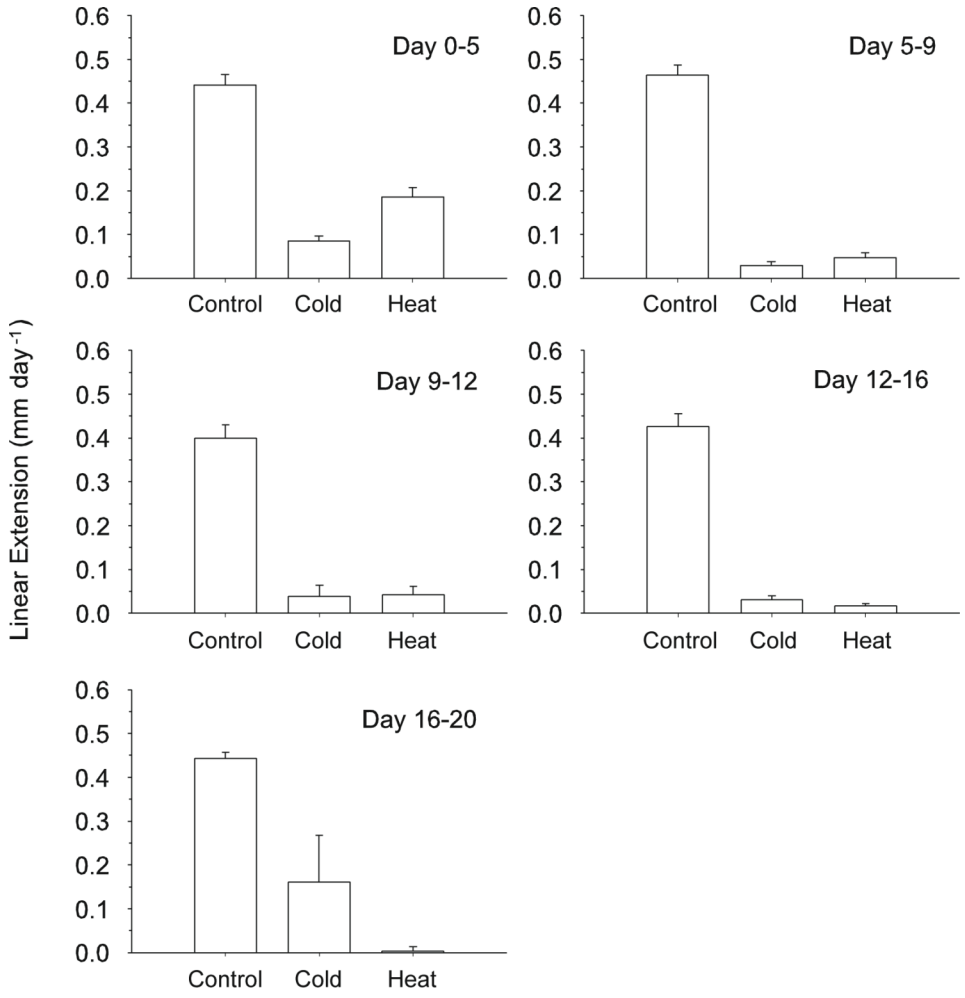


Figure 3-2. Effect of thermal shock on *Acropora yongei* growth rates. Values are means + SE. (cold shock and control treatments *N*=15 for 0-5d, *N*=10 for 5-9 d, *N*=5 for 9-12 d, 12-16 d, 16-20 d; heat shock treatment *N*=19 for 0-5 d, *N*=13 for 5-9 d, *N*=9 for 9-12 d, *N*=4 for 12-16 d, 16-20 d).

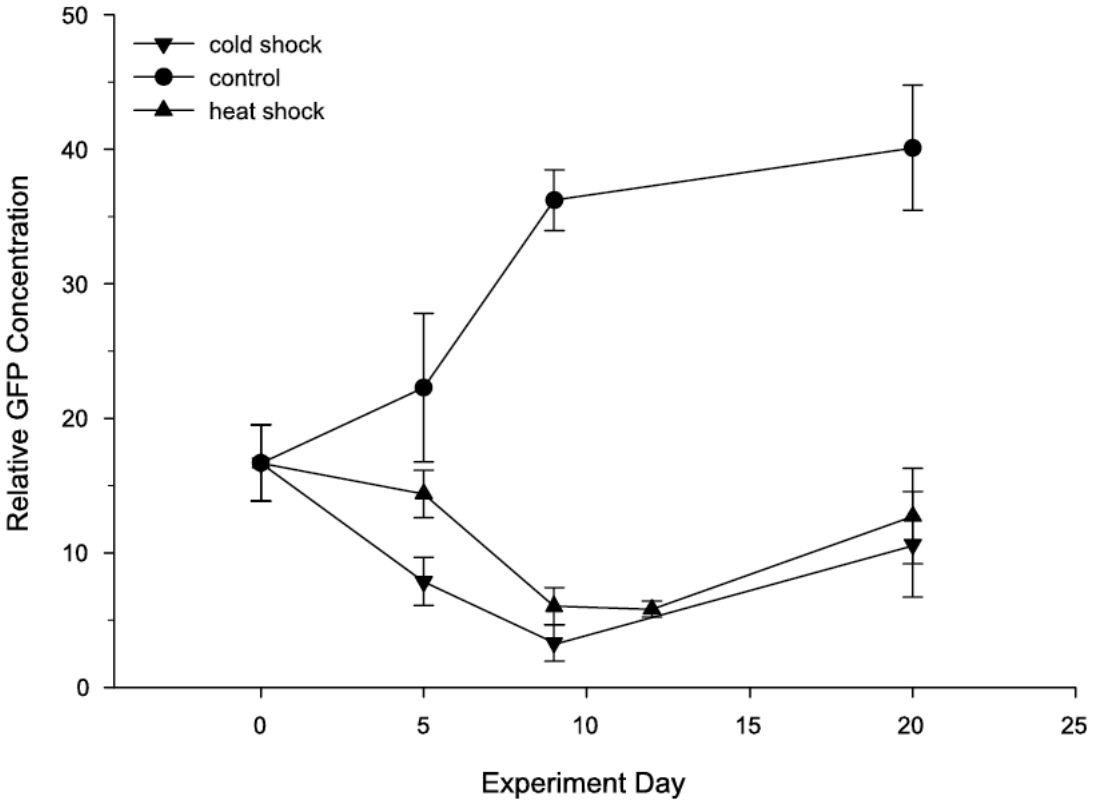


Figure 3-3. Effect of thermal shock on relative GFP concentration in *Acropora yongei*. Values are means ± SE (*N*=4-5).

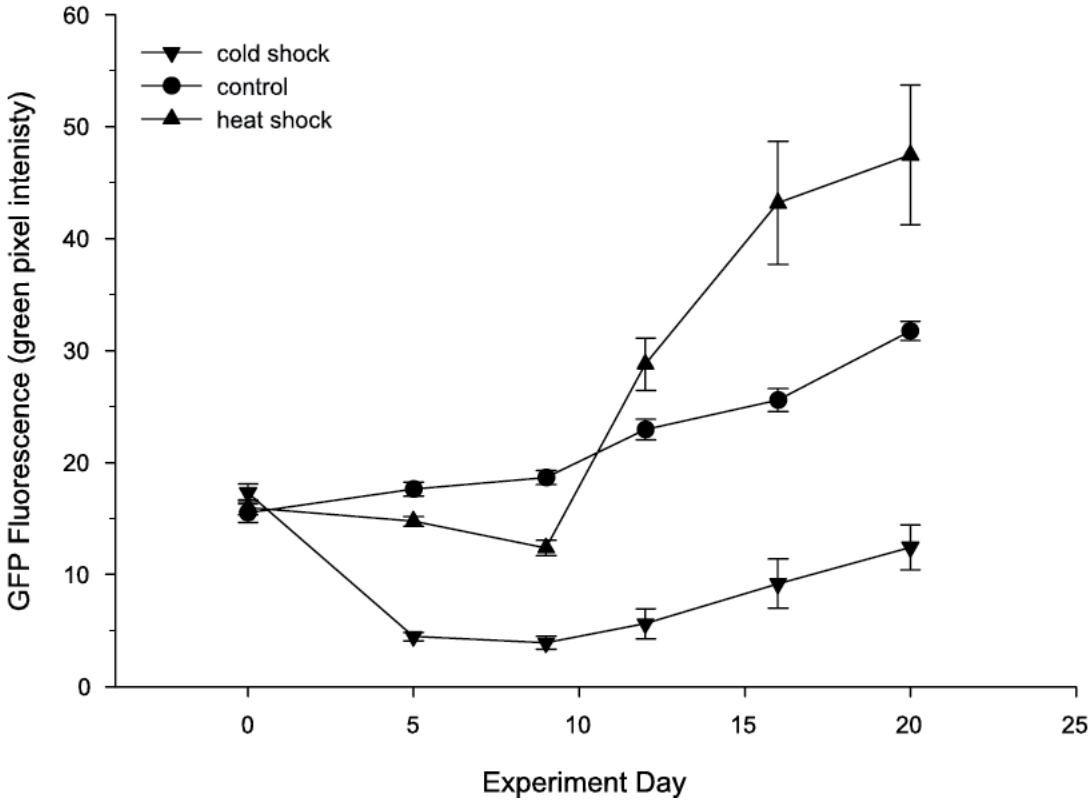


Figure 3-4. Effect of thermal shock on GFP fluorescence intensity (green pixel intensity) in *Acropora yongei*. Values are means ± SE (cold shock and control treatments *N*=15 for 0 d, 5d, *N*=10 for 9 d, *N*=5 for 12 d, 16 d, 20 d; heat shock treatment *N*=19 for 0, 5 d, *N*=14 for 9 d, *N*=9 12 d, *N*=4 for 16 d and 20 d).

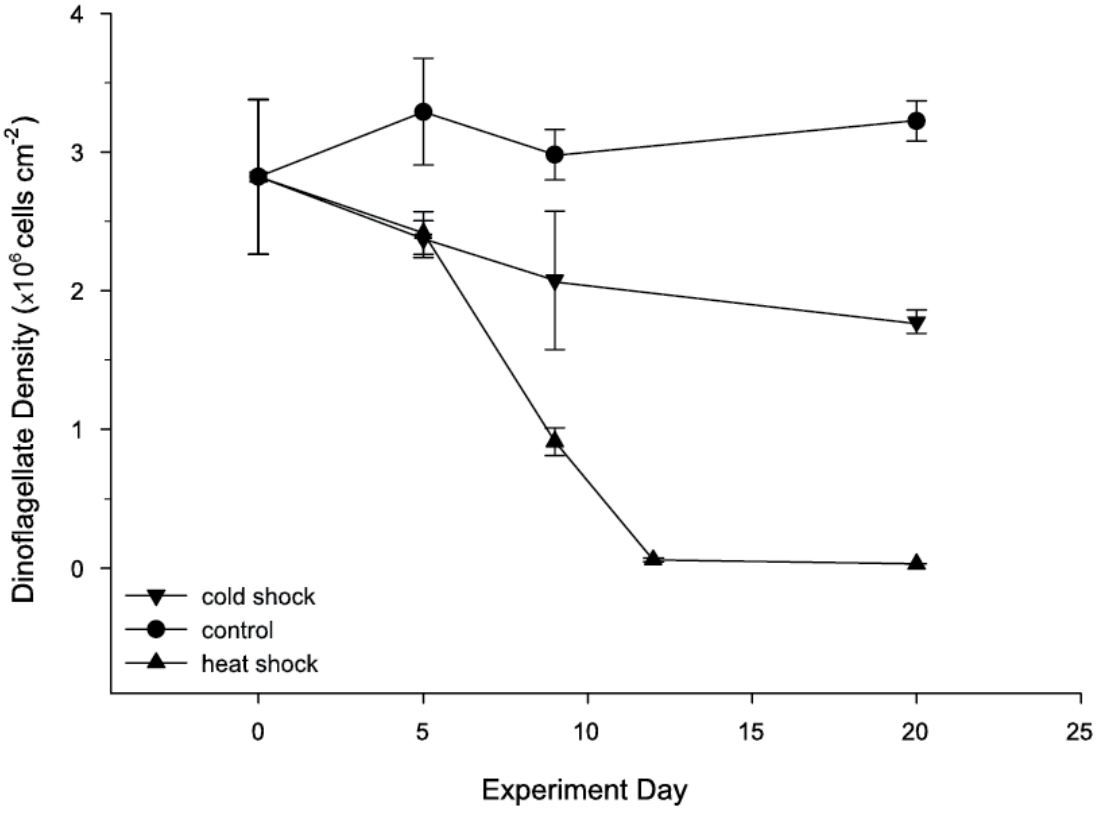
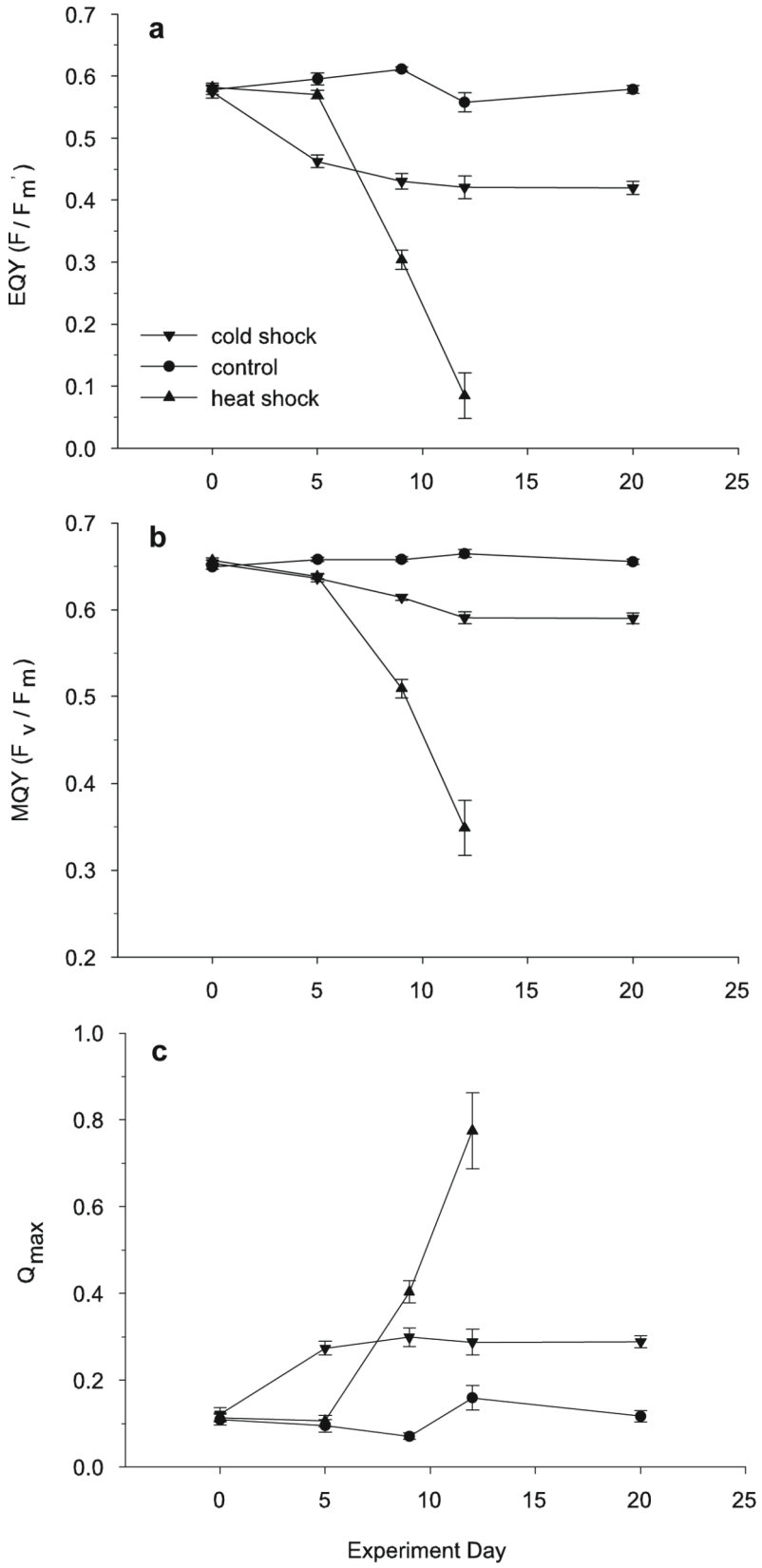


Figure 3-5. Effect of thermal shock on endosymbiotic dinoflagellate density in *Acropora yongei*. Values are means ± SE. (*N*=4-5).

Figure 3-6. Effect of thermal shock on photosynthetic efficiency in *Acropora yongei:* (**a**) effective quantum yield (EQY), (**b**) maximum quantum yield (MQY), and (**c**) pressure over photosystem II (Q_m). Values are means ± SE. (cold shock and control treatments *N*=15 for 0 d, 5d, *N*=10 for 9 d, *N*=5 for 12 d, 20 d; heat shock treatment *N*=19 for 0 d and 5 d, *N*=13 for 9 d, *N*=6 12 d, except for MQY where *N*=8 12 d).


a
b
c
EQY (F / F m ')
MQY (F v / F m)
Q max
cold shock
control
heat shock
Experiment Day
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
1.0
0
5
10
15
20
25

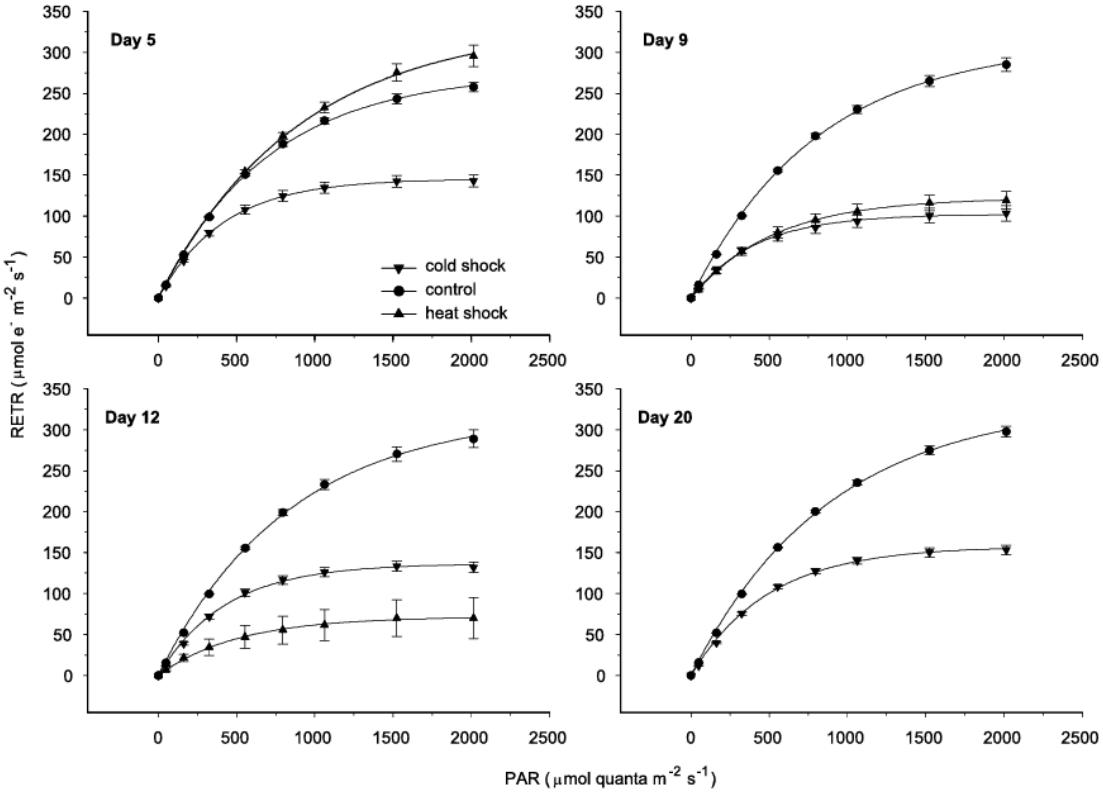


Figure 3-7. Effect of thermal shock on rapid light curves of *Acropora yongei*. Symbols represent means ± SE and curves represent models based on all data for each treatment. (*N*=5 for all treatments and time points, except for heat shock treatment 12 d where *N*=3).

Figure 3-8. Effect of thermal shock on photosynthetic parameters derived from rapid light curves of *Acropora yongei*: (**a**) $RETR_{max}$, (**b**) I_k (saturating irradiance), and (**c**) alpha. Values are means ± SE. (*N*=5 for all treatments and time points, except for heat shock treatment 12 d where *N*=3).

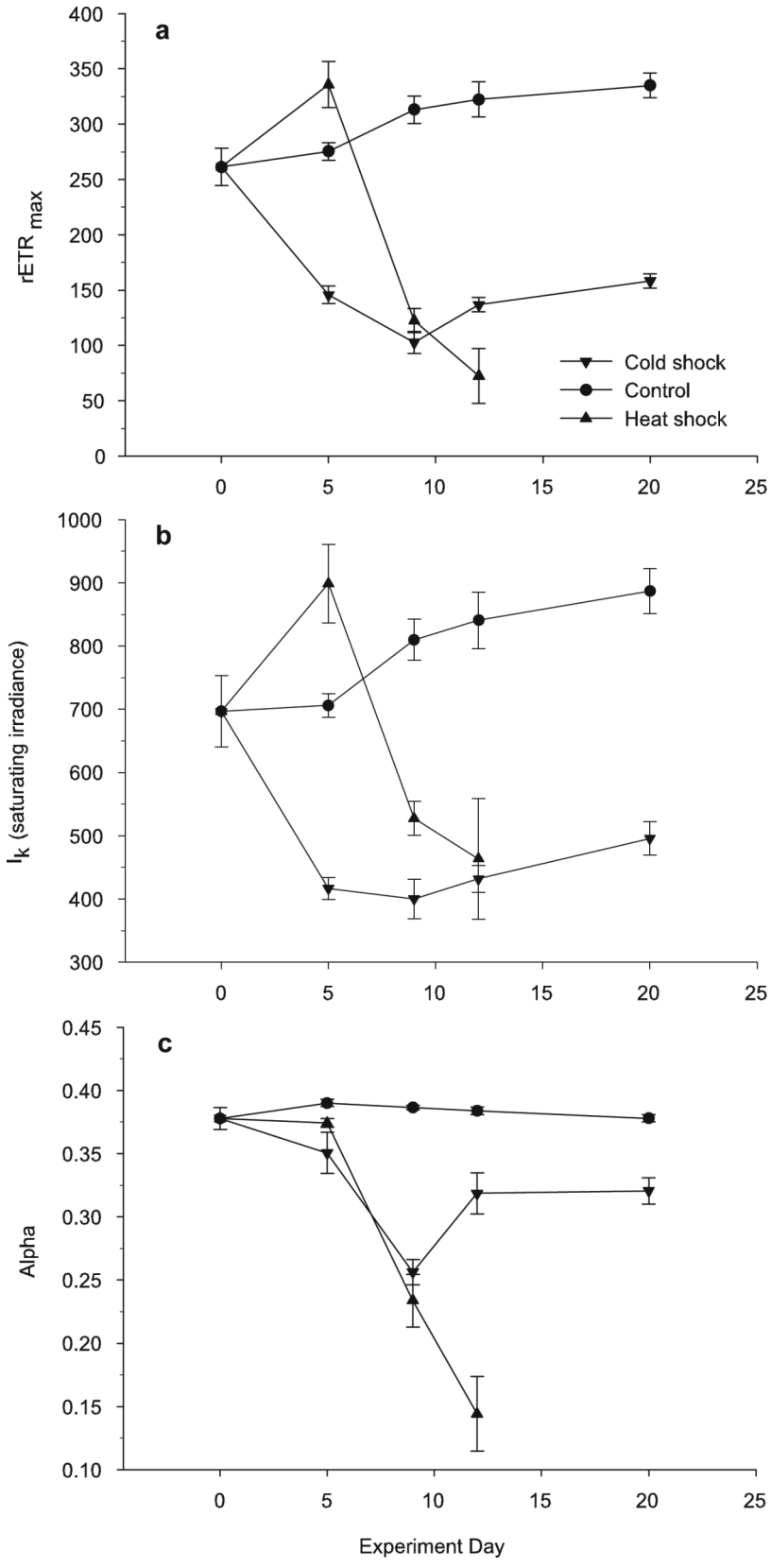
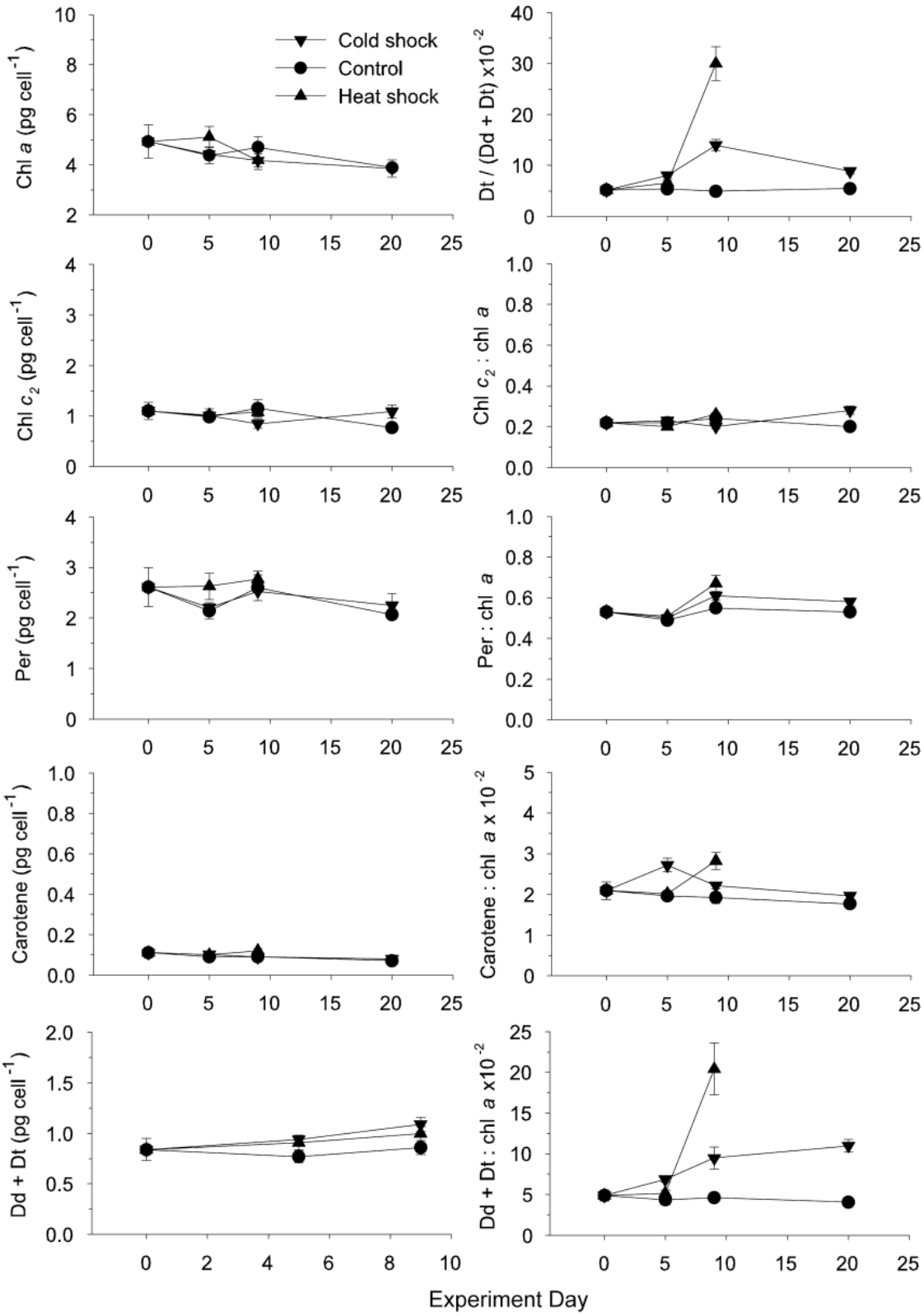

a
b
c
rETR max
Ik (saturating irradiance)
Alpha
Experiment Day
Cold shock
Control
Heat shock
400
350
300
250
200
150
100
50
0
1000
900
800
700
600
500
400
300
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0
5
10
15
20
25

Figure 3-9. Effect of thermal shock on photosynthetic pigment composition in *Acropora yongei*. Values are means ± SE (*N*=5). Heat treatment at 20 d were below detection limit of the methodology.


Cold shock
Control
Heat shock
Chl a (pg cell-1)
Dt / (Dd + Dt) x10-2
Chl c2 (pg cell-1)
Chl c2 : chl a
Per (pg cell-1)
Per : chl a
Carotene (pg cell-1)
Carotene : chl a x 10-2
Dd + Dt (pg cell-1)
Dd + Dt : chl a x10-2
Experiment Day

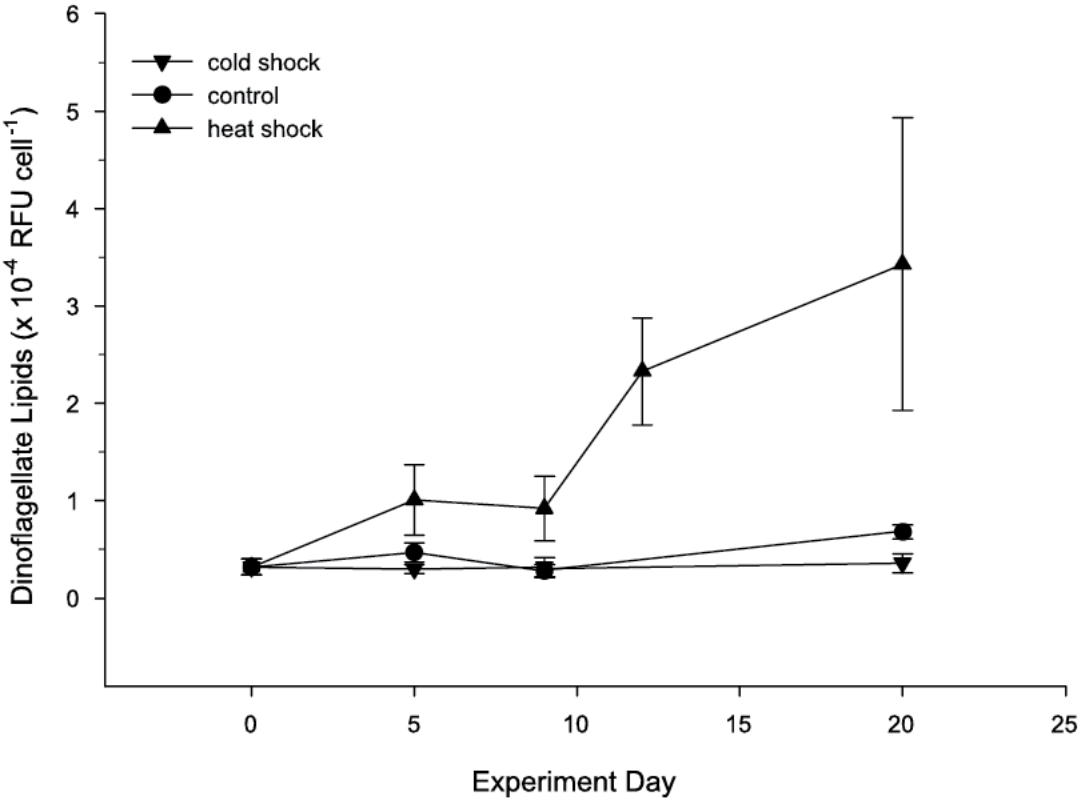


Figure 3-10. Effect of thermal shock on lipid concentration of endosymbiotic dinoflagellates in *Acropora yongei*. Values are means ± SE (*N*=4-5).

Table 3-1. Summary of two-way analysis of variance (ANOVA) statistics to test the effects of thermal treatment and time during the 20 d thermal shock experiment on *Acropora yongei*.

Parameter	Treatment			Time			Treatment x Time		
	F statistic	*P*	R^2	F statistic	*P*	R^2	F statistic	*P*	R^2
Coral									
GFP abundance	$F_{2,35}$=34.7	<0.0001	0.55	$F_{2,35}$=4.1	<0.05	0.07	$F_{4,35}$=3.2	<0.05	0.10
GFP fluorescence	$F_{2,115}$=208.9	<0.0001	0.33	$F_{5,115}$=69.5	<0.0001	0.27	$F_{10,115}$=32.1	<0.0001	0.25
Growth	$F_{2,69}$=107.0	<0.0001	0.55	$F_{4,69}$=3.8	<0.01	0.01	$F_{8,69}$=2.7	<0.05	0.00
Dinoflagellate									
Density	$F_{2,35}$=419.8	<0.0001	0.40	$F_{2,35}$=243.0	<0.0001	0.22	$F_{3,35}$=191.5	<0.0001	0.36
EQY	F2,90=232.5	<0.0001	0.30	F3,90=137.2	<0.0001	0.26	F6,90=88.9	<0.0001	0.34
MQY	$F_{2,92}$=192.8	<0.0001	0.33	$F_{3,92}$=88.9	<0.0001	0.23	$F_{6,92}$=63.4	<0.0001	0.32
Q_m	$F_{2,87}$=77.3	<0.0001	0.24	$F_{3,87}$=47.5	<0.0001	0.22	$F_{6,87}$=33.9	<0.0001	0.32
$RETR_{max}$	$F_{2,34}$=147.2	<0.0001	0.53	$F_{2,34}$=31.9	<0.0001	0.11	$F_{4,34}$=41.1	<0.0001	0.30
I_k	$F_{2,34}$=70.7	<0.0001	0.56	$F_{2,34}$=6.0	<0.05	0.05	$F_{4,34}$=16.0	<0.0001	0.26
Alpha	$F_{2,34}$=76.5	<0.0001	0.43	$F_{2,34}$=41.4	<0.0001	0.23	$F_{4,34}$=22.5	<0.0001	0.25
Chl *a*	$F_{2,24}$=0.6	0.57		$F_{1,24}$=1.0	0.32		$F_{2,24}$=1.7	0.2	
Chl c_2	$F_{2,24}$=0.9	0.42		$F_{1,24}$=0.0	0.9		$F_{2,24}$=1.2	0.31	
Chl c_2: chl *a*	$F_{2,24}$=0.9	0.43	0.04	$F_{1,24}$=2.3	0.14	0.06	$F_{2,24}$=5.7	<0.01	0.29
Per	$F_{2,24}$=1.9	0.17		$F_{1,24}$=3.8	0.06		$F_{2,24}$=0.3	0.73	
Per : chl *a*	$F_{2,24}$=5.6	<0.05	0.14	$F_{1,24}$=39.4	<0.0001	0.49	$F_{2,24}$=2.6	0.1	0.06
Carotene	$F_{2,24}$=2.7	0.08		$F_{1,24}$=0.3	0.61		$F_{2,24}$=0.7	0.5	
Carotene : chl *a*	$F_{2,24}$=7.2	<0.01	0.25	$F_{1,24}$=4.6	<0.05	0.08	$F_{2,24}$=6.8	<0.01	0.24
Dd + Dt	$F_{2,24}$=5.2	<0.05	0.26	$F_{1,24}$=4.9	<0.05	0.12	$F_{2,24}$=0.2	0.83	0.01
Dd + Dt : chl *a*	$F_{2,24}$=22.2	<0.0001	0.32	$F_{1,24}$=34.2	<0.0001	0.25	$F_{2,24}$=18.2	<0.0001	0.26
Dt / (Dd + Dt)	$F_{2,24}$=40.7	<0.0001	0.34	$F_{1,24}$=65.6	<0.0001	0.27	$F_{2,24}$=35.7	<0.0001	0.29
Neutral lipids	$F_{2,35}$=10.3	<0.001	0.27	$F_{2,35}$=4.7	<0.05	0.12	$F_{4,35}$=3.0	<0.05	0.16

Table 3-2. Summary of *t*-tests to test the differences between cold shock and control corals of *Acropora yongei* on 20 d.

Parameter	*t*	Degrees of Freedom	*P*
EQY	12.7	7	<0.0001
MQY	9.4	6	<0.0001
Q_m	-8.9	8	<0.0001
$RETR_{max}$	13.7	6	<0.0001
I_k	8.8	7	<0.0001
Apha	5.3	5	<0.001
Chl *a*	0.12	5	p=0.91
Chl c_2	-2.3	5	p=0.07
Chl c_2: chl *a*	-3.9	8	<0.01
Per	-0.75	8	0.47
Per : chl *a*	-2.1	8	0.07
Carotene	0.76	8	0.47
Carotene : chl *a*	-1.6	8	0.14
Dd + Dt	-2.8	5	<0.05
Dd + Dt : chl *a*	-19.1	8	<0.0001
Dt / (Dd + Dt)	-4.1	8	<0.01

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CHAPTER IV

Life history changes in coral fluorescence and the effects of ambient light intensity on larval physiology and settlement

ABSTRACT

Coral fluorescence, common in both adult and larval life history stages, is produced by fluorescent proteins that absorb high-energy wavelengths of light. This study investigates the changes of coral fluorescence in different life history stages and the effects of parental light environment on larval fluorescence, endosymbiotic dinoflagellate abundance, size and settlement in the brooding coral *Seriatopora hystrix*. Coral fluorescence changed during the development from green fluorescence in larvae to cyan fluorescence in adult colonies; this shifting corresponds with differences in the light environment of each life history stage and suggests a photoprotective function of fluorescent proteins. Coral larvae showed great variation in coral fluorescence and dinoflagellate abundance, and there was no relationship between green fluorescence intensity and dinoflagellate abundance or green fluorescence intensity and larval size. Larvae from parents collected from high and low light environments were similar in green fluorescence intensity, dinoflagellate abundance and size. However, larvae from high light parents exhibited small but significant differences from larvae from low light parents, having increased dinoflagellate abundance, larvae area and rates of settlement in higher light environments. Large variations in larvae fluorescence and dinoflagellate abundance and minimal effects of parental environment on larvae characteristics indicate that parental colonies produce larvae with a wide range of physiological characteristics.

INTRODUCTION

Understanding the reproductive biology of scleractinian corals has become increasingly important as more coral reefs become degraded and the global ecosystem is threatened (Hoegh-Guldberg et al., 2007; Wilkinson, 2008). Many common scleractinian corals are brooders (Richmond and Hunter, 1990), in which eggs are fertilized internally and larvae are capable of settling quickly, but also have to potential to spend significant amounts of time in the water column remaining competent for >100 d (Richmond, 1987; Harii et al., 2002). Because adult corals are sessile, the mobility of larvae and the location of settlement are of critical consequence. Coral larvae swim using cilia, until the larvae undergo metamorphosis and settle on the benthos (Rinkevich and Loya, 1979). The irradiance including both photosynthetically active radiation (400-700 nm, PAR) and ultraviolet radiation (UVR) decreases with depth, and the ocean absorbs wavelengths of light differently (Kirk, 1994; Shick et al., 1996). Small differences in depth, particularly at shallow depths, can have a very sharp gradient of light intensity (Dunne and Brown, 1996). For example, the offshore reefs of Belize, at midday the PAR at 6 m depth is about 60% of at 1.5 m depth (Shick et al., 1996). While coral reefs are often characterized as having oligotrophic waters, many reefs actually are near islands that have high rainfall with substantial terrigenous inputs. High amounts of sediment in the water column can have a strong affect on the light spectrum (Dunne and Brown, 1996).

Adult corals are often found at different depths, and the gradient of light environments affects coral physiology (Falkowski and Dubinsky, 1981). Because

corals obtain significant amounts of energy from primary production by their endosymbiotic dinoflagellates (Muscatine, 1990), the light environment can determine the corals' capacity for growth (Goreau and Goreau, 1959) and reproduction (Kojis and Quinn, 1984) as well as the potential for additional environmental stressors such as increased seawater temperature (Hoegh-Guldberg and Smith, 1989; Shick et al., 1996). The symbiosis physiology of brooded larvae is thought to be similar to those of adult corals (Gaither and Rowan, 2010). Some species of coral brooders release larvae containing endosymbiotic dinoflagellates (Rinkevich and Loya, 1979), and these symbionts can transfer significant quantities of photosynthetically-fixed carbon to the larvae (Gaither and Rowan, 2010).

The effects of parental environment on coral larvae settlement and survivorship is known only in the context of UVR absorbing molecules called mycosporine-like amino acids (MAAs) (Baker, 1995; Kuffner, 2001). In adult corals, MAA concentrations are correlated with depth (Shick et al., 1995). There were no effects of parental environment on larvae settlement and survivorship despite differences in abundances of MAA concentrations in larvae from parental colonies either collected from different depths (Kuffner, 2001) and the same depth and exposed to different UVR treatments for one month (Baker, 1995).

In addition to UVR absorbing compounds, corals also contain PAR-absorbing proteins. Corals have long been known to be fluorescent (Kawaguti, 1944; Catala-Stucki, 1959; Kawaguti, 1969), which results from a family of fluorescent proteins that absorb high-energy potentially damaging light and emit lower-energy less harmful

light (Dove et al., 1995; Dove et al., 2001; Alieva et al., 2008). A whole spectrum of fluorescent proteins has been identified in corals, but the green fluorescent protein (GFP) is most common and thought to be the ancestral protein (Alieva et al., 2008; Gruber et al., 2008). Fluorescent proteins are ubiquitous in scleractinian corals; nearly all reef-building corals in the shallows of the Great Barrier Reef have medium to high levels of fluorescent pigments (Salih et al., 2000; Dove et al., 2001). Fluorescent proteins also contribute to the color of corals (Dove et al., 2001). In addition to the many Cnidarians that have fluorescent proteins (Shagin et al. 2004), GFP-like homologues have been found in copepods (Shagin et al., 2004) and amphioxus (Deheyn et al., 2007; Bomati et al., 2009).

While fluorescent proteins have become a widely used tool in cellular biology (Tsien, 1998), the *in vivo* function of fluorescent proteins in corals remains unknown and controversial. Fluorescent proteins were originally proposed to have a photoprotective function (Kawaguti, 1944; Salih et al., 2000). However, this hypothesis is weakened by a lack of correlation between fluorescent proteins and depth (Vermeij et al., 2002; Mazel et al., 2003). Another early hypothesis, photosynthesis enhancement (Kawaguti, 1969), has been questioned because of the inefficient transfer of fluorescence emission to dinoflagellates (Gilmore et al., 2003; Mazel et al., 2003). Lack of clear evidence has led to alternative hypotheses including camouflage (Matz et al., 2006), antioxidant activity (Bou-Abdallah et al., 2006; Palmer et al., 2009a), regulation of symbiotic dinoflagellates (Field et al., 2006; Dove et al., 2008), modulation of the light environment within the coral cells (Roth et al. in

review), and part of an innate immune response (Palmer et al., 2009b). Recently, there has been increasing evidence that fluorescent proteins in corals are strongly influenced by light level and wavelength (D'Angelo et al., 2008; Dove et al., 2008; Bay et al., 2009; Roth et al. in review).

Fluorescence has primarily been studied in adult corals (all citations in above paragraph), with the exception of one study on gene expression (microarrays) during coral larvae thermal stress (Rodriguez-Lanetty et al., 2009). Fluorescence has been briefly noted for a variety of coral eggs (Hirose et al., 2000; Leutenegger et al., 2007; Roth et al., 2007), embryos in *Montastrea cavernosa* (Leutenegger et al., 2007), coral larvae in *Stylophora pistillata* (Rinkevich and Loya, 1979), first polyps in *Acropora millepora* (D'Angelo et al., 2008), and in many families and genera of recent recruits (Roth and Knowlton, 2009). Because of the pelagic nature of coral larvae where there is a potential for higher light intensities among a broader spectrum of light, fluorescent proteins could play an important role in larval survival.

The aim of this study was to investigate the fluorescence in different life history stages in the brooding coral *Seriatopora hystrix* and the effects of parental light environment on coral larvae fluorescence, dinoflagellate abundance, size and settlement behavior. This study also examined the relationships between these characteristics and the larvae variation within an individual parent colony. Understanding the patterns of fluorescence during different life history stages may give insight into the functions of fluorescent proteins in corals.

MATERIAL AND METHODS

Sample collection and aquarium design

Seriatopora hystrix is a common Indo-Pacific shallow water coral with many color and branching morphologies, which may actually represent many cryptic species (Veron and Pichon, 1976; Veron, 2000). Brown color morph *S. hystrix* were exclusively used in this study to prevent different physiologies due to different color morphs (Takabayashi and Hoegh-Guldberg, 1995). Adult colonies of *S. hystrix* (*N*=16, 14-22 cm in diameter) were collected from depths of 4-7 m Nanwan Bay in Kenting, Taiwan (21º 56' 29" N, 120º 44' 70" E). The Kenting coastal area has three river drainages and receives heavy rainfall throughout the year creating an ocean environment with high amounts of nutrients and suspended solids (Meng et al., 2008). In Taiwan, *S. hystrix* is a hermaphroditic brooder producing larvae monthly throughout the year and generally larvae peak release is around the first quarter to the full moon of the lunar cycle (Fan et al., 2002; Hsieh, 2009). Corals were collected six days after the full moon (13 July 2009) because peak release of larvae was predicted to be ~15 d later.

At the National Museum of Marine Biology and Aquarium, adult corals were placed in individual 10 l aquaria with flow through filtered seawater (~6 sec ml^{-1}) and an air bubbler. All parasitic snails were removed upon collection. The corals were maintained in an outdoor area (with plastic ceiling) with natural photoperiod and at ambient temperatures. The light in the tanks could reach 1200 µmol quanta m^{-2} s^{-1} at the peak day with clear skies. The outflow from each aquarium flowed through a

unique larval collection cup (Fan et al., 2006). The larvae were released pre-dawn (Fan et al., 2006) and the cups were collected ~0800 hrs and examined for larvae. Larvae used in this study were collected from 26-30 July 2009 (≥3 larvae per adult colony per day) and used to investigate the GFP fluorescence and endosymbiotic dinoflagellate abundance variation within an individual parent colony.

To test the effect of the parental environment on the GFP and dinoflagellate abundance, adult coral colonies were maintained under two light environments (*N*=8 for each). There was no further manipulation (as described above) for the high light treatment. The low light treatment was created using a neutral density shade cloth to reduce 85% of the sunlight; thus, light levels could reach 180 μmol quanta m^{-2} s^{-1} at peak day with clear skies. In brooding corals, oocytes are likely to be fertilized about two weeks before they release larvae (Permata et al., 2000). In this study, corals were in treatments for 13-17 d before larvae were collected ensuring that most of the larval development occurred while parent colonies were in different light treatments.

Spectral properties of coral fluorescence

The spectral characteristics of the coral adult and larval fluorescence were determined with a fluorescence spectrophotometer (F-2500, Hitachi). The coral tissue from a branch of the adult colony was removed with an artist's airbrush and filtered seawater. Larvae from the same parent colony were pooled to obtain fluorescence measurements; only a subset of adults produced enough larvae to make measurements. Pooled in tact live larvae in filtered seawater were measured. Excitation and emission

spectra were normalized to the highest peak in the spectrum. Additionally, the emission spectra of all adult colonies were measured using a spectrograph (Echelle SE200 Digital Spectrograph, Catalina Scientific) with a fiber optic probe placed about 2 mm from the live coral (Roth et al. in review) and blue light excitation (470 ± 90 nm, EXFO X-Cite 120 W mercury lamp).

Epifluorescence microscopy and image analyses

Adults, recruits and larvae were imaged using an epifluorescence stereoscope (Nikon SMZ1500 with EXFO X-Cite 120 W mercury lamp) coupled to a color digital camera (Retiga 2000R, QImaging). Each sample was observed with white light and three filter cube sets, DAPI (excitation 390 ± 22 nm, emission 460 ± 50 nm), cyan (excitation 436 ± 20 nm, emission 480 ± 40 nm), and blue (excitation 470 ± 90 nm, longpass emission ≥500 nm). No fluorescence was observed in any sample with the DAPI filter cube and therefore fluorescent images were obtained only from cyan and blue filter cubes.

All images were processed in ImageJ (NIH software). For an RGB image, green channel of a fluorescent image is correlated to GFP concentration (Roth et al. in review). To determine the GFP fluorescence of larvae, the green channel image obtained with the blue filter cube set was used to trace the outline of the larva and the average pixel intensity within that region was measured. The average background was also obtained for each image and subtracted from the average pixel intensity of each larva. Pixel resolution of fluorescence intensity was 8-bit, scaling from 0-255.

Images collected with white light illumination were used to determine the surface area of endosymbiotic dinoflagellates in each larva as a proxy for dinoflagellate abundance. The dinoflagellate percent surface area of each larva was determined by tracing the edge of the larva and individually thresholding the image so that the surface area of the larva with dinoflagellates was selected. An 8-bit image is required to threshold and the blue channel showed the most distinct separation between dinoflagellate coral and therefore was used (Figure 4-1). An example coral larva showing the steps of threshold technique is provided in electronic supplementary material to this new methodology (Figure 4-1). The measurement obtained is the surface area of the dinoflagellate area relative to the whole surface area of the larva.

Red fluorescence due to chlorophyll fluorescence abundance was also tested as a proxy for dinoflagellate abundance. Chlorophyll absorbs blue light and fluoresces red and thus fluorescence images captured with the blue filter set were used to quantify pixel intensity of the red channel. Chlorophyll fluorescence was calculated by the same methodology as described above for GFP fluorescence, except for the red channel of the image was used instead of the green. Larvae chlorophyll fluorescence was significantly correlated to the dinoflagellate percent surface area of a larva, yet the explained variation due to this factor was minimal (Figure 4-2, $F_{1,89}$=7.4, P< 0.01, R^2=0.08). This poor relationship can, however, be explained by the presence of GFP fluorescence and the optical capabilities of the camera. GFP fluorescence and chlorophyll fluorescence were negatively correlated (Figure 4-3, $F_{1,89}$=214.3, P<0.0001, R^2=0.71). Because GFP fluorescence is brighter than chlorophyll

fluorescence, more chlorophyll fluorescence can be observed when there is less GFP fluorescence. Therefore, a larger range of dinoflagellate surface area is observed with lower values of chlorophyll fluorescence, because less chlorophyll fluorescence could result from either less dinoflagellate abundance or more GFP fluorescence. Moreover, larvae that showed very little chlorophyll fluorescence were double-checked using the white images, and some did have high abundances of dinoflagellates. Therefore, chlorophyll fluorescence is not an adequate methodology for a proxy for dinoflagellate abundance because it is affected by the GFP fluorescence.

White light images were also used to determine the length, width, and 2-dimensional area of each larva. The length of the larva was measured as the longest distance from the oral to aboral end and the width of the larva was measured at the widest part of the larva orthogonal to the length. The area of a larva was calculated as the area of an ellipse $A = \pi ab$, where a is ½ length and b is ½ width.

Settlement behavior experiment

Larvae settlement behavior experiments were performed in pre-soaked polystyrene 6- well culture plates. Half of each well (35 mm in diameter) was exposed to light and half was covered in black tape that was impenetrable to the light. The culture plates were placed under fluorescent light bulbs with a PAR irradiance of 230 μmol quanta m^{-2} s^{-1} on a 12:12 h light:dark photoperiod and in water baths (27ºC) to maintain constant temperature. Ten larvae from a single parent colony were placed in a well with 13 ml of filtered seawater. After 24 hrs, the location of larval settlement

was characterized as the following: settled in high light (exposed half), settled in low light (covered half), settled along the light/dark border (~2mm on either side of the border), unattached (under went metamorphosis but not connected to the substrate), and swimming (no metamorphosis). The larvae that settled in high and low light habitats were of primary focus in this experiment to determine whether the larvae had settlement preferences for lighter or darker habitats.

Data analysis

All statistical analyses were conducted using JMP version 8.0 (SAS Institute, Inc.). T-tests were used to test the effect of the parental environment on GFP fluorescence, dinoflagellate abundance, larval width, larval length, larval size and also to compare the adult fluorescence emission peaks as determined by the spectrophotometer and spectrograph (see above). These data sets were tested for assumptions of normality and homoscedasticity, and data were transformed accordingly prior to analyses. Simple correlation analysis was used to test the relationship between GFP and dinoflagellate abundance and larvae area and dinoflagellate abundance. The results of the settlement experiment were presented as the percentage settled in high and low light by wells (with ~10 larvae per well). Pearson's chi-square test of the contingency table on the raw larvae data (non-percentage) was used to test the effect of the parental environment on the settlement experiment. Fisher's exact test (1-tailed) was used to further examine the association between the larvae settlement and the parental environment. Averages represent

arithmetic means ± standard errors. Statistical differences were significant at the $\alpha = 0.05$ level.

RESULTS

Seriatopora hystrix showed different fluorescence patterns at different life history stages, most likely a result of different FP expression (Figure 4-1, Figure 4-2). The adult colonies of *S. hystrix* display cyan fluorescence throughout the coenosarc and the polyp, while the larvae expressed green fluorescence throughout the whole larvae, with perhaps higher concentrations at the oral and aboral ends. The adult colonies of *S. hystrix* displayed a single cyan fluorescent protein (CFP) with an excitation peak of 459.8 ± 0.3 nm and an emission peak of 486.5 ± 0.5 nm (N=2). These results are similar to the emission peak measured on all the live adult colonies of 484.3 ± 0.4 nm (N=16) with no significant difference between the two instruments (t_{16}=2.0, P=0.07). In contrast the larvae expressed two green fluorescent proteins (GFPs) with excitation peaks at 489.5 ± 0.5 nm and 504 ± 0 nm and emission peaks 499.3 ± 0.3 nm and 512.7 ± 0.3 nm, respectively (hundreds of larvae per adult and five adults included). Larvae were collected from high and low light parents and the two types of GFP were observed in larvae from both parental treatments. The recruits also expressed GFP (Figure 4-1f), but a full spectral characterization was not possible because they were attached to the substrate and there were too few samples. *S. hystrix* displayed an ontogenetic development pattern of coral fluorescence.

The larvae exhibited a nearly 15-fold range of GFP fluorescence and even within larvae from the same individual parent colony expressed a large range (Figure 4-3a, *N*=91). Most larvae had a high abundance of dinoflagellates and the abundance ranged 4-fold (Figure 4-3b, *N*=91). There was no relationship observed between larvae GFP fluorescence and dinoflagellate abundance (Figure 4-3c) based on a least-squares linear regression ($F_{1,89}$=0.3, *P*=0.60, R^2=0.003). Three larvae released by an individual parent colony on the same night exemplify the amount of variation in dinoflagellate abundance and the lack of correlation with GFP fluorescence (Figure 4-4). Larval size and dinoflagellate abundance were not significantly correlated based on a least-squares linear regression (Figure 4-3d; $F_{1,89}$=1.9, *P*=0.17, R^2=0.02). There was no significant relationship between GFP fluorescence and larval size based on a least-squares linear regression ($F_{1,89}$=1.89, *P*=0.17, R^2=0.02).

In general, larval characteristics of fluorescence, dinoflagellate abundance, and size were very similar regardless of their parental environment. The larval GFP fluorescence from high light parents (141.9 ± 6.9) and low light parents (144.8 ± 7.0) were not significantly different (t_{150}=-0.77, *P*=0.78). There was a small but significant difference between larval dinoflagellate abundance (t_{150}=2.3, *P*<0.05) from high light parents (54.8 ± 1.4% larva surface area) and low light parents (49.3 ± 1.9% larva surface area). The larval length from high light parents (822 ± 19 μm) and low light parents (773 ± 19 μm) was not significantly different (t_{150}=1.8, *N*=0.08). The larval width from high light parents (441 ± 11 μm) and low light parents (413 ± 12 μm) was not significantly different (t_{150}=1.7, *N*=0.09). However, there was a small but

significant difference (t_{150}=2.34, P<0.05) in larval size from high light parents (0.28 ± 0.009 mm^2) and low light parents (0.25 ± 0.01 mm^2). In the low light larvae, there was no relationship between GFP fluorescence and larval size ($F_{1,59}$=0.12, P=0.74, R^2=0.002), and no relationship between dinoflagellate abundance and larval size ($F_{1,59}$=2.0, P=0.17, R^2=0.03).

A total of 719 larvae were used in the settlement experiment. The larvae from the high light parents (N=391, 39 wells) settled relatively equally between high (N=154) and low light (N=150) environments, and the remaining larvae settled in the border (N=57), were unattached (N=1), or still swimming (N=29). Larvae from low light parents (N=328, 33 wells) preferentially settled in low light (N=145) rather than in high light (N=105); and the remaining larvae settled in the border (N=26), were unattached (N=7), or still swimming (N=45). The larvae settlement experiment showed differences between the larvae from parents in different environments (Figure 4-5). The larvae brooded under high light conditions showed no preference between settling in high (49.7%) and low light (50.3%) environments. The larvae brooded under low light conditions settled more under low light (57.9%) than under high light (42.1%). The frequencies of larvae settlement in low and high light habitats were significantly different between the parental environments (χ^2=4.1, N=554, P<0.05). There was a greater probability for larvae to settle in low light habitats if the parent was under a low light environment (Fisher's Exact test, P<0.05).

DISCUSSION

Seriatopora hystrix displayed changes in fluorescence with life history stage. Larvae displayed bright green fluorescence and contained two GFPs, whereas adult corals expressed cyan fluorescence and accordingly had a single CFP. The fluorescent proteins exhibited by the adult corals were shifted towards higher energy wavelengths of light, which transmit further in the ocean. The high turbidity in Nanwan Bay (Meng et al., 2008) most likely causes rapid light attenuation and more drastic changes in the light spectrum. Therefore, although the adult colonies were collected from shallow depths (4-7 m), in these conditions this represents a considerably different light environment than the water column. Light is an important cue for the release of larvae, and the peak larvae release of *S. hystrix* is 0400-0600 hrs (Fan et al., 2006). Therefore, larvae that do not settle very quickly (in minutes) will be exposed to high levels of sunlight as they swim through the water column. The light environment in the water column has proportionally more green light in comparison to what the adults receive, and consequently the larvae expressed two GFPs. The shift in fluorescent proteins during life stages in corals to match the light environment is consistent with a photoprotective type function of fluorescent proteins where the fluorescent proteins could help buffer the coral as well as its symbionts.

The life history pattern of fluorescence of *S. hystrix* differs from what has previously been briefly described in broadcast-spawning coral species. The emission spectra of eggs and adults are similar in *Montipora capitata* (Roth et al., 2007) and *Montastrea cavernosa* (Leutenegger et al., 2007), suggesting that there is no change in

fluorescent proteins expressed in these life history stages. In comparison to *S. hystrix*, which releases in the morning, the Caribbean coral *M. cavernosa* releases gametes at night (Wyers et al., 1991) as does the Pacific coral *M. capitata* (Roth et al., 2007). No fluorescence was observed in the sperm of *M. capitata* (Roth et al., 2007), suggesting that they do not live for very long.

The most comprehensive investigation of fluorescent proteins in different life history stages was conducted on the cephalochordate amphioxus. In distinction to corals, amphioxus has 16 closely related GFP-like proteins with emissions ranging from 495-524 nm (Deheyn et al., 2007; Bomati et al., 2009). Many stages of amphioxus including unfertilized eggs, larvae and adults have many of the same GFPs, but at different occurrences, as determined by genetic sequencing (Deheyn et al., 2007; Bomati et al., 2009). In amphioxus, the earlier life history stages tend to have more occurrences of GFPs than the adult stage (Bomati et al., 2009). The spatial pattern and location of expressed GFP differs in amphioxus larvae and adults (Deheyn et al., 2007), which is similar to the case for *S. hystrix*.

S. hystrix larvae showed large ranges of coral fluorescence and dinoflagellate abundance, even among larvae produced by the same parent colony or from the same parent light environment. High light larvae ranged 15 fold in green fluorescence intensity and 4 fold in dinoflagellate abundance, although most larvae had fairly high dinoflagellate abundance. This study showed that the fluorescence had no relationship with larval size or dinoflagellate abundance. The large variation of dinoflagellate abundance in larvae in this study is similar to what has been observed in brooding

corals (Isomura and Nishihira, 2001; Gaither and Rowan, 2010) including a >4 fold range in *S. hystrix* (Isomura and Nishihira, 2001). While larvae with less dinoflagellates initially have lower rates of photosynthesis, the number of dinoflagellates increases quickly so that there is no difference with larvae with high abundances of larvae after 3 weeks (Gaither and Rowan, 2010), indicating dinoflagellate abundance upon larval release may not be a critical factor.

There are several possible explanations high variability in fluorescence and dinoflagellate abundance in larvae. Within an adult brooding coral, there are many microhabitats of different light regimes created by branching where light can differ by 50 fold, which causes differences in productivity (Titlyanov, 1991). Moreover, the age of the branch can also have large consequences on the density and size of polyps, dinoflagellate abundances and photosynthetic capacity (Titlyanov, 1991). Healthy adult corals living at the same depths can also show a large range of dinoflagellate pigment concentration (1.5-10 fold) and dinoflagellate abundance (1.3-8.8 fold) (Apprill et al., 2007). Additionally, there can be high genetic variability in larvae released from an individual coral colony because fertilization can result from multiple sires as well as selfing in *S. hystrix* (Sherman, 2008). These environmental and genetic differences within an individual parent colony may contribute to the variation in coral larvae.

Because of the large amount of variation in coral larvae, it was not surprising that there were relatively few differences in larvae from high and low light parent colonies. There was no difference between the green fluorescence intensity, length or

width of larvae from parental treatments. Additionally, both types of GFP were found in larvae from each parental light environment, perhaps suggesting that expression of the two GFPs are fundamental for the larvae. Given all the larvae variation, the small but significant differences between the larvae from different light treatments are important. The larvae from high light parents were slightly larger and had slightly higher dinoflagellate abundance than the larvae from low light parents. One advantage to size is that larger larvae have higher survivorship rates than smaller larvae (Isomura and Nishihira, 2001). Because the variation in larval size increases as there are more larvae (Isomura and Nishihira, 2001) and larvae frequently change shape (Rinkevich and Loya, 1979), it places greater importance on the measured differences in size between larvae from different parental treatments. Moreover, the larvae from high light parents were more likely to settle in higher light environments than the larvae from low light parents. If the rates of survivorship are equal, the larvae that settle in higher light environments may grow more quickly. It is also possible the differences in larvae settlement would have been more pronounced if high light intensities were used. Previous studies have shown that there was no effect of parents from different PAR and UVR light environments on larvae settlement or mortality (Baker, 1995; Kuffner, 2001). However, the light intensity and spectral quality of light can have important consequences for larvae settlement depending on the parent depth distribution (Mundy and Babcock, 1998). In Southern Taiwan, *S. hystrix* is more typically found at depths of 4-12 m, while *Pocillopora damicornis* is more common at shallower depths (1-5 m) (T. Fan, unpubl. data), but it is unknown whether settlement

preferences or differential post-settlement mortality is the cause of such distribution patterns. The implications of the great variation in the larvae and the subtle differences of larvae from different parental environments found in this study suggest that the adult coral colonies are producing larvae that have the physiological capacity to settle in a variety of habitats, but that larvae may settlement preferences similar toward their parental environment**.**

This characterization of fluorescence in larvae and adult corals provided insights into the functions of fluorescent proteins. The shift in fluorescence to more closely correspond the light environment of the particular life history stage suggests that the expression of fluorescent proteins in corals is related to the light environment and consistent with a photoprotective function. Large variations in larvae fluorescence and dinoflagellate abundance and minimal effects of parental environment on larvae characteristics could indicate that parental colonies are producing larvae with a range of physiological capacities, but show small preferences for settling in similar habitats as their parents.

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Chapter IV, in full, will be submitted for publication as: Roth, Melissa; Fan, Tung-Yung; Deheyn, Dimitri. “Life history changes in coral fluorescence and effects of ambient light intensity on larval physiology and settlement”. The dissertation author was the primary investigator and author of this paper.

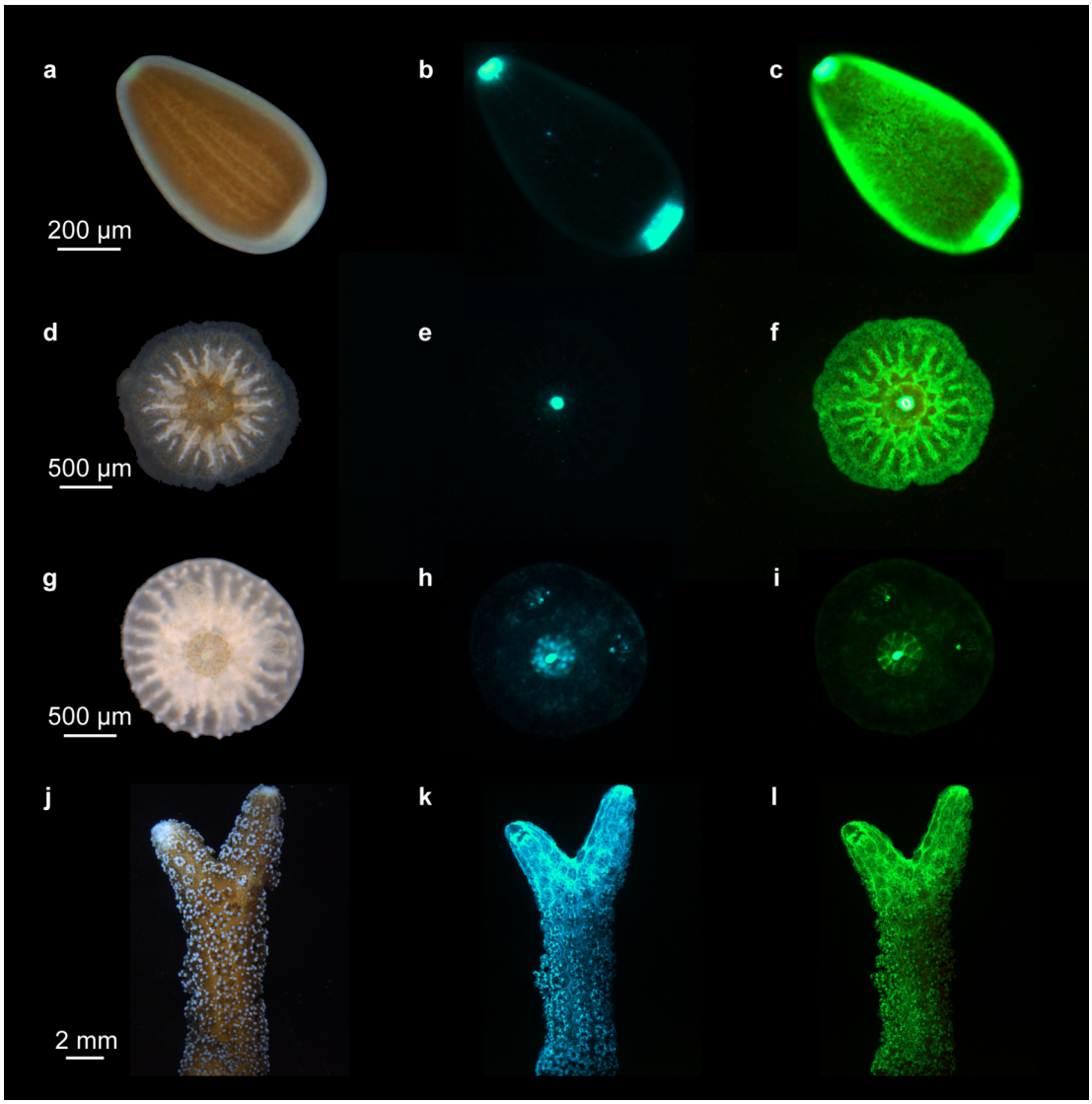

Figure 4-1. Images representing life history stages of (**a-c**) *Seriatopora hystrix* including larva, (**d-f**) 1 d recruit, (**g-i**) 14 d recruit, and (**j-l**) adult under (**a**, **d**, **g**, **j**) white light, (**b**, **e**, **h**, **k**) cyan light (excitation 436 ± 20 nm and interference filter 480 ± 40); and (**c**, **f**, **i**, **l**) blue light (excitation 470 ± 40 nm and longpass emission filter ≥500 nm).

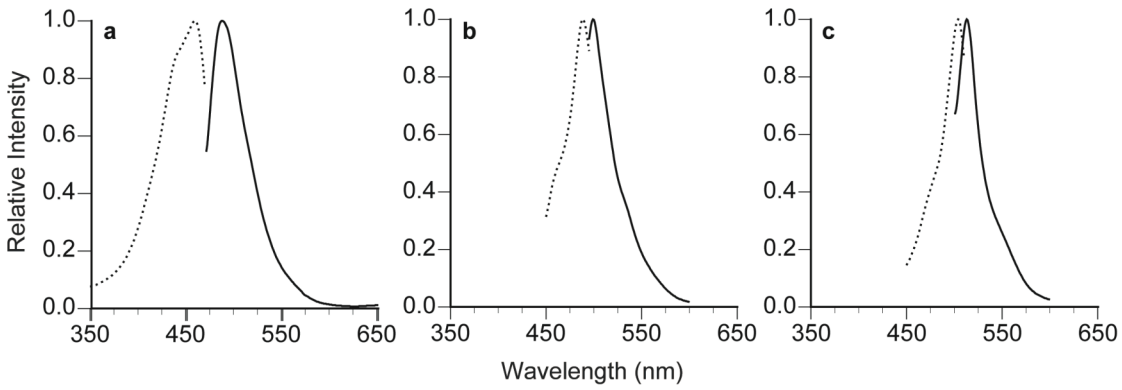

Figure 4-2. Spectral characteristics of fluorescence in (**a**) *Seriatopora hystrix* adult corals and (**b**-**c**) coral larvae. Dotted line represents excitation spectra; solid line represents emission spectra.

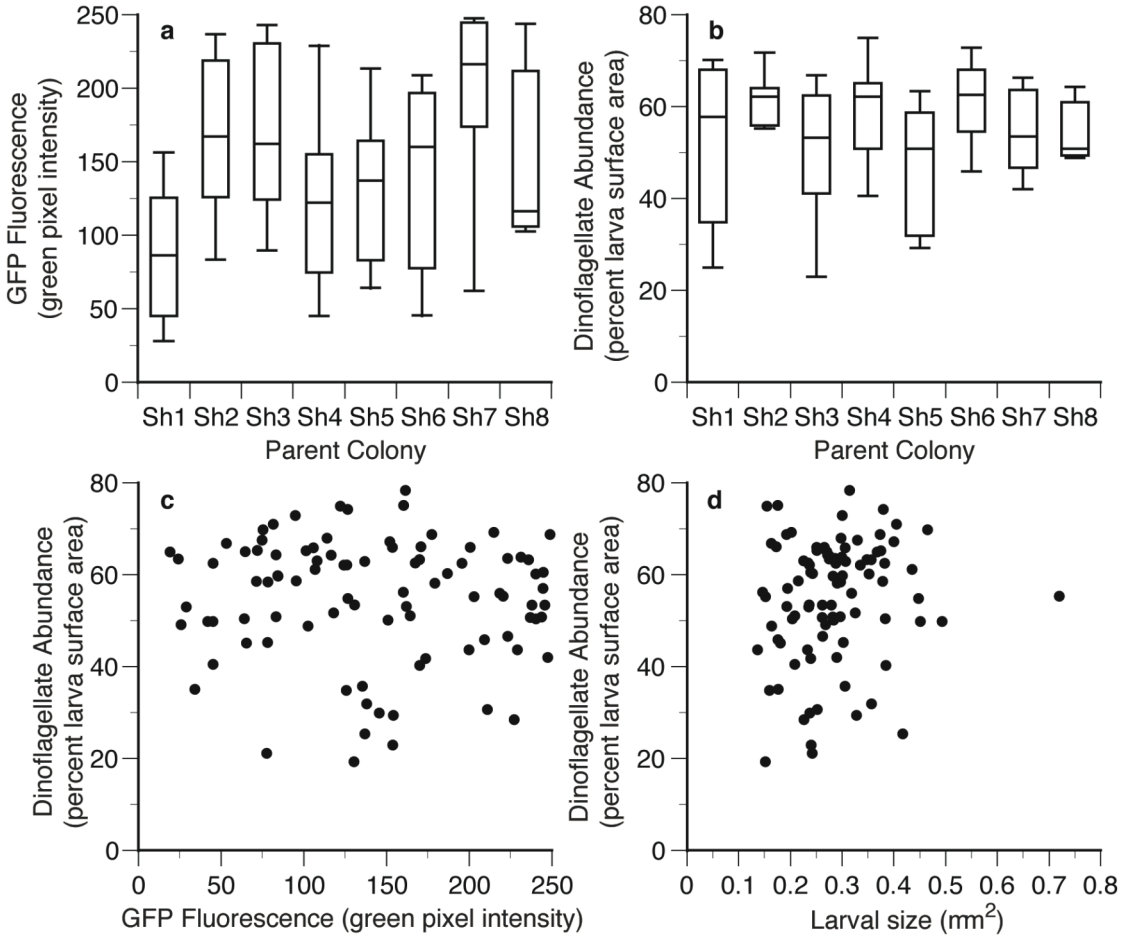

Figure 4-3. *Seriatopora hystrix* larval characteristics. Box plot of GFP fluorescence (**a**) and dinoflagellate abundance (**b**) in larvae produced by eight parent colonies (*N*=14 for Sh1, 13 for Sh2, 9 for Sh3, 15 for Sh4, 14 for Sh5, 9 for Sh6, 14 for Sh7, and 3 for Sh8). Errors bars represent deciles (10^{th} and 90^{th} percentiles), boxes represent quartiles (25^{th}, 50^{th}, and 75^{th} percentiles). (**c**) Larva fluorescence is not related to dinoflagellate abundance. Each point represents an individual larva ($F_{1,89}$=0.3, *P*=0.60, R^2=0.003). (**d**) Dinoflagellate abundance is not related to larval size ($F_{1,89}$=1.9, *P*=0.17, R^2=0.02).

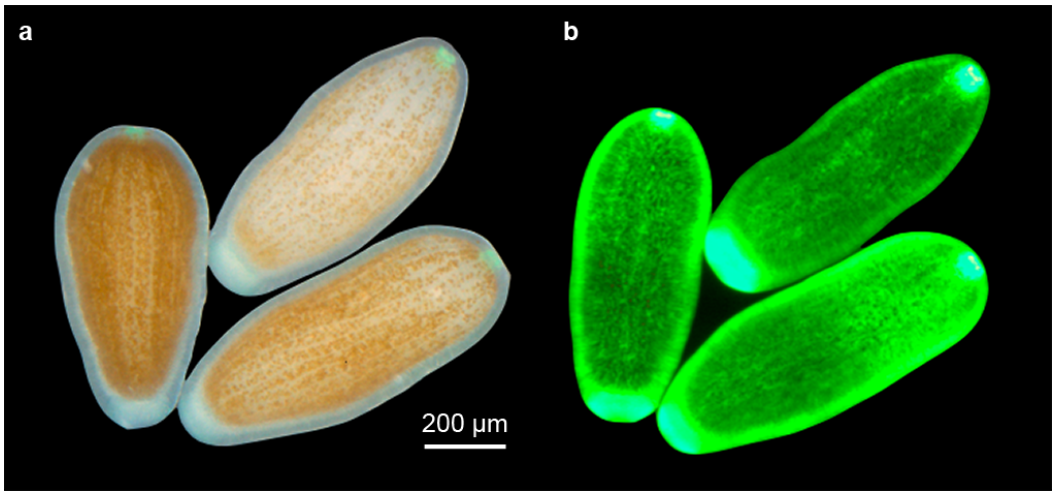

Figure 4-4. *Seriatopora hystrix* coral larvae from one parent colony under white (**a**) and blue light (**b**) showed similar levels of GFP fluorescence (left to right: 153, 185, 138 green pixel intensity) regardless of dinoflagellate abundance (left to right: 71, 15, 44% larva surface area).

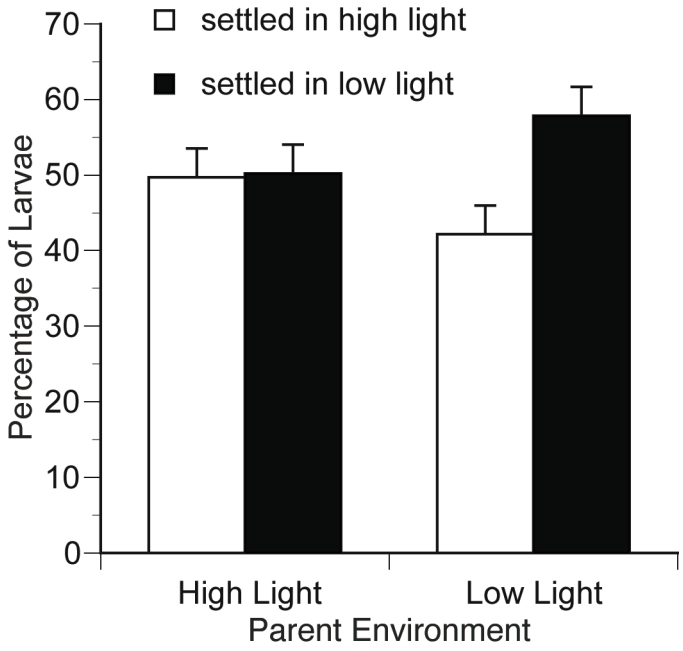

Figure 4-5. *Seriatopora hystrix* coral larvae settlement in high light and low light conditions from parent colonies acclimated to high (*N*=39 wells) or low light (*N*=33 wells) environments. Chi square test indicated significantly different larvae settlement frequency distributions depending on parent environment.

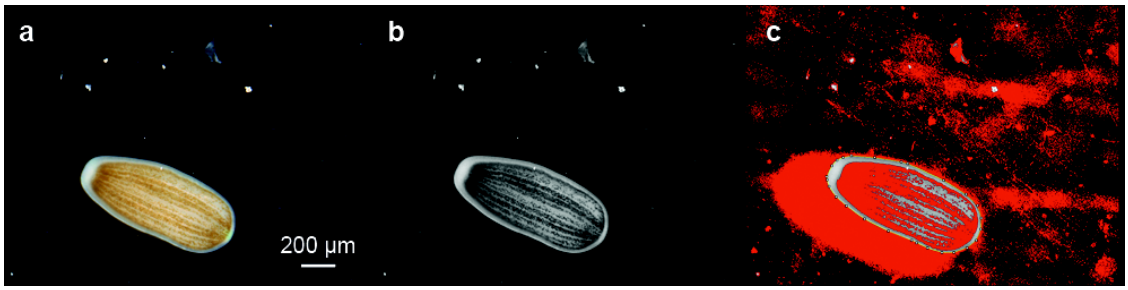

Figure 4-A1. Representative *Seriatopora hystrix* coral larva showing the methodology to quantify the dinoflagellate larva percent surface area as a proxy for dinoflagellate abundance in a single larva. (**a**) Image of larva under white illumination, (**b**) the blue channel image, and (**c**) the blue channel image with outline the larva traced (in yellow) and the threshold adjusted to quantify the percentage of the dinoflagellate abundance, in this case 61% larva surface area.

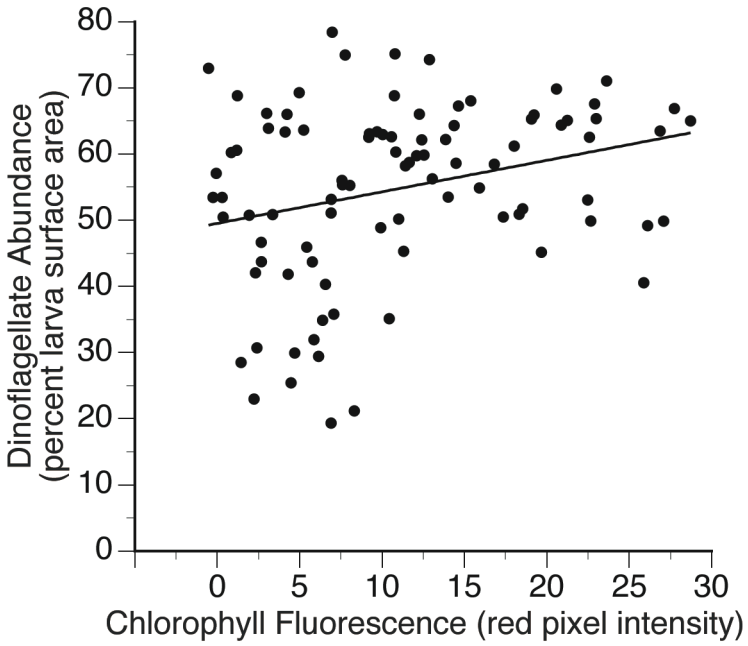

Figure 4-A2. The relationship between dinoflagellate abundance and chlorophyll fluorescence in *Seriatopora hystrix* coral larvae ($F_{1,89}$=7.4, P< 0.01, R^2=0.08). The line represents the least-squares linear regression (y = 49.5 + 0.48x).

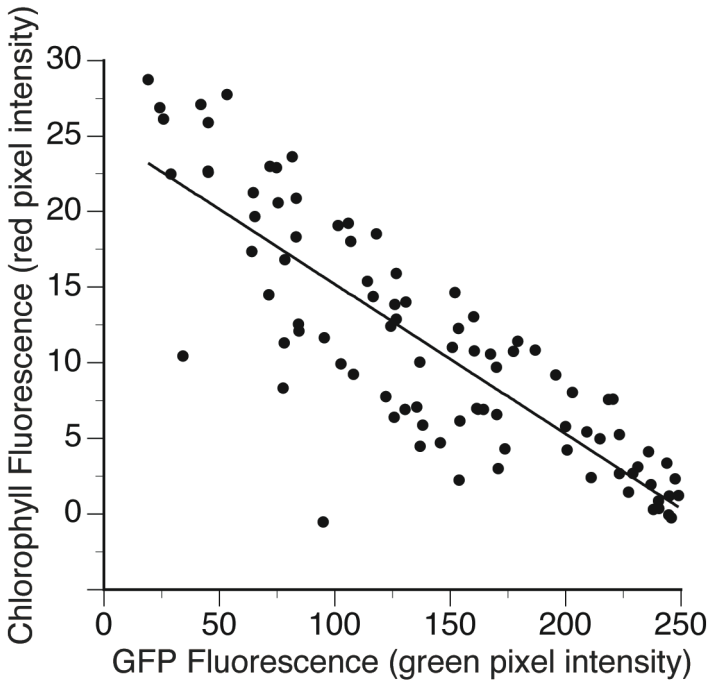

Figure 4-A3. The relationship between chlorophyll fluorescence and GFP fluorescence in *Seriatopora hystrix* coral larvae ($F_{1,89}$=214.3, P<0.0001, R^2=0.71). The line represents the least-squares linear regression (y = 25.1 – 0.10x).

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CHAPTER V

Distribution, abundance, and microhabitat characterization of small juvenile corals at Palmyra Atoll

ABSTRACT

(as it appears in the manuscript)

Juvenile corals are an important component of the population dynamics of corals, but little is known about the ecology and natural history of their early life-history stages. In demographic surveys, small juvenile corals are often grouped with larger and older corals or overlooked entirely due to their small size and cryptic nature. This study describes the distribution, abundance, and microhabitat of small juvenile corals, defined as post-settlement corals ≤5 mm in diameter, at Palmyra Atoll, Central Pacific. A diver-operated pulsating blue light and filter system was used to enhance innate coral fluorescence during daylight to aid in detecting small juvenile corals. Juvenile densities ranged from 0 to 59.5 m^{-2} and were more than 9 times higher on the fore reef (21.9 ± 0.8 m^{-2}) than on the back reef (2.4 ± 0.3 m^{-2}). The highest juvenile densities were observed in the middle of the sampled range at 14 m depth on the fore reef. Juvenile corals accounted for >31% of coral colonies in all habitats and depths, which resulted in positively skewed size-frequency distributions. The microhabitat of juvenile corals on coral rubble was best described as a convex surface covered with crustose coralline algae that lacked another coral within a 20 mm radius. This study provides basic ecology and natural history information of small juvenile corals and shows the feasibility of surveying corals ≤5 mm in diameter as a method for monitoring coral populations and assessing environmental changes on a coral reef.

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Distribution, abundance, and microhabitat characterization of small juvenile corals at Palmyra Atoll

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ABSTRACT: Juvenile corals are an important component of the population dynamics of corals, but little is known about the ecology and natural history of their early life-history stages. In demographic surveys, small juvenile corals are often grouped with larger and older corals or overlooked entirely due to their small size and cryptic nature. This study describes the distribution, abundance, and microhabitat of small juvenile corals, defined as post-settlement corals ≤5 mm in diameter, at Palmyra Atoll, Central Pacific. A diver-operated pulsating blue light and filter system was used to enhance innate coral fluorescence during daylight to aid in detecting small juvenile corals. Juvenile densities ranged from 0 to 59.5 m^{-2} and were more than 9 times higher on the fore reef (21.9 ± 0.8 m^{-2}) than on the back reef (2.4 ± 0.3 m^{-2}). The highest juvenile densities were observed in the middle of the sampled range at 14 m depth on the fore reef. Juvenile corals accounted for >31% of coral colonies in all habitats and depths, which resulted in positively skewed size-frequency distributions. The microhabitat of juvenile corals on coral rubble was best described as a convex surface covered with crustose coralline algae that lacked another coral within a 20 mm radius. This study provides basic ecology and natural history information of small juvenile corals and shows the feasibility of surveying corals ≤5 mm in diameter as a method for monitoring coral populations and assessing environmental changes on a coral reef.

KEY WORDS: Juvenile corals · Ecology · Distribution · Abundance · Microhabitat · Recruit · Palmyra Atoll

INTRODUCTION

Coral reefs are in a state of global decline because of multiple stressors, including overfishing, poor water quality, and rising concentrations of carbon dioxide (Hughes 1994, Jackson 1997, Pandolfi et al. 2003, Bellwood et al. 2004, Hoegh-Guldberg et al. 2007). Therefore, a better understanding of coral population biology is needed for coral reef assessment and management. Population dynamics and demographic analyses require accurate ecological information about all life-history stages, but little is known about the ecology of small juvenile corals (e.g. ≤5 mm in diameter). Small juvenile corals, also termed recruits in some studies, are critical to the future of coral reefs because they become the foundation of a reef ecosystem, and are an important component of resiliency, a reef's ability to recover from disturbance (Bellwood et al. 2004).

Coral population data can be used to assess the health of coral reefs. For example, coral size-frequency distributions are used to infer the future trajectory of coral populations (Bak & Meesters 1998, 1999, Hughes & Tanner 2000, Smith et al. 2005). In demographic models, varying recruitment rates can change whether a coral population increases or decreases (Hughes &

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Mar Ecol Prog Ser 376: 133–142, 2009

Tanner 2000, Edmunds & Elahi 2007). The ecology and population dynamics of early post-settlement corals are poorly understood because of challenges in surveying juvenile corals. Coral recruits are often ≤1 mm (Babcock et al. 2003), making them difficult to detect on a coral reef (Miller et al. 2000). A lack of data on the abundance of juvenile corals can lead to erroneous size-frequency distributions and inaccurate population models.

There are 3 principal reasons why previous juvenile coral studies provide inadequate information on the earliest life-history stage of corals: (1) Many studies defined juvenile corals broadly, grouping small and large juveniles together. In studies that randomly surveyed juvenile coral densities on natural substrates (e.g. Bak & Engel 1979, Rogers et al. 1984, Edmunds & Carpenter 2001), the most common definition for a juvenile coral was a coral ≤40 mm in diameter (Fig. 1). Occasionally, size detection limits are reported as low as 5 mm (Miller et al. 2000) or even 1 mm (Glassom & Chadwick 2006), but such small individuals are difficult to detect and are almost certainly underrepresented in these studies, as previously acknowledged (Miller et al. 2000, Glassom & Chadwick 2006). (2) Artificial substrates such as ceramic tiles are often used to study post-settlement recruits (Hughes et al. 1999, Mundy 2000, Fox 2004). Population dynamics on artificial substrates are substantially different than on natural reef substrates (Rylaarsdam 1983) because artificial substrates often lack appropriate chemical cues from biofilms and/or crustose coralline algae to which juvenile corals respond (Morse & Morse 1996). Furthermore, mortality on artificial substrates is much higher than on natural substrates (Rylaarsdam 1983). Hence, basic measurements of distribution and abundance on artificial substrates may differ from measurements on natural reef substrates because the population dynamics are likely to be dissimilar between the 2 environments. (3) Most previous studies of juvenile corals have been conducted on degraded coral reefs. Over 70% of the previous density studies, including the 2 previous studies that included a ≤5 mm size class (Smith 1992, Mumby 1999), were carried out in the Caribbean (Fig. 1), where significant reductions in the abundance of herbivores and carnivores began centuries ago, followed by major losses of architectural species beginning in the 1970s (Jackson 1997, Aronson et al. 2002, Gardner et al. 2003, Pandolfi et al. 2003). Thus, the importance of the present study was to provide ecological data on small juvenile corals on natural substrates of a healthy coral reef, thereby addressing the limitations of previous studies.

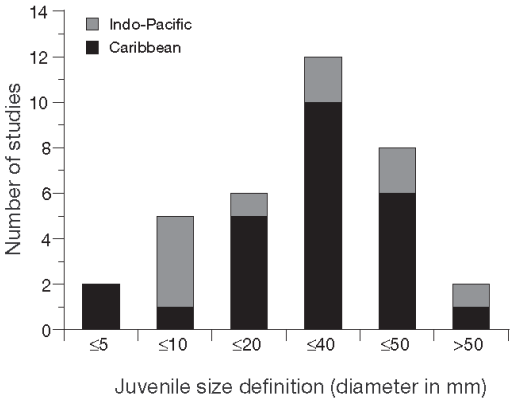

Fig. 1. The smallest juvenile size definition used in previous studies of juvenile coral density on natural substrates (n = 35). Studies were conducted in the Caribbean and Indo-Pacific. The definition was given as a diameter in the literature except for Smith (1992). For Smith (1992), the smallest size class (≤0.25 cm^2) was converted to equivalent spherical diameter of ≤5.6 mm, which was placed in ≤5 mm category. (Sources: ≤5 mm: Smith 1992, Mumby 1999; ≤10 mm: Connell 1973, Harriott 1985, Babcock 1991, Hughes & Tanner 2000, Brown et al. 2002; ≤20 mm: Smith 1997, Kramer 2003, Ruiz-Zarate & Arias-Gonzalez 2004, Piniak et al. 2005, Smith et al. 2005, Vidal et al. 2005; ≤40 mm: Bak & Engel 1979, Colgan 1981, Rogers et al. 1984, Wittenberg & Hunte 1992, Chiappone & Sullivan 1996, Edmunds & Bruno 1996, Edmunds 2000, Edmunds & Carpenter 2001, Edmunds et al. 2004, Carpenter & Edmunds 2006, Glassom & Chadwick 2006, Edmunds & Elahi 2007; ≤50 mm: Rylaarsdam 1983, Bak 1987, Edmunds et al. 1998, van Woesik et al. 1999, Miller et al. 2000, Webster & Smith 2002, Moulding 2005, Sandin et al. 2008; >50 mm: Dustan 1977, Tamelander 2002)

The objective of the present study was to characterize the distribution, abundance, and microhabitat of corals ≤5 mm in diameter using a novel modification of fluorescence techniques. Coral fluorescence has been used recently to help observe coral recruits, because both corals and their symbiotic dinoflagellates autofluoresce when excited with specific wavelengths of light. Past studies have shown that nighttime use of fluorescence techniques results in recruits being more easily observed than in traditional daytime searches (Piniak et al. 2005). Nighttime surveys using fluorescence techniques located 20 to 50% more coral recruits and smaller recruits compared to traditional daytime searches (Baird et al. 2006). However, night diving is logistically more complicated than day diving. The present study modified the fluorescence technique by adding a pulsating excitation light so that small corals would be easily detected during the day.

The present study was conducted at Palmyra Atoll (5° 52′ N, 162° 06′ W), a US territory in the Northern Line Islands, Central Pacific (Fig. 2). Palmyra is characterized as a healthy coral reef community with a com-

plete food web, a predominance of crustose coralline algae and hard coral cover, and low biomass of fleshy algae (Sandin et al. 2008). There are 36 genera and 176 species of scleractinians found on Palmyra (Maragos et al. 2008b). Because of Palmyra's geographic isolation, restricted human presence, and recent protection by the US Fish and Wildlife Service, many localized sources of anthropogenic stress, such as overfishing, are absent. Despite some physical alterations to the atoll during World War II (Dawson 1959, Maragos et al. 2008a), Palmyra ranks as exceptionally healthy compared to most reefs (Knowlton & Jackson 2008).

MATERIALS AND METHODS

Study locations. Palmyra Atoll is exposed to considerable wave action and strong currents. Depending on the positing of the Intertropical Convergence Zone, Palmyra lies within the North Equatorial Current or the North Equatorial Countercurrent (Hamann et al. 2004, Maragos et al. 2008b). The present study included atoll-wide surveys in both the fore reef and the back reef in August and September 2006 (Fig. 2). The fore reef was dominated by *Montipora* spp., *Pocillopora* spp., *Fungia* spp., *Pavona* spp., and *Porites* spp., while the back reef was dominated by *Montipora* spp., *Pocillopora* spp., *Pavona* spp., and *Acropora* spp. (M. S. Roth unpubl. data). Previous studies found 20 and 45% live coral cover on the fore reef (Sandin et al. 2008 and NOAA data reported therein). Eight fore reef sites were haphazardly chosen to span the north and south sides of the atoll, encompassing all sloping fore reef habitat. Each fore reef site was surveyed at 3 depths: 10, 14, and 18 m. Because of wave exposure, it was not feasible to survey shallower depths on the fore reef, and that area was devoid of corals. The back reef primarily consisted of habitat on the western side of the atoll with 2 shallow pools on the eastern side of the atoll. Sites were haphazardly chosen to span the western side of the atoll, and at sites of interest including the Longline Wreck (Site D, Fig. 2) and the Northeast and Southeast Coral Gardens (Sites G and H, Fig. 2). On the shallow back reef, it was only possible to survey at 1 depth at each site, which varied from 1.5 to 4.9 m.

Detection of juvenile corals using pulsating fluorescence-aided visualization. In the present study, a small juvenile coral was defined as a post-settlement coral with its longest diameter ≤5 mm. Hereafter, small juvenile corals are referred to as juvenile corals. Any corals ≤40 mm are most likely to arise from sexual reproduction rather than asexual reproduction (Bak & Engel 1979) and were easy to distinguish from corals produced through asexual reproduction by growth and morphological characteristics.

When excited with blue light, both corals and their symbiotic dinoflagellates autofluoresce. Coral autofluorescence, which is primarily green but can also be other colors, is due to the presence of fluorescent proteins, while dinoflagellate red autofluorescence is due to chlorophyll. Autofluorescence was detected with a prototype lighting system (NightSea). The light (UK C4) was modified so that both bulbs were fitted with a custom blue interference filter to induce autofluorescence. In an improvement over previous fluorescence methods, one of the bulbs was customized to flash. A diving mask yellow barrier filter, which blocked the excitation light to the viewer, enabled the easy detection of corals including those on all sides of surfaces

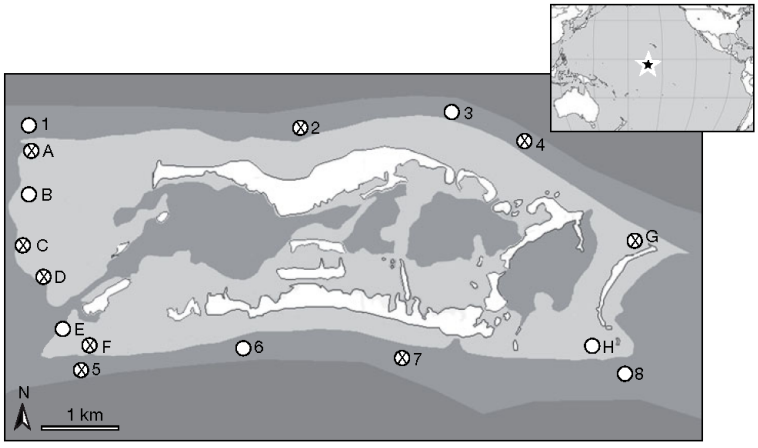

Fig. 2. Study sites at Palmyra Atoll (5°52′ N, 162°06′ W). Transect and quadrat surveys were conducted at all sites (○), and rubble surveys were conducted on a subset of sites (×). Fore reef sites are labeled from west to east: (1: Tortugonas, 2: North Beach, 3: Aviation, and 4: Quail on the north side of the atoll, and 5: Penguin Spit, 6: Paradise, 7: North South Causeway, and 8: Southeast Corner on the south side of the atoll). Back reef sites were labeled from north to south: (A: Tortugonas, B: Far North of Longline Wreck, C: North of Longline Wreck, D: Longline Wreck, E: Penguin Spit Permanent, F: Penguin Spit Mid on the west side of the atoll, G: Northeast Coral Gardens, and H: Southeast Coral Gardens on the east side of the atoll)

Mar Ecol Prog Ser 376: 133–142, 2009

and in crevices. The pulsating excitation light caused corals <1 mm in diameter to 'blink' conspicuously, even in daylight. Because other reef organisms fluoresce, it was necessary to discriminate small corals from similar-looking organisms such as anemones and zoanthids, which were soft when probed compared to corals. The only coral species observed without autofluorescence was the azooxanthellate *Tubastrea* sp.; additionally hydrocorals in the genus *Stylaster* lacked autofluorescence. There have been observations of non-fluorescent morphs of coral species (Salih et al. 2000, Baird et al. 2006).

Transect and quadrat surveys. The fore reef and back reef of Palmyra Atoll were surveyed using transects and quadrats. Quadrats with a circular area of 0.24 m^2 were placed every 5 m along 50 m transects of constant depth. All scleractinian corals inside or partially inside a quadrat were counted and binned into size classes using longest diameter as the size criterion. As described in the previous paragraph, a pulsating blue light with a barrier filter was used during the daytime to facilitate detection of the smallest corals. Because the lighting system was intended to enhance detection of juvenile corals, corals were counted whether they were detected under blue light or sunlight. Because it was not possible to taxonomically identify small juvenile corals, surveys represent the whole coral community. At each fore reef site 9 to 11 quadrats were sampled, and 11 quadrats at each back reef site.

Rubble surveys. To determine the microhabitat characteristics of juvenile corals, coral rubble pieces were collected and carefully examined for juvenile corals using white and blue light and a stereomicroscope. Two 5 gallon (19 l) buckets of rubble from the top ~10 cm of the reef were collected at each site, and the volume of rubble collected determined by water displacement. A subset of back reef and fore reef sites (Fig. 2) were surveyed for logistical reasons. All sides of the rubble were examined but it was not feasible to record the orientation of each piece of rubble. The size of each coral was measured with vernier calipers and the coral microhabitat was described by the surface geometry, substrate material and proximity to other corals. The geometry was defined by the surface plane on which the coral was lying: convex surface, depression (concave surface), hole/crevice (cavity in the surface), or cryptic (other non-exposed surfaces such as between branches). The substrate material was recorded as anything underneath the coral; when a coral was lying on crustose coralline algae and bare space, both were included. A coral was categorized as having a coral neighbor if there was a coral within 20 mm of its perimeter. The microhabitat characteristics of 2 size classes of corals (≤1 and 4–5 mm) were compared using the frequency distributions of microhabitat data.

Statistical analyses. Statistical analyses were conducted using JMP version 7.0 and R software version 2.2.1. A 2-way ANOVA (site and depth) was used to test for differences in juvenile densities on the fore reef. A 1-way ANOVA (site) was used to test for differences in juvenile densities in the back reef. To compare the fore reef and back reef communities, data from fore reef (all depths) and back reef were used in statistical analyses. Unpaired *t*-tests were used to compare fore reef and back reef juvenile densities. Juvenile densities were expressed as mean ± SE. Size-frequency histograms were created from ln-transformed data, and compared to normal distributions based on the characteristics of the data using the Kolmogorov-Smirnov test (Bak & Meesters 1998, 1999). Skewness was calculated from transformed data and its significance was determined by a comparison with the skewness of 10000 replicates of resampling a normal distribution with the same sample size. Pearson's χ^2 tests were used to test for differences in microhabitat (geometry and substrate) frequency among juveniles from different depths on the fore reef. Pearson's χ^2 test with Yates continuity correction was used to compare juvenile neighbor microhabitat data from different depths on the fore reef. Additionally, Pearson's χ^2 test was used to test for differences in microhabitat between ≤1 and 4–5 mm corals. Back reef juvenile corals in the rubble surveys were not included in statistical analyses because of low sample size. Statistical significance was based on an $\alpha = 0.05$ level.

RESULTS

Coral size-frequency distribution

Juvenile corals constituted a substantial proportion of the total coral population (Table 1). The proportion of juveniles was much larger on the fore reef (34.7 ± 0.9%) than the back reef (8.6 ± 0.1%). The coral population at Palmyra Atoll was dominated by smaller corals (Fig. 3). Logarithmic transformations of neither the fore reef nor the back reef coral population size frequencies were normally distributed ($p < 0.001$ for each); both were significantly right- (positive) skewed, with skewness values of +1.22 ($p < 0.001$) and +0.72 ($p < 0.001$), respectively. On the fore reef, the size distribution was significantly right-skewed at each depth examined: +1.13 ($p < 0.001$) at 10 m, +1.29 ($p < 0.001$) at 14 m, and +1.21 ($p < 0.001$) at 18 m. These results indicate that each habitat contained more small corals than would be predicted by a normal distribution (Fig. 3).

Table 1. Juvenile coral (≤5 mm) density and percentage of total corals. N: number of quadrats. Data are mean ± SE

Habitat	N	Density (m^{-2})	% of total corals
10 m fore reef	86	17.05 ± 1.06	33.7 ± 0.7
14 m fore reef	82	30.13 ± 1.64	36.7 ± 0.6
18 m fore reef	78	18.60 ± 1.10	32.6 ± 0.7
Total fore reef	246	21.90 ± 0.77	34.7 ± 0.9
Back reef	88	2.41 ± 0.30	8.6 ± 0.1
Total	334	16.77 ± 0.61	31.1 ± 0.3

Juvenile coral distribution and abundance

In the transect and quadrat surveys, a total of 1338 juvenile corals were counted in 80.16 m^2 reef sampled. Average juvenile density on the fore reef was over 9 times higher and statistically different than the back reef (t_{332} = 7.4, p < 0.001; Table 1). On the fore reef, both depth ($F_{2,222}$ = 7.4, p < 0.001) and site ($F_{7,222}$ = 4.4, p < 0.001) had a significant effect on the juvenile density, but the interaction between depth and site was not significant ($F_{14,222}$ = 1.2, p = 0.26; Fig. 4A). Average density was highest at mid-depths (14 m), with shallower (10 m) and deeper (18 m) depths having densities of 43 and 38% respectively of densities observed at mid-depths. The highest recorded juvenile density was 59.5 ± 8.3 m^{-2} at Site 5 (Penguin Spit) at a depth of 14 m. The southern sites (Sites 5 to 8, Fig. 2) had higher densities of juvenile corals that were significantly different from the northern sites (Sites 1 to 4; t_{244} = 2.8, p < 0.01), but there was no difference in densities between western (Sites 1, 2, 5, and 6) and eastern sites (Sites 3, 4, 7, and 8; t_{244} = –0.6, p = 0.52). On the back reef, there were 2 sites without juvenile corals. There were no significant differences in juvenile densities among back reef sites ($F_{7,80}$ = 2.1, p = 0.05; Fig. 4B).

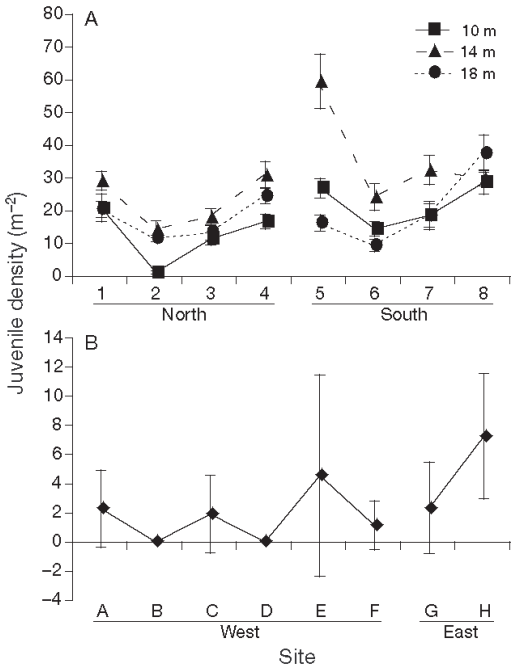

Fig. 4. Juvenile coral (≤5 mm in diameter) densities at the (A) fore reef by depth and site and (B) back reef by site. Data are mean ± SE. See Fig. 2 legend for site codes

The coral rubble surveys showed similar juvenile distribution and abundance patterns to the transect and quadrat surveys. We sampled 114 l of coral rubble

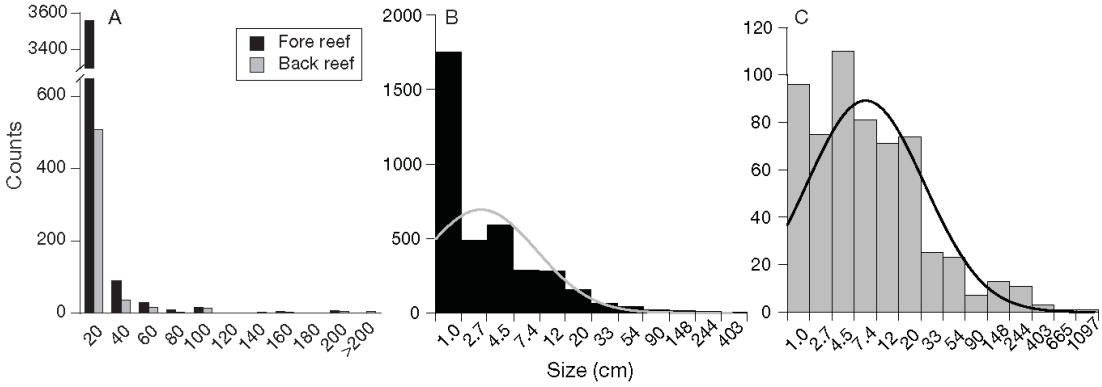

Fig. 3. Size distribution of corals. (A) Untransformed size-frequency distribution on the fore reef (n = 3713) and the back reef (n = 591). Size-frequency distribution of logarithmically transformed data on (B) the fore reef and (C) the back reef. In (B) and (C), the curve represents the normal distribution. Coral distributions were significantly right-skewed (p < 0.001) and significantly different from the normal distribution (p < 0.001 for each). Labels on the x-axis were represented by their endpoints (e.g. 20 represents 0–20 and 40 represents 20.01–40)

Mar Ecol Prog Ser 376: 133–142, 2009

from 4 fore reef sites and 5 back reef sites. On the fore reef, most juvenile corals (45.7%) were found at the mid-depth of 14 m, followed by 18 m (40.9%) and 10 m (13.4%). As with the density data, more juveniles were observed in the fore reef rubble (n = 314) than the back reef rubble (n = 8).

Juvenile coral microhabitat

Based on microhabitat analysis of 314 juvenile corals obtained from the fore reef coral rubble, most juvenile corals were found on convex surfaces, rather than in depressions, holes/crevices, or other cryptic locations (Fig. 5A), a pattern that was not affected by depth (χ^2 = 6.7, p = 0.35). Corals were predominately located on crustose coralline algae, followed by bare space at all depths (Fig. 5B). Surprisingly, >12% of corals were found on the brown encrusting alga *Lobophora* sp. The substrate characteristic patterns were not significantly different between samples collected from 14 and 18 m (χ^2 = 3.0, p = 0.38). These patterns were generally similar to substrate characteristics from corals collected from 10 m, but the crustose coralline algae microhabitat was less dominant (χ^2 = 24.2, p < 0.001). Just under half (48%) of the corals had a coral neighbor within a 20 mm radius.

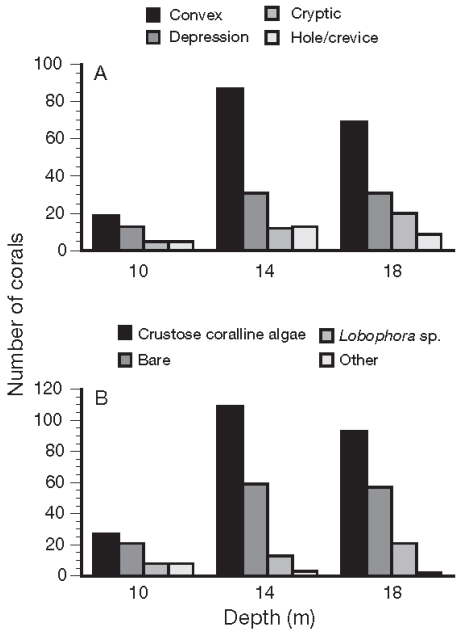

Fig. 5. Microhabitat characteristics, (A) geometry and (B) substrate, of juvenile corals (≤5 mm in diameter) on coral rubble from the fore reef. See 'Materials and methods' for description of surface geometry

The microhabitats of corals ≤1 and 4–5 mm were not statistically different. The ratio of substrate geometries (convex:depression:hole/crevice:other cryptic) for ≤1 mm corals was 41:21:10:11 and for 4–5 mm corals was 42:11:8:5 (χ^2 = 3.7, p = 0.29). The distribution of substrates (crustose coralline algae:bare:*Lobophora* sp.: other) for ≤1 mm corals was 58:32:10:2 and for 4–5 mm corals was 48:34:10:6 (χ^2 = 2.9, p = 0.40). The distribution of neighbors (with:without) for ≤1 mm corals was 37:46 and for 4–5 mm corals was 29:36 (χ^2 = 0.03, p = 0.87).

Too few juveniles were found on the back reef for statistical analyses of their microhabitat characteristics. Qualitatively, back reef juvenile corals appeared to have similar microhabitat distributions as fore reef corals.

DISCUSSION

The present study used a modified fluorescence technique to make extensive daytime observations of juvenile corals. The equipment required was low-tech, easy to use while SCUBA diving, and affordable for scientists and managers. Coral species that have non-fluorescent morphs may be more difficult to detect and could be underrepresented in these surveys. However, this methodology significantly enhanced the ability to detect small corals that are otherwise difficult to observe and enabled a thorough investigation of the distribution, abundance, and microhabitat of small corals.

The microhabitat data confirmed what is well known—that juvenile corals prefer crustose coralline algae and bare space (Morse & Morse 1996, Harrington et al. 2004)—but provided some surprising results. Most intriguing was that *Lobophora* sp. was chosen as a substrate for coral settlement and that large (4–5 mm) and small juveniles (≤1 mm) had similar distributions in this regard. This suggests that *Lobophora* sp. did not affect juvenile survivorship at these size classes, perhaps questioning the negative effects of macroalgae on juvenile corals on some reefs. Evidence of these negative effects includes macroalgae decreasing juvenile coral settlement, growth rates, and survivorship in some species (Lirman 2001, Birrell et al. 2005, Box & Mumby 2007). However, neither settlement rates nor survivorship of coral larvae from *Favia fragrum* were negatively affected by settling on the green alga *Halimeda opuntia*, nor was development of the recruits affected over 5 d (Nugues & Szmant 2006). It is likely that the competition between coral and algae could be affected by nutrients and herbivory (Jompa & McCook 2002).

In contradiction to what was anticipated, the distribution of microhabitat substrates was identical between ≤1 and 4–5 mm corals. These results may challenge previous assumptions of the effects of microhabitat on recruit survivorship (Caley et al. 1996), including geometry (Sammarco 1980), substrate (Harrington et al. 2004), and competition (Sammarco 1991). Other studies have also reported a lack of strong correlation between microhabitat (orientation of substrate) and survivorship in the Caribbean (Edmunds et al. 2004) and on the Great Barrier Reef (Babcock & Mundy 1996), despite selection for particular microhabitats.

Juvenile coral (≤5 mm) densities on the fore reef at Palmyra Atoll were highest at mid-depths of 14 m, possibly representing a balance between considerable wave exposure at shallower depths and less solar irradiance at deeper depths. Palmyra receives different currents depending on the time of year (Hamann et al. 2004, Maragos et al. 2008b), which may have contributed to a more homogeneous environment. Studies on small-scale flow around Palmyra during coral spawning times could elucidate the spatial patterns of coral recruitment, particularly because coral recruitment can vary considerably in space and time (Hughes et al. 1999). The much lower densities on the back reef compared to the fore reef may have resulted from differences in biological communities or environmental conditions including human alterations to the hydrodynamics due to prior construction during World War II (Dawson 1959, Maragos et al. 2008a).

The 2 other studies of juvenile corals ≤5 mm, which were conducted in the Caribbean (Fig. 1), reported lower densities than those observed on the reefs of Palmyra Atoll (Smith 1992, Mumby 1999). Smith (1992) recorded juvenile densities up to ~24 m^{-2} in Bermuda (data estimated from size-class distribution in Smith 1992, their Fig. 2); Mumby (1999) reported juvenile densities up to ~0.6 m^{-2} in Belize, compared to 59.5 m^{-2} in the present study. The disparity in juvenile densities between the present study and these 2 previous studies could have many causes, including the state of reef degradation surveyed, the equipment used to detect juvenile corals, and the ocean basin.

To compare juvenile density estimates with previous studies that have defined juvenile corals as ≤40 mm in diameter, results were re-calculated using the same size class. At Palmyra, densities of corals ≤40 mm on the back reef (13.4 ± 1.1 m^{-2}) and fore reef (50.7 ± 1.3 m^{-2}) were significantly different (t_{332} = 7.7, $p < 0.0001$; Fig. 6). Corals ≤5 mm in diameter represent nearly 20% and over 40% of the juveniles (≤40 mm) on the back reef and fore reef, respectively. The densities of juveniles (≤40 mm) on the fore reef of Palmyra (41.5 to 67.0 m^{-2}) were among the highest recorded in the literature (Table 2). Another study that reported high densities of juvenile corals (42 to 173 m^{-2}) at sites selected with medium-to-low adult coral cover was in the Red Sea (Glassom & Chadwick 2006). Interestingly, the proportion of juvenile corals to the total coral population was ~10 to 27%, similar to that of the present study.

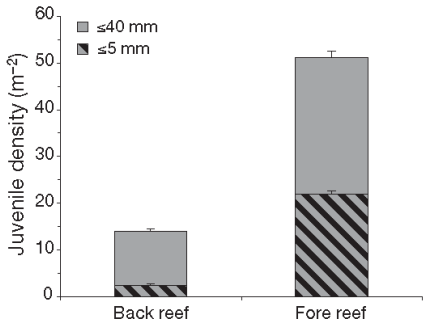

Fig. 6. Densities of juvenile corals using size criteria ≤40 mm and ≤5 mm. Data are mean ± SE

Juvenile corals accounted for a substantial proportion of the coral population; corals on the fore reef ≤5 mm in diameter represented over one-third of all corals. Correspondingly, size-frequency distributions from these data provide a perspective on what a healthy coral reef atoll may look like when all corals including the smallest size classes are surveyed. The size frequencies have a significant positive skewness, indicating a high proportion of small corals, which was also found during a recent study at Palmyra Atoll (Sandin et al. 2008). Surveys of healthy and degraded reefs in the Line Archipelago showed the same patterns in size-frequency distributions of corals (Sandin et al. 2008). However, the most degraded reef had fewer corals in the smallest size class and zero corals in the largest size class, but these distinctions were not captured in the size-frequency distribution analyses.

However, positive skewness of size distributions can be difficult to interpret. It may reflect a high proportion of juvenile corals, which is likely to be an important component of coral reef health, but it could also represent a declining population. Mortality and partial mortality of corals caused by disease, bleaching, or predation reduces colony size and causes fission of large corals. Positive skewing of coral populations in degraded habitats has been reported in the Caribbean (Hughes & Tanner 2000, Edmunds & Elahi 2007) and in the Indian Ocean (McClanahan et al. 2008), and predicted from *Acanthaster planci* predation disturbance models of the eastern Pacific (Fong & Glynn 1998). In contrast, Bak & Meesters (1998, 1999) proposed that a negatively skewed size-frequency distribution mod-

Table 2. Comparative table of mean juvenile coral densities using the standard definition of juvenile corals of ≤40 mm in diameter (in descending order of density). C. = central, USVI = US Virgin Islands, W. = western

Juvenile density (m^{-2})	Depth (m)	Reef habitat	Location	Ocean/sea	Source
67.0	14	Fore reef	Palmyra Atoll	C. Pacific	Present study
43.6	18	Fore reef	Palmyra Atoll	C. Pacific	Present study
42–173	5	Patch reef	Eilat, Israel	Red Sea	Glassom & Chadwick (2006)
41.5	10	Fore reef	Palmyra Atoll	C. Pacific	Present study
~24	4.5–8.5	'Urchin' zone	Jamaica	Caribbean	Edmunds & Carpenter (2001)
~17	3–37	Drop-off zone	Netherlands Antilles	Caribbean	Bak & Engel (1979)
13.3	1.5–4.9	Back reef	Palmyra Atoll	C. Pacific	Present study
13, 33	9	Submarine canyon	St. Croix, USVI	Caribbean	Rogers et al. (1984)
10.5–18.1	6	Fringe reef	St. John, USVI	Caribbean	Edmunds & Bruno (1996, pers. comm.)
~8[a]	14–26	Exposed fore reef	Florida	Caribbean	Edmunds et al. (2004, pers. comm.)
6.3–43.7	1–6	Fringing reef	Barbados	Caribbean	Wittenberg & Hunte (1992)
5.35–12.3	6–16	Submarine terrace	Guam	W. Pacific	Colgan (1981)
5.2–17.9	10	Fore reef	Jamaica	Caribbean	Edmunds & Bruno (1996)
4.5–12.7	16–33	Seaward slope	Guam	W. Pacific	Colgan (1981)
~4–32[a]	5	Fringing reef	St. John, USVI	Caribbean	Edmunds (2000)
3.2–7.75	1–6	Reef front	Guam	W. Pacific	Colgan (1981)
3–42	18, 27, 37	Submarine canyon	St. Croix, USVI	Caribbean	Rogers et al. (1984)
2.5–32.3	<6	Outer fore reef	St. Croix, Barbados, Bonaire, Belize, Jamaica, Grenada	Caribbean	Carpenter & Edmunds (2006)
1.18–3.74	2.7–18.6	Spur/groove, reef flat	Florida	Caribbean	Chiappone & Sullivan (1996)
0–0.15	5, 9	Fringing reef	St. John, USVI	Caribbean	Edmunds & Elahi (2007)

[a]Mean density was estimated from graphs given in 0.25 m^{-2}

eled by a log-normal distribution inferred an unhealthy coral population. They hypothesized that disturbances increased mortality of smaller colonies and coupled with recruitment failure resulted in the predominance of larger colonies. Positively skewed size-frequency distributions were observed in the present study because we surveyed a healthy coral reef using a novel method to aid in detecting small juveniles. These data suggest that healthy reefs may be positively skewed, and with degradation become normally distributed and with further degradation become negatively skewed.

Because changes in coral size-frequency distributions can result from multiple factors, purely examining relative size frequencies to infer status of a coral population can be misleading. We suggest that, in addition to relative size-frequency data, it is important to compare the absolute numbers in each size class with particular attention to the smallest and the largest size class of corals. In addition, distinguishing recent settlers from the small remains of once-large colonies can help determine the processes underlying the size patterns observed.

Juvenile coral investigations not only provide relevant ecological data for coral population dynamics, but also could be useful as an indicator of environmental changes. Size-frequency distributions could be particularly relevant for coral reefs that do not have a well-established baseline of coral populations, yet still provide insight into a reef's resiliency and its future. A standardized procedure for surveying juvenile corals is needed so that data between locations will be comparable (Abelson & Gaines 2005). Daytime coral fluorescence surveys are effective, technologically simple, and affordable, providing a new capability for investigations of coral reef biology. Using fluorescence to aid finding juvenile corals should be part of the standardized technique to ensure reliable data on the smallest life-history stage.

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CHAPTER VI

Conclusion of the dissertation

INTRODUCTION

The main goal of my dissertation was to provide insights into the understanding of the coral photophysiology and assess the development of novel methodologies that could be used to monitor coral health and coral reef health. My dissertation focused on the fluorescent protein because it is an abundant protein in corals, has a strong potential to play a critical role in the photobiology of the coral holobiont, and has optical properties that may be used to develop nondestructive *in situ* monitoring technologies. However, despite such attractive factors, the dynamics of coral fluorescent proteins in response to changes in environmental parameters are poorly understood and the functions of fluorescent proteins are unknown.

Because of the vulnerable position of corals on account of climate change and local stressors, there is a need for greater knowledge and awareness of coral physiology when generating a management plan and course of action to conserve coral reefs. Within this chapter, I will summarize the contributions of my dissertation from the individual chapters and insights from cross-chapter syntheses. I will focus this summary on the three main themes that represent the backbone of my dissertation: 1) implications on the function of fluorescent proteins; 2) implications for using coral fluorescence as an indicator of coral physiology; and 3) implications for using coral fluorescence to monitor coral reef health.

IMPLICATIONS ON THE FUNCTION OF FLUORESCENT PROTEINS

Regulation of GFP during photoacclimation supports a photoprotective function of fluorescent proteins. Similar to ultraviolet absorbing mycosporine-like amino acids (Dunlap and Chalker, 1986; Shick et al., 1995; Shick, 2004) and antioxidant concentrations (Shick et al., 1995), green fluorescent protein (GFP) abundance was positively correlated with light intensity. In Chapter II, increasing light intensity increased GFP abundance within 5 d, while reducing light intensity decreased GFP abundance in *Acropora yongei*. Because GFP absorbs high-energy blue light, a larger concentration of the GFP suggests that the protein would absorb more light. GFP has the potential to protect the coral as well as the dinoflagellates from excess high-energy light. The dinoflagellate response was relatively minor in comparison to the magnitude of changes of GFP concentration, suggesting that GFP modulates the internal light environment. The positive correlation between light and GFP is consistent to what was observed in *Acropora millepora* and *Montipora digitata* over 6 wks of photoacclimation (D'Angelo et al., 2008), and in *A. millepora* where a decrease in GFP-related gene expression was observed in response to decreases in light, but not GFP abundance in 10 d (Bay et al., 2009). As described in Chapter II, the reduction of light intensity in the low light treatment in phase I and in the high light treatment in phase II required >10 d for decreases in green fluorescence to be observed in *A. yongei*. The intense green fluorescence was observed in both the coenosarc and polyps in *A. yongei*, which would provide photoprotection throughout the coral. The

experiments in Chapter II confirm the response of GFP to light intensity and elucidate the time scale of the response. Different colors of fluorescent proteins have different relationships with light (D'Angelo et al., 2008), suggesting that different fluorescent proteins may have distinct roles within corals.

Active degradation of GFP during thermal shock supports a photoprotective function of fluorescent proteins. The reduction of GFP gene expression, which can result from a non-stressful situation such as a decrease in light intensity (Bay et al., 2009), requires >10 d to be observed as a change in GFP concentration (Bay et al., 2009; Roth et al., in review; Chapter II). Immediate (≤6 h) reductions in gene expression and abundance of GFP-like homologues were observed in *A. millepora* and *Montipora monasteriata* (Dove et al., 2006; Smith-Keune and Dove, 2008). Similarly, in Chapter III, GFP abundance decreased by 5 d in both heat and cold shock treatments in *A. yongei*. GFP abundance in both treatments reached a low by 9 d, and by 20 d GFP concentrations were similar to 5 d. This rapid reduction of GFP suggests active degradation or depletion of the protein, which contrasts to a decrease in production and a slow decline of abundance that seems to occur over time scales >10 d. Photoprotective mechanisms, including increasing xanthophyll cycling and decreasing photosynthetic efficiency, were observed confirming that the coral holobiont was under photostress. The rapid reduction of GFP may support that the protein had a role in photoprotection, and it is also possible that the GFP was used as an antioxidant. Indeed, antioxidant type activities have been observed in coral

fluorescent proteins (Palmer et al., 2009) and jellyfish GFP *in vitro* (Bou-Abdallah et al., 2006).

Life history changes in coral fluorescence support a photoprotective function of fluorescent proteins. The life history of brooding corals, as well as broadcasting species, includes different stages that live in different habitats. Like many marine invertebrates, adult corals are benthic and have pelagic larvae, which swim in the water column until they are ready to settle on the reef substrata. Larvae may be competent to settle shortly after release, but also have the potential to remain in the water column for long periods of time (competent for ≥100 d; Richmond, 1987; Harii et al., 2002). Coral fluorescence has been identified in many life history stages of corals including eggs (Hirose et al., 2000; Leutenegger et al., 2007; Roth et al., 2007), embryos in *Montastrea cavernosa* (Leutenegger et al., 2007), first polyps in *Acropora millepora* (D'Angelo et al., 2008), and in many families and genera of recent recruits (Roth and Knowlton, 2009; Chapter V). Chapter IV characterized coral fluorescence in adult corals and their larvae and recent recruits of the brooding coral *Seriatopora hystrix* in Nanwan Bay of Kenting, Taiwan. Adult colonies of *S. hystrix* had one type of cyan fluorescent protein (CFP), and the larvae they released had two types of GFP. Blue light, specifically 440-490 nm, transmits the deepest in oligotrophic waters (Falkowski et al., 1990). Even in clear waters of coral reefs, light intensity declines rapidly within the first 5 m (Dunne and Brown, 1996; Shick et al., 1996). The waters of Nanwan Bay are characterized by high concentrations of nutrients and suspended solids because of terrigenous input (Meng et al., 2008). The high turbidity

environment in waters in Nanwan Bay would therefore dramatically reduce not only the light intensity, but also shift the light spectrum, such as has been observed in Thailand (Dunne and Brown, 1996). Although the adult corals in this study were collected from the shallow reef (4-7 m), it is likely that under these conditions this habitat represents a considerably different optical environment than the water column. Hence, it is probable that the benthic environment would be blue-shifted compared to the water column, corresponding to the shift observed in the different life history stages of the corals. Because the fluorescent proteins in the different life history stages of corals matched the light regime where they inhabit, it would enable the fluorescent protein to have a greater photoprotective role.

Taken together, the responses of fluorescent proteins both within photoacclimation and temperature shock experiments, and the patterns of coral fluorescence during life history stages are all consistent with a photoprotective function of fluorescent proteins. This research also supports an antioxidant type role of fluorescent proteins because increased light intensities caused increased GFP concentrations, and under thermal shock GFP was depleted or actively degraded. However, there may be other functions of fluorescent proteins in corals, particularly with different types of fluorescent proteins.

IMPLICATIONS FOR USING CORAL FLUORESCENCE AS AN INDICATOR OF CORAL PHYSIOLOGY

The intensifying pressure on the coral reefs only amplifies the need for a nondestructive tool to monitor coral health. The optical properties of fluorescent proteins can be sampled noninvasively. To develop coral fluorescence as a monitoring methodology three criteria must be met: changes in fluorescence need to be correlated with fluorescent protein abundance, changes in fluorescence need to have a physiological significance, and changes in fluorescence must be measured reliably in the field.

Changes in coral fluorescence are positively correlated with changes fluorescent protein abundance. Chapter II, during the photoacclimation experiment, showed that green fluorescence and GFP abundance were correlated in *A. yongei* (R^2=0.59, P<0.001). Additionally, the temperature shock experiment showed a small but significant positive correlation between green fluorescence and GFP abundance (R^2=0.14, P<0.01). Moreover, if the temperature stressed samples from the end of the experiment, which represent extreme physiological conditions (heat treatment 12 and 20 d and cold treatment 20 d), are removed from the comparison, the correlation becomes much stronger (R^2=0.69, P<0.0001). These data also suggest that the green fluorescence is affected by the dinoflagellate abundance as well. In the temperature treatment samples from the end of the experiment, the green fluorescence had a strong inverse correlation with dinoflagellate density (R^2=0.83, P<0.0001), which was not observed with GFP abundance and dinoflagellate density (R^2=0.02, P=0.74). The

dinoflagellate abundance could affect the fluorescence of GFP either through shading GFP and decreasing the amount of light that excites GFP, similar to the "package effect" (Kirk, 1994), and/or through absorbing or blocking the fluorescence emitted by GFP; these changes would be amplified by increased light scattering from the coral skeleton. The result is that a severe reduction in dinoflagellate density causes a perceived increase in green fluorescence. Therefore, experiments during photoacclimation and thermal shock show that GFP abundance and green fluorescence are positively correlated except during coral bleaching. Establishing a relationship between fluorescent protein concentration and fluorescence is the first advancement necessary for developing fluorescence as an indicator of coral health.

GFP is positively correlated to the coral growth rate. Growth, like reproduction, is considered a higher-level physiological process, with a much narrower threshold range than survival (Hofmann and Todgham, 2010). The growth of corals will be particularly valuable because sea level rise will require corals faster while ocean acidification may also decrease coral growth. However, coral growth rates on a reef are difficult to obtain because they require repeated measurements at the very least, and at the worst, destructive sampling such as in the case of using alizarin dye as a growth band marker in the skeleton.

Chapter II, the photoacclimation experiment, showed that coral green fluorescence and GFP abundance were both correlated with coral growth rates in *A. yongei* (respectively R^2=0.39, P<0.001 and R^2=0.41, P<0.01).). Additionally Chapter III, the thermal shock experiment, showed that GFP abundance and coral growth rate

were positively correlated in *A. yongei* (R^2=0.49, P<0.0001). Green fluorescence and coral growth rate, excluding heat treatment 12 and 20 d and cold treatment 20 d, were also significantly correlated (R^2=0.61, P<0.0001). Anecdotally, more intense green fluorescence has been observed on the tips of *A. yongei*, which is why the tips were avoided during these experiments. These data therefore support the use of coral fluorescence as an indicator of the growth of corals and only require a single time point of data collection. Moreover, in the thermal shock experiment, the correlation between fluorescence and growth was only observed prior to bleaching; and thus fluorescence (and lack thereof) could be used as a precursor to bleaching and thus an early signal of stress in *A. yongei*.

Suggestions for reliable field measurements of fluorescence. In this dissertation, coral fluorescence was primarily measured in a laboratory setting (Chapters II-IV). For coral fluorescence to be used as a physiological indicator of coral health, reliable field measurements must be obtainable. These studies under carefully controlled conditions in the laboratory provided essential requirements and guidance on the methodology most likely to succeed in the field. As a result, it appears that hyperspectral imaging would provide the most accurate and complete optical information from coral fluorescence. Reef-building corals can have multiple fluorescent proteins (Alieva et al., 2008), and at the very least, their endosymbiotic dinoflagellates have chlorophyll, which fluoresces in the far-red. GFP-like homologues and chlorophyll-derived pigments can interfere with each other and create false readings when using full color imaging processing (as opposed to RGB

channels). For example in Chapter III, severe coral bleaching caused an apparent increase in green fluorescence, which caused the dinoflagellate density and green fluorescence to be negatively correlated, when actual GFP abundance showed no correlation to dinoflagellate density. In the same manner, an inverse relationship between green fluorescence and red fluorescence was observed in coral larvae in Chapter IV. In that particular case, green fluorescence was much brighter than the chlorophyll fluorescence, so that greater chlorophyll fluorescence was observed simply when there was less green fluorescence present.

While monitoring coral fluorescence can provide important physiological information, the detection system must be able to collect wavelengths of light separately, such as a hyperspectral imaging system. The spatial information that hyperspectral imaging would supply will be important because coral fluorescence can have spatial patterns (Gruber et al., 2008). For example, *A. yongei* exhibited uniform fluorescence in the coenosarc and polyps, but some corals show fluorescence concentrated at the oral disk and/or tentacles (Gruber et al., 2008). Corals that have more than one fluorescent protein often show complementary fluorescence where different regions express different fluorescence (Gruber et al., 2008). A hyperspectral imaging system, however, currently does not exist for underwater and fluorescence measurements, and clearly more engineering and laboratory testing and calibration research needs to be conducted on the use of white light and fluorescence hyperspectral imaging. The development of this methodology will benefit from careful

laboratory studies expanding off this dissertation research, such as including corals with different colors of fluorescent proteins and more species of corals.

IMPLICATIONS FOR USING CORAL FLUORESCENCE TO MONITOR CORAL REEF HEALTH

Coral recruits are an important component of coral reef resiliency, the ability of the reef to recover from disturbances (Bellwood et al., 2004). Chapter IV provided evidence to support that adult corals produce larvae with a wide range of physiological capacities, which will be important for populating corals reefs after large disturbances. Scientists and managers use coral demography to assess the health of coral reefs. Coral size-frequency distributions can be used to infer the future trajectory of coral populations (Bak and Meesters, 1998; Bak and Meesters, 1999; Hughes and Tanner, 2000; Smith et al., 2005). Chapter V developed a new technique to accurately sample coral recruit populations on natural substrata. The methodology involves a simple diver-operated system using a pulsating blue light and filter to enhance the innate fluorescence of juvenile corals, making the detection of corals only 1 or 2 mm possible even under daylight conditions (Figure 6-1). Juvenile corals constitute a significant portion of the total population at Palmyra Atoll, a healthy coral reef in the central Pacific. The large abundance of juvenile corals leads to positively skewed sized distributions. However, positively skewed size distributions can also result from degraded populations (Hughes and Tanner, 2000; Edmunds and Elahi, 2007; McClanahan et al., 2008) because mortality and partial mortality of corals can reduce

colony size and cause fission of large corals. This dissertation suggests that in addition to population size frequency, absolute densities of each size class, in particular the largest and smallest, give valuable information for the state of a coral reef. Combining these types of datasets will be especially useful for coral reefs that do not have well-established baselines of the coral populations, but can still provide insight into a reef's resiliency and future. The diver-operated fluorescence methodology should be included as a standard protocol for accurately and reliably surveying juvenile corals.

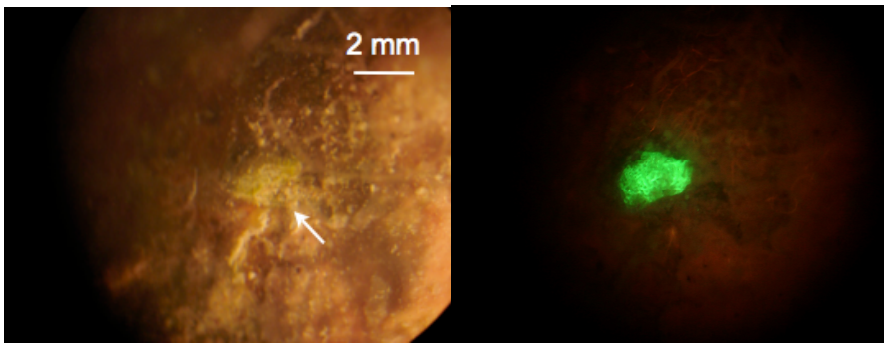

Figure 6-1. Coral recruit under the microscope with white light (left panel) and blue light stimulating autofluorescence (right panel).

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