The Journal of Experimental Biology 213, 3644-3655

doi:10.1242/jeb.040881

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

Melissa S. Roth[1,*], Michael I. Latz[1], Ralf Goericke[2] and Dimitri D. Deheyn[1]

[1]Marine Biology Research Division, Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA 92093-0202, USA and [2]Integrative Oceanography Division, Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA 92093-0218, USA

*Author for correspondence (melissasroth@gmail.com)

Accepted 31 August 2010

SUMMARY

Reef-building corals inhabit high light environments and are dependent on photosynthetic endosymbiotic dinoflagellates for nutrition. While photoacclimation 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 day 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 days of the photoacclimation experiment led to a ~1.6 times increase in GFP concentration for high light corals (900 μmol quanta $m^{-2} s^{-1}$) and a ~4 times decrease in GFP concentration for low light corals (30 μmol quanta $m^{-2} s^{-1}$) compared with medium light corals (300 μmol quanta $m^{-2} s^{-1}$). Green fluorescence increased ~1.9 times in high light corals and decreased ~1.9 times in low light corals compared with medium light corals. GFP concentration and green fluorescence intensity were significantly correlated. Typical photoacclimation responses in the dinoflagellates were observed including changes in density, photosynthetic pigment concentration and photosynthetic efficiency. 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 and support the hypothesis that GFP has a photoprotective function. 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.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/213/21/3644/DC1

Key words: acclimation, dinoflagellate, fluorescence, GFP, light, photoprotection, *Symbiodinium*, symbiosis.

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, 1990; Muscatine and Porter, 1977). 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, 1996; Lesser, 1997; Lesser, 2006; Lesser and Shick, 1989; Lesser et al., 1990). High irradiance, often accompanied by elevated seawater temperatures, can lead to coral bleaching, the breakdown of the coral–dinoflagellate symbiosis (Hoegh-Guldberg, 1999; Lesser, 1997; Weis, 2008) that can cause coral health to decline 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 PSII; increases in dinoflagellate density, except in cases of extreme low light, which decreases density (Brown et al., 1999; Falkowski and Dubinsky, 1981; McCloskey and Muscatine, 1984; Porter et al., 1984; Shick et al., 1995; Ulstrup et al., 2008; Warner et al., 2002); 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, 2004; Shick et al., 1995); 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 (Alieva et al., 2008; Gruber et al., 2008; Salih et al., 2000) 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 (Bou-Abdallah et al., 2006; Palmer et al., 2009b), regulation of symbiotic dinoflagellates (Dove et al., 2008; Field et al., 2006) and as 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 (Dove, 2004; Mazel et al., 2003; Vermeij et al., 2002). 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 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. Endosymbiotic dinoflagellate responses included changes in cell density, photosynthetic pigment concentrations and photochemical efficiency. These results support the hypothesis that GFP has a photoprotective function.

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. *Acropora 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 (Fig. 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 (Fig. 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 an 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 individual 1 l glass aquaria. 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 may be distributed locally by spreading broken branches upon high physical disturbances. The limited genetic variability of replicate ramets was a strength of the study, allowing a 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 ~0.71 min^{-1}. Corals were maintained under a photoperiod of 12 h:12 h light:dark at a seawater temperature of 26.5±1°C. 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+ 54 W Powerchrome fluorescent lamps, Sunlight Supply, Vancouver, WA, USA). Light

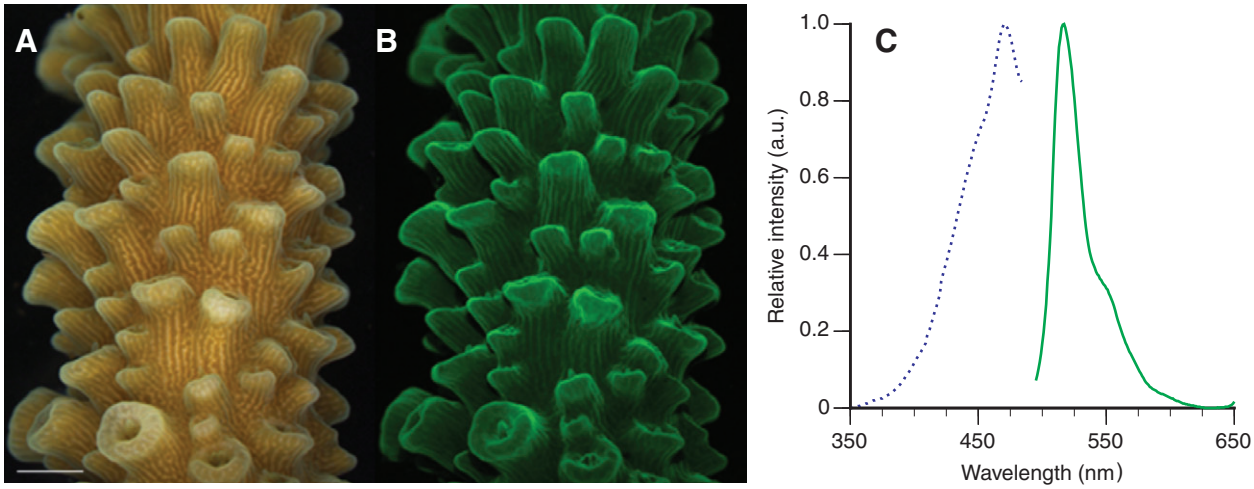

Fig. 1. Optical properties of *Acropora yongei*. Images with (A) white light illumination and (B) blue light excitation (470 nm) with a long-pass filter (transmission >500 nm). Scale bar represents 2 mm. (C) Spectral characteristics showing the excitation spectrum (dotted line) of emission at 517 nm,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

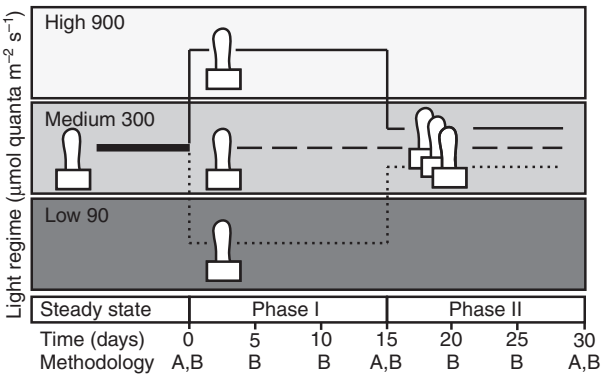

Fig. 2. Schematic diagram of the photoacclimation experiment design. At the beginning of the steady-state phase, corals were fragmented and placed under medium light for 2–4 weeks. At the beginning of phase I, corals were divided into three light intensity treatments for 15 days. At the beginning of phase II, corals were returned to medium light for 15 days. 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 photosystem II (PSII) and growth rate.

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 (Fig. 2). At the beginning of phase I, corals were placed in three light treatments for 15 days (days 0–15): 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 days (days 15–30). Every 5 days, corals (day 0: N=40, days 5–15: N=11–12 per treatment; days 20–30: N=5–6 per treatment) were non-destructively measured for fluorescence emission, green fluorescence intensity, frequency distribution of green fluorescence, maximum quantum yield of PSII, and growth. A subset of coral samples (N=5–6) was collected at 0, 15 and 30 days 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 –80°C until further analyses.

Quantitative western blots (immunoblot)

Custom-made 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 (amino acids 80–97) and the result was a specific antibody that cross-reacted with a ~27 kDa protein (see supplementary material Fig. S1A), 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 mmol l^{-1} Tris-HCl (pH 6.8), 2% w/v SDS, 25 mmol l^{-1} dithiothreitol, 10 mmol l^{-1} EDTA, 4% w/v polyvinylpolypyrrolidone, 1% DMSO] and protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA). The homogenate was vortexed and incubated twice at 90°C 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 (supplementary material Fig. S1B). 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 membranes 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) 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 immunochemistry. Every 5 days during the experiment, the coral was imaged with an epifluorescence stereoscope (Nikon SMZ1500 with 100 W mercury lamp and filter cube with excitation at 450–490 nm and longpass emission barrier >500 nm; Melville, NY, USA) coupled to a Retiga 2000R color digital camera (Fig. 1; QImaging, Surrey, Canada). Images were taken under the same settings, which included an exposure time 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 Inc., 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 to 255. To examine whether changes in green fluorescence were the result of a particular region or the entire area of the 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 5 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). For two 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 using 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 450 ***g*** at 4°C for 10 min. The supernatant was removed and the dinoflagellate pellet resuspended in filtered seawater. The surface area of the underlying skeleton was calculated from its measured height and diameter 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 an Elzone II 5390 particle counter (Micromeritics, Norcross, GA, USA). The volume of ~2000 particles between 7 and 12 µm diameter was determined. The dinoflagellate concentration was normalized by the surface area of the coral piece, 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*, chlorophyll c_2, peridinin, 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 450 ***g*** at 4°C for 10 min. The supernatant was discarded and samples were extracted for 18 h at 4°C 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 mm×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 mol l^{-1}, 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 are reported in pg $cell^{-1}$.

Photochemical efficiency of PS 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; where F_v is variable fluorescence and F_m is maximum fluorescence) was measured pre-dawn similar to previously described methods (Rodrigues et al., 2008; Warner et al., 1996). 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 from which the spectroscopy and optical fluorescence measurements were taken. Measurements were taken every 5 days during phases I and II of the experiment.

Linear extension growth

The growth rate of the corals was determined by linear extension from the tip of the coral. Digital images were captured through the stereoscope (Nikon SMZ1500) under white light every 5 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 in extension 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 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 of light treatments on green fluorescence intensity, frequency distribution of the 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 α=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 the others (Table 1; Fig. 3A; supplementary material Fig. S2). The GFP concentration of the HL corals increased ~1.6 times compared with the ML control corals, while that of the LL corals decreased ~4.0 times compared with the ML corals. At the end of phase II, GFP concentrations of HL and

LL returned to similar levels to those of ML corals and these differences were no longer significant.

Green fluorescence was dynamic and reversible under the 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 the others (Table 1; Fig. 3B; supplementary material Fig. S2). The green fluorescence of HL corals increased ~1.9 times compared to ML corals, while that of LL corals decreased ~1.9 times compared with ML corals. At the end of phase II, the green fluorescence pixel intensity in phase I LL and ML corals was similar while that of the HL corals remained significantly different and higher.

Green fluorescence intensity at the end of phase I was significantly correlated with GFP concentration (Fig. 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 showed a change in GFP concentration as opposed to the change occurring in only a small part of the coral (Fig. 4). The frequency distributions were unimodal. There was a shift in the peak 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 with the ML corals (Fig. 4B). The peak pixel intensities among the treatments were significantly different from each other (Table 1). The HL corals peaked at a 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 from the ML and LL corals (Fig. 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.R., 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 whose green channel distribution curves were smooth (M.S.R., unpublished). Therefore, the jagged pattern in the curves is not likely to be caused by the coral anatomy, but rather is a

Table 1. Summary of analysis of variance (ANOVA) statistics to test differences among treatment groups at the end of phase I (day 15) and phase II (day 30)

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
	II	30	17.6	2,13	0.06			
Green fluorescence pixel intensity	I	15	117.7	2,31	***	A	B	C
	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
	II	30	6.6	2,13	*	A	A	B
FWHM	I	15	0.1	2,29	0.94			
	II	30	0.6	2,13	0.57			
Growth rate	I	15	14.2	2,31	***	A	B	B
	II	30	0.6	2,13	0.66			
Conjugated ubiquitin concentration	I	15	0.3	2,15	0.95			
	II	30	1.3	2,13	0.80			
Dinoflagellate								
Maximum quantum yield of PS II	I	15	30.0	2,31	***	A	B	C
	II	30	5.4	2,13	*	A,B	A	B
Dinoflagellate density	I	15	106.8	2,15	***	A	B	B
	II	30	120.7	2,13	**	A	A,B	B
Chlorophyll *a*	I	15	4.8	2,15	*	A	A	B
	II	30	1.3	2,13	0.30			
Chlorophyll c_2	I	15	6.2	2,15	*	A	A,B	B
	II	30	2.1	2,13	0.16			
Chlorophyll c_2:chlorophyll *a*	I	15	2.2	2,15	0.14			
	II	30	1.5	2,13	0.27			
Peridinin	I	15	5.8	2,15	*	A	A	B
	II	30	1.1	2,13	0.35			
Peridinin:chlorophyll *a*	I	15	2.1	2,15	0.15			
	II	30	0.3	2,13	0.77			
Carotene	I	15	1.4	2,15	0.27			
	II	30	1.5	2,13	0.27			
Carotene:chlorophyll *a*	I	15	13.2	2,15	***	A	A	B
	II	30	0.2	2,13	0.83			
Dd+Dt:chlorophyll *a*	I	15	13.6	2,15	***	A	A	B
	II	30	1.0	2,13	0.40			

Post-hoc pairwise grouping represent the results from Tukey pairwise comparisons with differences P<0.05.
*P<0.05; **P<0.01; ***P<0.001.
Dd, diadinoxanthin; Dt, diatoxanthin.

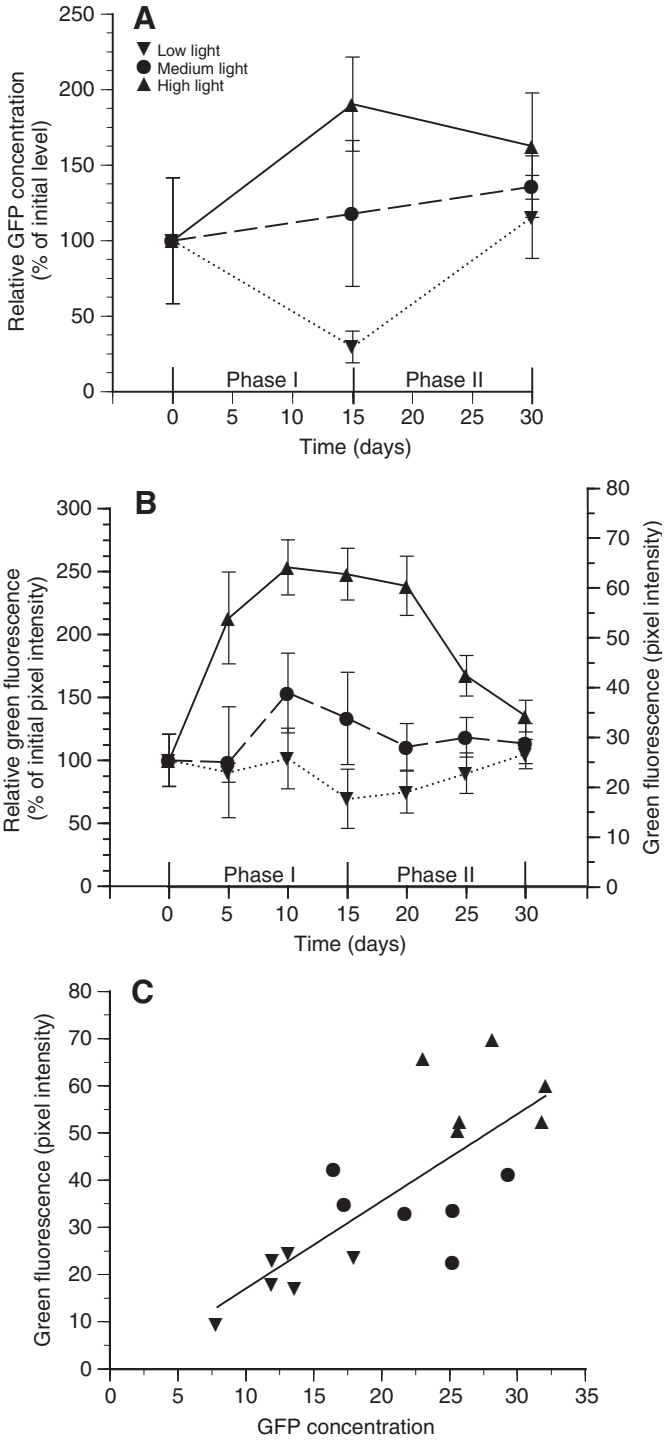

Fig. 3. Photoacclimation of the green fluorescent protein of *A. yongei*. (A) GFP concentration represented as treatment means ± s.d. (*N*=5–6) as relative to initial level at day 0. (B) Green fluorescence pixel intensity represented as treatment means ± s.d. (*N*=40 for day 0, *N*=11–12 for days 5–15, *N*=5–6 for days 20–30). (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.

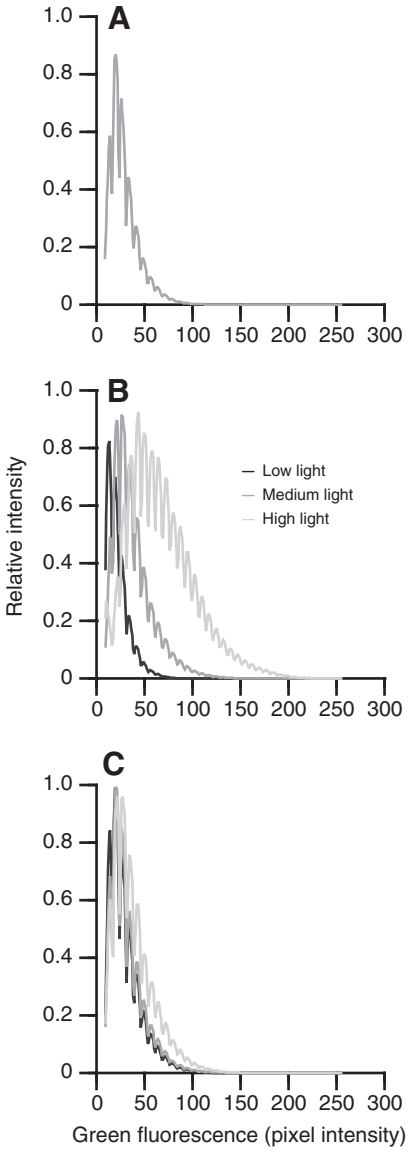

Fig. 4. Frequency distribution of green fluorescence intensity of *A. yongei*. Average pixel intensity frequency distribution from images taken of corals at (A) the end of steady-state phase (day 0, *N*=40), (B) the end of phase I (day 15, *N*=11–12), and (C) the end of phase II (day 30, *N*=5–6).

property of FPs in corals, perhaps from the protein arrangement or position in the coral.

The fluorescence emission spectrum (Fig. 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 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 with ML corals (Fig. 5); each treatment was significantly different from the others (Table 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 changed significantly during the experiment (Table 2). At the end of phase I, there was a significant

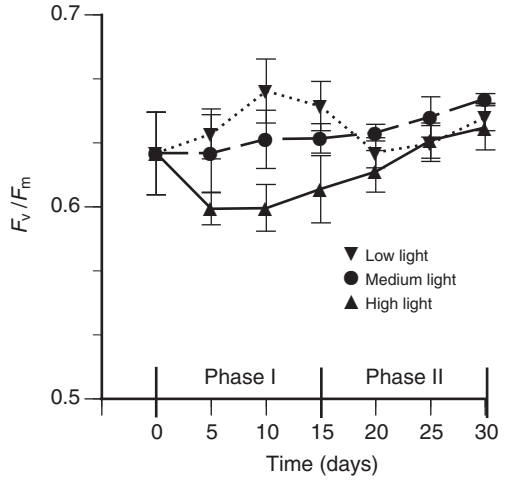

Fig. 5. Photoacclimation of the photochemical efficiency of PSII of *A. yongei*, measured as pre-dawn maximum quantum yield (F_v/F_m). Values are means ± s.d. (N=38 for day 0, N=11–12 for days 5–15, N=5–6 for days 20–30).

difference in dinoflagellate density among treatments (Table 1); the density in LL corals was 36% lower and significantly different from 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 1 and 2). There was a trend of a higher ratio of chlorophyll c_2 to chlorophyll *a* and of peridinin to chlorophyll *a* in the HL treatment, but the differences were not significant. At the end of phase II, none of the observed differences were significant. Because of the changes in chlorophyll *a*, the ratio of carotene to chlorophyll *a* and of Dd+Dt to chlorophyll *a* was significantly different at the end of phase I, when the corals showed slightly greater relative concentrations of photoprotective pigments under HL (Tables 1 and 2). At the end of phase II of the light treatment, these ratios had returned to their initial values and showed no significant difference among treatment groups. 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).

Coral condition during photoacclimation

All corals survived and grew measurably during phases I and II regardless of treatment. From days 10 to 15 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 from and lower than that of ML and HL corals (Table 1). At the end of phase II (days 25–30), 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 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 (Fig. 6). Both the correlation between growth rate and relative GFP concentration ($F_{1,16}$=11.2, P<0.01, R^2=0.41) and that between 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 the 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

Table 2. Dinoflagellate density and photosynthetic pigment composition from *Acropora yongei* during photoacclimation experiment

	Pre-experiment (day 0)	Phase I (day 15)			Phase II (day 30)		
Light regime	ML	LL	ML	HL	LL	ML	HL
Density ($\times10^6$ 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)
Chlorophyll *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)
Chlorophyll 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)
Chlorophyll c_2:chlorophyll *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)
Peridinin (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)
Peridinin:chlorophyll *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:chlorophyll *a* $\times10^{-2}$	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:chlorophyll *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) $\times10^{-2}$	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)

Values are treatment means (s.d.) (N=5–6).

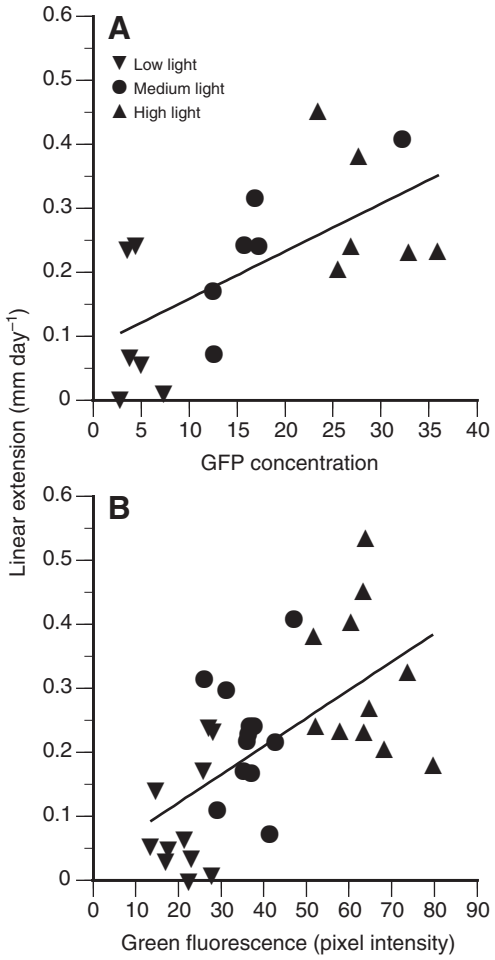

Fig. 6. Positive correlations between green fluorescent protein and coral growth rate. Lines represent simple correlations between linear extension (from days 10 to 15) and (A) relative GFP concentration at day 15 and (B) green fluorescence at day 15.

optimize photosynthesis and provide photoprotection to both the coral and its symbionts. This study provides evidence that suggests GFP may be important to modulate photosynthesis and supports the hypothesis that GFP has 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 *A. yongei*. Both an increase and a 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 are 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. The results are also consistent with lower green fluorescence in corals under low light (80–100 µmol quanta m^{-2} s^{-1}) compared with higher light conditions (400 µmol quanta m^{-2} s^{-1}) (D'Angelo et al., 2008). The study of D'Angelo and co-authors (D'Angelo et al., 2008) revealed no difference in green fluorescence at a light intensity >400 µmol quanta m^{-2} s^{-1}, whereas this study found a ~1.9 times increase in green fluorescence between ML and HL corals. A 10 day translocation experiment from the field to the laboratory (corresponding to a higher to lower light treatment) triggered 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. However, in that case, GFP-like proteins have a positive correlation with light intensity over long time scales of months (D'Angelo et al., 2008; Dove et al., 2008; Takabayashi and Hoegh-Guldberg, 1995). Corals with abundant blue-absorbing GFP-like protein (at 420 nm) appear much less efficient at producing O_2, implying a decreased photosynthetic rate with blue light illumination (Dove et al., 2008). The correlations of fluorescent proteins and GFP-like proteins with ambient light characteristics suggests that the light absorption properties of the fluorescent proteins is a critical component for contributions of these proteins in the photoacclimation response.

The increase in green fluorescence occurred in <5 days (within the 5 day resolution of the experiment) after an increase in light intensity, while decreases in green fluorescence were observed 10–15 days after the reduction in light intensity for the LL corals and 5–10 days 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 a 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 an 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 (Kuhl et al., 1995) and enhanced irradiance (Enriquez et al., 2005) within their cells. 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} (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 a shift to 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).

HL corals increased green fluorescence (~1.9 times) and GFP concentration (~1.6 times) in nearly a 1:1 relationship. However, there was a discrepancy between the ~1.9 times decrease in green fluorescence and ~4.0 times 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 the coral tissue becoming more transparent. 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 are 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.

In this study, only a single fluorescent pigment, with maximum emission at 516 nm, was found to be present in all corals irrespective of treatment. Corals that have multiple FPs can have FPs with differing responses, such that 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 with 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. The ratios of peridinin and chlorophyll c_2 to chlorophyll *a* did not shown any significant change, suggesting that the proportions of antennae and cores of the photosynthetic apparatus remained the same across treatments, thus confirming that the different light levels were not stressful. However, significant changes were observed with regards to the photoprotective pigments, thus reflecting photoacclimation; indeed, the ratios of carotene and total xanthophyll pool to chlorophyll *a* increased in HL corals. The recovery of 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 shallow coral reefs (Lesser et al., 1990; Lesser et al., 2000). Therefore, this study shows that GFP concentrations change in the absence of exposure of the photosynthetic apparatus to damaging light and reinforces the notion that GFP concentration can be regulated as a photoacclimation strategy, and not a stress response.

Photoacclimation response by endosymbiotic dinoflagellates

In contrast to most previous coral symbiont photoacclimation studies, which have examined corals from a natural light gradient due to their depth of occurrence, field transplant studies, seasonality and outside tank experiments (Falkowski and Dubinsky, 1981; McCloskey and Muscatine, 1984; Titlyanov et al., 2001; Ulstrup et al., 2008; Warner et al., 2002), this study involved a highly controlled laboratory experiment with careful manipulations of light intensity. We also used as a model *A. yongei*, a shallow-water coral that 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 the 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 the level present in ML control corals during the 15 days 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).

In the present study, the increase in maximum quantum yield in LL corals and decrease in HL corals during phase I was 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 reversibility in maximum quantum yield when corals were returned to original light conditions in phase II indicated rapid photoacclimation and that the HL corals had not experienced long-term photodamage during phase I.

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

Although the changes in light intensity between treatments were considerable, the photoacclimation response of the photosynthetic efficiency of PSII was rather limited in comparison to the *in vitro* photoacclimation responses of cultured dinoflagellates. Photoacclimation in cultured endosymbiotic dinoflagellates provide the most relevant reference to assess the capacity and extent of the changes observed in the photosynthetic system. In this study, endosymbiotic dinoflagellates in the HL corals had 22% less chlorophyll *a* per cell than those 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 in LL treatments (Hennige et al., 2009; Iglesias-Prieto and Trench, 1994; Robison and Warner, 2006). Additionally, those experiments had a smaller difference between their light treatments (6 times) than what was used in this study (30 times). 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 those in the LL corals, while dinoflagellates in culture have 51% less chlorophyll c_2 per cell and 52% less peridinin per cell than those in 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 from 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* (Hennige et al., 2009; Warner et al., 2002). Responses of the photosynthetic pigment and photochemical efficiency observed in our study were reduced compared with those in culture described in the literature (Hennige et al., 2009; Iglesias-Prieto and Trench, 1994; Robison and Warner, 2006). This study supports the hypothesis that coral GFP modifies 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, is 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 was examined in comparison to the 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 light of the same wavelength. 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 ratio of chlorophyll c_2 to chlorophyll *a* and of peridinin to chlorophyll *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* (Hennige et al., 2009; Iglesias-Prieto and Trench, 1994). If endosymbiotic dinoflagellates maintain relatively more peridinin and chlorophyll c_2 to chlorophyll *a* under HL than dinoflagellates in culture, there is a greater opportunity for GFP to play a photoprotective role. However, changes in photosynthetic pigments with irradiance are non-linear (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 (Anthony et al., 2008; Hoegh-Guldberg et al., 2007). Scientists and coral reef 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 adds to the complexity of using fluorescence as a coral health indicator at this stage, but warrants further characterization of such a possibility.

As a start for such a characterization, fluorescence changes in corals have 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 among 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 with 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 the global health of corals through large and long time scale environmental changes. In conclusion, this study describes the possible range of variation of GFP for a model coral under 'normal' non-stressful conditions. 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 explore the use of fluorescence as a tool for monitoring coral health. In particular, studies on the response of coral fluorescence to different environmental stressors will be instrumental in deciding whether, how and for which coral species fluorescence could be used as an indicator of coral health. To date it has been found that the expression of GFP homologs is down-regulated with heat stress (Smith-Keune and Dove, 2008), and bleached corals in the field have reduced green fluorescence (Salih et al., 2000). Additional controlled laboratory studies on coral fluorescence under various environmental conditions will provide results that, when combined with measurements made directly in the field, will further validate (or not) the use 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 of 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. Levels of both fluorescent proteins and their non-fluorescing homologs are correlated with light intensity (this study) (D'Angelo et al., 2008; Dove et al., 2008; Takabayashi and Hoegh-Guldberg, 1995), suggesting that light absorption is a critical aspect of the GFP function in corals and the responses of FPs are consistent with photoprotective-type functions. 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 density, that occur in 8–40 days (Titlyanov et al., 2001). This study provides evidence that 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 may involve a hierarchy of responses, where the primary response is by the host coral and does not involve changes in photosynthetic 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

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
LL	low light treatment
ML	medium light treatment
PAM	pulse amplitude modulated
PSII	photosystem II

ACKNOWLEDGEMENTS

This research was supported by a National Science Foundation Graduate Research Fellowship (M.S.R.); the Scripps Institution of Oceanography's John Dove Isaacs Professorship of Natural Philosophy (Nancy Knowlton); the Air Force Office of Scientific Research Biomimetics, Biomaterials, and Biointerfacial Sciences program (grant no. FA9550-07-1-0027; D.D.D. and M.I.L.); and the Department of Scripps Institution of Oceanography (M.S.R.). The authors would like to thank M. Roadman for assistance with HPLC, E. Kisfaludy and F. Nosratpour for aquarium support, the Birch Aquarium at the Scripps Institution of Oceanography for providing corals, V. Vacquier and M. Landry for use of equipment, G. Moy for antibody development support, B. Magit for general assistance, S. Bornheimer and anonymous reviewers for comments on the manuscript.

REFERENCES

Alieva, N. O., Konzen, K. A., Field, S. F., Meleshkevitch, E. A., Hunt, M. E., Beltran-Ramirez, V., Miller, D. J., Wiedenmann, J., Salih, A. and Matz, M. V. (2008). Diversity and evolution of coral fluorescent proteins. *PLoS ONE* **3**, e2680.

Anthony, K. R. N., Kline, D. I., Diaz-Pulido, G., Dove, S. and Hoegh-Guldberg, O. (2008). Ocean acidification causes bleaching and productivity loss in coral reef builders. *Proc. Natl. Acad. Sci. USA* **105**, 17442-17446.

Bay, L. K., Ulstrup, K. E., Nielsen, H. B., Jarmer, H., Goffard, N., Willis, B. L., Miller, D. J. and Van Oppen, M. J. H. (2009). Microarray analysis reveals transcriptional plasticity in the reef building coral *Acropora millepora*. *Mol. Ecol.* **18**, 3062-3075.

Bou-Abdallah, F., Chasteen, N. D. and Lesser, M. P. (2006). Quenching of superoxide radicals by green fluorescent protein. *Biochim. Biophys. Acta* **1760**, 1690-1695.

Brown, B. E. and Suharsono (1990). Damage and recovery of coral reefs affected by El Niño related seawater warming in the Thousand Islands, Indonesia. *Coral Reefs* **8**, 163-170.

Brown, B. E., Dunne, R. P., Ambarsari, I., Le Tissier, M. D. A. and Satapoomin, U. (1999). Seasonal fluctuations in environmental factors and variations in symbiotic algae and chlorophyll pigments in four Indo-Pacific coral species. *Mar. Ecol. Prog. Ser.* **191**, 53-69.

Bulina, M. E., Chudakov, D. M., Mudrik, N. N. and Lukyanov, K. A. (2002). Interconversion of Anthozoa GFP-like fluorescent and non-fluorescent proteins by mutagenesis. *BMC Biochem.* **3**, 7.

Chang, S. S., Prezelin, B. B. and Trench, R. K. (1983). Mechanisms of photoadaptation in 3 strains of the symbiotic dinoflagellate *Symbiodinium microadriaticum*. *Mar. Biol.* **76**, 219-229.

D'Angelo, C., Denzel, A., Vogt, A., Matz, M. V., Oswald, F., Salih, A., Nienhaus, G. U. and Wiedenmann, J. (2008). Blue light regulation of host pigment in reef-building corals. *Mar. Ecol. Prog. Ser.* **364**, 97-106.

Dove, S. (2004). Scleractinian corals with photoprotective host pigments are hypersensitive to thermal bleaching. *Mar. Ecol. Prog. Ser.* **272**, 99-116.

Dove, S. G., Takabayashi, M. and HoeghGuldberg, O. (1995). Isolation and partial characterization of the pink and blue pigments of pocilloporid and acroporid corals. *Biol. Bull.* **189**, 288-297.

Dove, S. G., Hoegh-Guldberg, O. and Ranganathan, S. (2001). Major colour patterns of reef-building corals are due to a family of GFP-like proteins. *Coral Reefs* **19**, 197-204.

Dove, S. G., Lovell, C., Fine, M., Deckenback, J., Hoegh-Guldberg, O., Iglesias-Prieto, R. and Anthony, K. R. N. (2008). Host pigments: potential facilitators of photosynthesis in coral symbioses. *Plant Cell Environ.* **31**, 1523-1533.

Dubinsky, Z. and Jokiel, P. L. (1994). The ratio of energy and nutrient fluxes regulates the symbiosis between zooxanthellae and corals. *Pac. Sci.* **48**, 313-324.

Dunlap, W. C. and Chalker, B. E. (1986). Identification and quantitation of near-UV absorbing compounds (S-320) in a hermatypic scleractinian. *Coral Reefs* **5**, 155-159.

Enriquez, S., Mendez, E. R. and Iglesias-Prieto, R. (2005). Multiple scattering on coral skeletons enhances light absorption by symbiotic algae. *Limnol. Oceanogr.* **50**, 1025-1032.

Falkowski, P. G. and Dubinsky, Z. (1981). Light-shade adaptation of *Stylophora pistillata*, a hermatypic coral from the Gulf of Eilat. *Nature* **289**, 172-174.

Falkowski, P. G., Jokiel, P. L. and Kinzie, R. A. (1990). Irradiance and corals. In *Ecosystems of the World: Coral Reefs*, Vol. 25 (ed. Z. Dubinsky), pp. 89-107. Amsterdam: Elsevier.

Field, S. F., Bulina, M. Y., Kelmanson, I. V., Bielawski, J. P. and Matz, M. V. (2006). Adaptive evolution of multicolored fluorescent proteins in reef-building corals. *J. Mol. Evol.* **62**, 332-339.

Fitt, W. K. and Warner, M. E. (1995). Bleaching patterns of four species of Caribbean reef corals. *Biol. Bull.* **189**, 298-307.

Goericke, R. and Welschmeyer, N. A. (1992). Pigment turnover in the marine diatom *Thalassiosira wesisflogii*. 2. The $^{14}CO_2$-labeling kinetics of carotenoids. *J. Phycol.* **28**, 507-517.

Gorbunov, M. Y. and Falkowski, P. G. (2002). Photoreceptors in the cnidarian hosts allow symbiotic corals to sense blue moonlight. *Limnol. Oceanogr.* **47**, 309-315.

Goreau, T. F. (1959). The physiology of skeleton formation in corals. 1. A method for measuring the rate of calcium deposition by corals under different conditions. *Biol. Bull.* **116**, 59-75.

Gruber, D. F., Kao, H. T., Janoschka, S., Tsai, J. and Pieribone, V. A. (2008). Patterns of fluorescent protein expression in scleractinian corals. *Biol. Bull.* **215**, 143-154.

Heim, R., Prasher, D. C. and Tsien, R. Y. (1994). Wavelength mutations and posttranslational autoxidation of green fluorescent protein. *Proc. Natl. Acad. Sci. USA* **91**, 12501-12504.

Hennige, S. J., Suggett, D. J., Warner, M. E., McDougall, K. E. and Smith, D. J. (2009). Photobiology of *Symbiodinium* revisited: bio-physical and bio-optical signatures. *Coral Reefs* **28**, 179-195.

Hoegh-Guldberg, O. (1999). Climate change, coral bleaching and the future of the world's coral reefs. *Mar. Freshw. Res.* **50**, 839-866.

Hoegh-Guldberg, O., Mumby, P. J., Hooten, A. J., Steneck, R. S., Greenfield, P., Gomez, E., Harvell, C. D., Sale, P. F., Edwards, A. J., Caldeira, K. et al. (2007). Coral reefs under rapid climate change and ocean acidification. *Science* **318**, 1737-1742.

Iglesias-Prieto, R. and Trench, R. K. (1994). Acclimation and adaptation to irradiance in symbiotic dinoflagellates.1. Responses of the photosynthetic unit to changes in photon flux-density. *Mar. Ecol. Prog. Ser.* **113**, 163-175.

Kaniewska, P., Campbell, P. R., Fine, M. and Hoegh-Guldberg, O. (2009). Phototropic growth in a reef flat acroporid branching coral species. *J. Exp. Biol.* **212**, 662-667.

Kawaguti, S. (1944). On the physiology of reef corals. VI. Study on the pigments. *Contrib. Palao Trop. Biol. Station* **2**, 616-673.

Kawaguti, S. (1969). The effect of green fluorescent pigment on the productivity of the reef corals. *Micronesica* **5**, 313.

Kinzie, R. A., Jokiel, P. L. and York, R. (1984). Effects of light of altered spectral composition on coral zooxanthellae associations and on zooxanthellae *in vitro*. *Mar. Biol.* **78**, 239-248.

Kuhl, M., Cohen, Y., Dalsgaard, T., Jorgensen, B. B. and Revsbech, N. P. (1995). Microenvironment and photosynthesis of zooxanthellae in scleractinian corals studied with microsensors for O_2, pH and light. *Mar. Ecol. Prog. Ser.* **117**, 159-172.

Labas, Y. A., Gurskaya, N. G., Yanushevich, Y. G., Fradkov, A. F., Lukyanov, K. A., Lukyanov, S. A. and Matz, M. V. (2002). Diversity and evolution of the green fluorescent protein family. *Proc. Natl. Acad. Sci. USA* **99**, 4256-4261.

Lesser, M. P. (1996). Elevated temperatures and ultraviolet radiation cause oxidative stress and inhibit photosynthesis in symbiotic dinoflagellates. *Limnol. Oceanogr.* **41**, 271-283.

Lesser, M. P. (1997). Oxidative stress causes coral bleaching during exposure to elevated temperatures. *Coral Reefs* **16**, 187-192.

Lesser, M. P. (2006). Oxidative stress in marine environments: Biochemistry and physiological ecology. *Annu. Rev. Physiol.* **68**, 253-278.

Lesser, M. P. and Farrell, J. H. (2004). Exposure to solar radiation increases damage to both host tissues and algal symbionts of corals during thermal stress. *Coral Reefs* **23**, 367-377.

Lesser, M. P. and Shick, J. M. (1989). Effects of irradiance and ultraviolet radiation on photoadaptation in the zooxanthellae of *Aiptasia pallida*: Primary production, photoinhibition, and enzymic defences against oxygen toxicity. *Mar. Biol.* **102**, 243-255.

Lesser, M. P., Stochaj, W. R., Tapley, D. W. and Shick, J. M. (1990). Bleaching in coral reef anthozoans: Effects of irradiance, ultraviolet radiation, and temperature on the activities of protective enzymens against active oxygen. *Coral Reefs* **8**, 225-232.

Lesser, M. P., Mazel, C., Phinney, D. and Yentsch, C. S. (2000). Light absorption and utilization by colonies of the congeneric hermatypic corals *Montastraea faveolata* and *Montastraea cavernosa*. *Limnol. Oceanogr.* **45**, 76-86.

Leutenegger, A., D'Angelo, C., Matz, M. V., Denzel, A., Oswald, F., Salih, A., Nienhaus, G. U. and Wiedenmann, J. (2007). It's cheap to be colorful – Anthozoans show a slow turnover of GFP-like proteins. *FEBS J.* **274**, 2496-2505.

Levy, O., Dubinsky, Z. and Achituv, Y. (2003). Photobehavior of stony corals: responses to light spectra and intensity. *J. Exp. Biol.* **206**, 4041-4049.

Levy, O., Achituv, Y., Yacobi, Y. Z., Dubinsky, Z. and Stambler, N. (2006a). Diel 'tuning' of coral metabolism: physiological responses to light cues. *J. Exp. Biol.* **209**, 273-283.

Levy, O., Achituv, Y., Yacobi, Y. Z., Stambler, N. and Dubinsky, Z. (2006b). The impact of spectral composition and light periodicity on the activity of two antioxidant enzymes (SOD and CAT) in the coral *Favia favus*. *J. Exp. Mar. Biol. Ecol.* **328**, 35-46.

Levy, O., Appelbaum, L., Leggat, W., Gothlif, Y., Hayward, D. C., Miller, D. J. and Hoegh-Guldberg, O. (2007). Light-responsive cryptochromes from a simple multicellular animal, the coral *Acropora millepora*. *Science* **318**, 467-470.

Matz, M. V., Marshall, N. J. and Vorobyev, M. (2006). Symposium-in-print: green fluorescent protein and homologs. *Photochem. Photobiol.* **82**, 345-350.

Mazel, C. H. (1995). Spectral measurements of fluorescence emission in Caribbean cnidarians. *Mar. Ecol. Prog. Ser.* **120**, 185-191.

Mazel, C. H., Lesser, M. P., Gorbunov, M. Y., Barry, T. M., Farrell, J. H., Wyman, K. D. and Falkowski, P. G. (2003). Green-fluorescent proteins in Caribbean corals. *Limnol. Oceanogr.* **48**, 402-411.

McCloskey, L. R. and Muscatine, L. (1984). Production and respiration in the Red Sea coral *Stylophora pistillata* as a function of depth. *Proc. R. Soc. Lond. B Biol. Sci.* **222**, 215-230.

Muko, S., Kawasaki, K., Sakai, K., Takasu, F. and Shigesada, N. (2000). Morphological plasticity in the coral *Porites sillimaniani* and its adaptive significance. *Bull. Mar. Sci.* **66**, 225-239.

Muscatine, L. (1990). The role of symbiotic algae in carbon and energy flux in reef corals. In *Ecosystems of the World*, Vol. 25 (ed. Z. Dubinsky), pp. 75-87. Amsterdam: Elsevier.
Muscatine, L. and Porter, J. W. (1977). Reef corals: Mutualistic symbioses adapted to nutrient-poor environments. *Bioscience* **27**, 454-460.
Myers, M. R., Hardy, J. T., Mazel, C. H. and Dustan, P. (1999). Optical spectra and pigmentation of Caribbean reef corals and macroalgae. *Coral Reefs* **18**, 179-186.
Nishiyama, Y., Allakhverdiev, S. I. and Murata, N. (2006). A new paradigm for the action of reactive oxygen species in the photoinhibition of photosystem II. *Biochim. Biophys. Acta* **1757**, 742-749.
Oswald, F., Schmitt, F., Leutenegger, A., Ivanchenko, S., D'Angelo, C., Salih, A., Maslakova, S., Bulina, M., Schirmbeck, R., Nienhaus, G. U. et al. (2007). Contributions of host and symbiont pigments to the coloration of reef corals. *FEBS J.* **274**, 1102-1109.
Palmer, C. V., Roth, M. S. and Gates, R. D. (2009a). Red fluorescent protein responsible for pigmentation in trematode-infected *Porites compressa* tissues. *Biol. Bull.* **216**, 68-74.
Palmer, C. V., Modi, C. K. and Mydlarz, L. D. (2009b). Coral fluorescent proteins as antioxidants. *PLoS ONE* **4**, e7298.
Porter, J. W., Muscatine, L., Dubinsky, Z. and Falkowski, P. G. (1984). Primary production and photoadaptation in light-adapted and shade-adapted colonies of the symbiotic coral, *Stylophora pistillata*. *Proc. R. Soc. Lond. B Biol. Sci.* **222**, 161-180.
Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G. and Cormier, M. J. (1992). Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* **111**, 229-233.
Robison, J. D. and Warner, M. E. (2006). Differential impacts of photoacclimation and thermal stress on the photobiology of four different phylotypes of *Symbiodinium* (Pyrrhophyta). *J. Phycol.* **42**, 568-579.
Rodrigues, L. J., Grottoli, A. G. and Lesser, M. P. (2008). Long-term changes in the chlorophyll fluorescence of bleached and recovering corals from Hawaii. *J. Exp. Biol.* **211**, 2502-2509.
Salih, A., Larkum, A., Cox, G., Kuhl, M. and Hoegh-Guldberg, O. (2000). Fluorescent pigments in corals are photoprotective. *Nature* **408**, 850-853.
Shick, J. M. (2004). The continuity and intensity of ultraviolet irradiation affect the kinetics of biosynthesis, accumulation, and conversion of mycosporine-like amino acids (MAAS) in the coral *Stylophora pistillata*. *Limnol. Oceanogr.* **49**, 442-458.
Shick, J. M., Lesser, M. P., Dunlap, W. C., Stochaj, W. R., Chalker, B. E. and Won, J. W. (1995). Depth-dependent responses to solar ultraviolet-radiation and oxidative stress in the zooxanthellate coral *Acropora microphthalma*. *Mar. Biol.* **122**, 41-51.
Smith-Keune, C. and Dove, S. (2008). Gene expression of a green fluorescent protein homolog as a host-specific biomarker of heat stress within a reef-building coral. *Mar. Biotechnol.* **10**, 166-180.
Sosik, H. M., Chisholm, S. W. and Olson, R. J. (1989). Chlorophyll fluorescence from single cells: interpretation of flow cytometric signals. *Limnol. Oceanogr.* **34**, 1749-1761.
Takabayashi, M. and Hoegh-Guldberg, O. (1995). Ecological and physiological differences between two colour morphs of the coral *Pocillopora damicornis*. *Mar. Biol.* **123**, 705-714.
Titlyanov, E. A., Titlyanova, T. V., Yamazato, K. and van Woesik, R. (2001). Photo-acclimation dynamics of the coral *Stylophora pistillata* to low and extremely low light. *J. Exp. Mar. Biol. Ecol.* **263**, 211-225.
Tsien, R. Y. (1998). The green fluorescent protein. *Annu. Rev. Biochem.* **67**, 509-544.
Ulstrup, K. E., Hill, R., van Oppen, M. J. H., Larkum, A. W. D. and Ralph, P. J. (2008). Seasonal variation in the photo-physiology of homogeneous and heterogeneous *Symbiodinium* consortia in two scleractinian corals. *Mar. Ecol. Prog. Ser.* **361**, 139-150.
Vermeij, M. J. A., Delvoye, L., Nieuwland, G. and Bak, R. P. M. (2002). Patterns in fluorescence over a Caribbean reef slope: The coral genus *Madracis*. *Photosynthetica* **40**, 423-429.
Warner, M. E., Fitt, W. K. and Schmidt, G. W. (1996). The effects of elevated temperature on the photosynthetic efficiency of zooxanthellae in hospite from four different species of reef coral: a novel approach. *Plant Cell Environ.* **19**, 291-299.
Warner, M. E., Chilcoat, G. C., McFarland, F. K. and Fitt, W. K. (2002). Seasonal fluctuations in the photosynthetic capacity of photosystem II in symbiotic dinoflagellates in the Caribbean reef-building coral *Montastraea*. *Mar. Biol.* **141**, 31-38.
Weis, V. M. (2008). Cellular mechanisms of Cnidarian bleaching: stress causes the collapse of symbiosis. *J. Exp. Biol.* **211**, 3059-3066.
Yellowlees, D. and Warner, M. (2003). Photosynthesis in symbiotic algae. In *Photosynthesis in Algae. Advances in Photosynthesis and Respiration*, Vol. 14 (ed. A. W. D. Larkum, S. E. Douglas and J. A. Raven), pp. 437-455. Dordrecht: Springer.