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Effects of cold stress and heat stress on coral fluorescence in reef-building corals

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Widespread temperature stress has caused catastrophic coral bleaching events that have been devastating for coral reefs. Here, we evaluate whether coral fluorescence could be utilized as a noninvasive assessment for coral health. We conducted cold and heat stress treatments on the branching coral *Acropora yongei*, and found that green fluorescent protein (GFP) concentration and fluorescence decreased with declining coral health, prior to initiation of bleaching. Ultimately, cold-treated corals acclimated and GFP concentration and fluorescence recovered. In contrast, heat-treated corals eventually bleached but showed strong fluorescence despite reduced GFP concentration, likely resulting from the large reduction in shading from decreased dinoflagellate density. Consequently, GFP concentration and fluorescence showed distinct correlations in non-bleached and bleached corals. Green fluorescence was positively correlated with dinoflagellate photobiology, but its closest correlation was with coral growth suggesting that green fluorescence could be used as a physiological proxy for health in some corals.

Coral reefs are not only one of the most diverse and productive ecosystems in the world, but also provide economic goods and ecosystem services valued at almost 400 billion a year[1]. Despite their great biological, economic, and societal importance, coral reefs are in worldwide decline as a result of both global (e.g. warming and ocean acidification) and local (e.g. pollution and over-fishing) stressors[2,3]. Stressors that co-occur could act in synergy, and thus possibly accelerate the decline of corals locally. Over the last 20 years there has been an annual loss of 1–2% coral cover throughout the Indo-Pacific[4]; yet such widespread chronic loss of corals is often punctuated by large mortality events when temperature anomalies occur[5].

Catastrophic coral bleaching events most often occur during periods of elevated temperature and high incident light[5,6]. However, low temperatures can also cause widespread coral bleaching and mortality[7,8]. Coral bleaching, which is the paling of corals as a result of the dissociation of corals and their endosymbiotic dinoflagellates, is caused by severe oxidative stress in the dinoflagellate photosynthetic apparatus and leads to damage in both the symbiont and the coral[9,10]. Bleaching indicates an unstable state and reef-building corals die if not re-populated with dinoflagellate symbionts in a timely manner. Bleached corals have reduced tissue regeneration[11], decreased growth[12,13], and even higher mortality[14]. Moreover, elevated temperature alone can cause lower calcification[14] and reproduction in corals[15,16]. Therefore, temperature stress and the susceptibility of coral to bleach are important factors to consider when assessing the health and future of coral reefs.

Today, observers and coastal managers evaluate coral bleaching in the field using change of coral color[17]. This methodology provides a good assessment of the extent of the coral bleaching, but cannot give any indications of coral health until the bleaching process has been initiated, and most often already well underway. Therefore, indicators of declining coral health prior to bleaching would be advantageous. Recent research on methods to determine coral health pre-bleaching includes destructive sampling of corals to measure specific cellular and molecular markers of stress[18] or measuring endosymbiotic dinoflagellate chlorophyll fluorescence representative of overall functionality of their photosynthetic ability[19,20]. Because of the increasingly high number of reefs at risk, there is a great incentive for scientists and coral reef managers to develop noninvasive tools permitting direct monitoring of coral growth and compromised health prior to potentially-lethal coral bleaching state, without destructive physical collection of the corals.

Corals produce large amounts of fluorescent proteins (FPs), homologous to the popular green fluorescent protein (GFP)[21]. FPs are ubiquitous in shallow reef-building corals[22,23] and can make up a significant portion of the total soluble protein in a coral[24]. FPs absorb potentially harmful high-energy photons of light and re-emit light

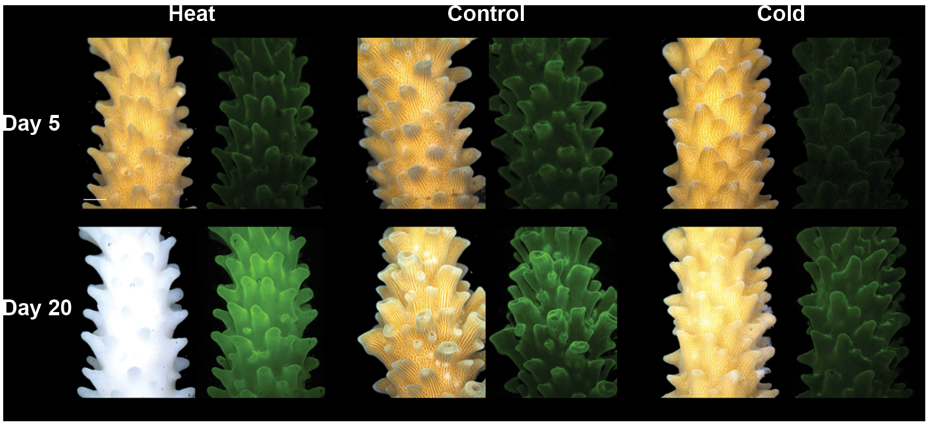

Figure 1 | Representative *Acropora yongei* samples from different treatments and time points during temperature change experiment. Each sample includes an image under white light (left panel) and blue light (excitation 470 ± 40 nm and longpass emission filter ≥500 nm; right panel); the same coral sample from each treatment is shown through time. Scale bar represents 2 mm.

with lower energy. A large diversity of FPs has been observed in corals[23], including ones that absorb light but do not re-emit in fluorescence, called GFP-like homologues or chromoproteins[25,26].

Despite the prevalence of fluorescent proteins in corals, their function remains ambiguous and controversial. The most parsimonious hypothesis is photoprotection[27], and experimentally, corals photoacclimate by changing fluorescent proteins concentrations[28,29]; however, in the field there is a lack of correlation between fluorescence intensity and level of ambient light (depth)[30–32]. A role in photosynthetic enhancement seems unlikely due to inefficient energy transfer between endosymbiotic dinoflagellates and host proteins[33]. Additional hypotheses include antioxidant capacity[34,35], regulation of dinoflagellate population[36,37], coral innate immune response[38,39], and camouflage[40]. It is probable that different fluorescent proteins will have different functions, thus providing corals with a mosaic of functional opportunities, such as suggested for other organisms[41]. However, the evolution of GFP-like homologues seems to indicate that the absorption properties of fluorescent proteins might be critical to their ecological function and/or that distinct types of fluorescent proteins could have different and possibly complementary functions. While the role of fluorescent proteins in corals remains unresolved, changes in fluorescent protein expression are one of the quickest and largest responses to changes in light[42] and heat[43,44]. This study explored the relationship between coral fluorescence and conventional assays usually considered to assess coral health.

Because of the inherent visual nature of the light signal emitted by FPs, they can be spectrally characterized and quantified in live corals non-invasively[28,45,46]. We conducted a cold (−5°C from maintenance temperature) and heat (+5°C from maintenance temperature) stress experiment for 20 d on the common Indo-Pacific reef-building coral *Acropora yongei*. Because *Acropora* corals are susceptible to coral bleaching[6] and *A. yongei* produces GFP abundantly and homogeneously with no spectral change during photoacclimation[28], *A. yongei* is a model organism to study the effects of temperature stress and coral fluorescence. In this study, we investigated the changes on GFP concentration and green fluorescence along with the temperature stress, correlated GFP concentration and green fluorescence, and correlated green fluorescence with various physiological parameters of the corals and endosymbiotic dinoflagellates. This study complements our previous work[13] that showed that cold stress was acutely more stressful than the heat stress, but that heat stress was ultimately more damaging for *A. yongei* where growth ceased, photosynthesis terminated, and the coral-algal symbiosis collapsed. The heat-treated corals, then bleached, offered the opportunity to examine the effect of bleaching on the level of green fluorescence over time. In this study, we focus on coral growth, dinoflagellate abundance, and photosynthetic parameters as indicators of coral health. Because FPs are amongst the most dynamic proteins responsive to environmental change[42–44], they might have the potential to help understand the adaptive physiology of corals to environmental constraints and possibly be used to non-invasively monitor coral health[28,45,46].

Results

Effect of temperature change on GFP. Temperature stress affected GFP fluorescence and concentration in addition to coral color (Fig. 1, Fig. 2). A decrease in GFP concentration was observed in both cold- and heat-treated corals, while an increase in GFP concentration was observed in control corals (Fig. 2). At 5 d, GFP concentration was 35% less in heat-treated corals and 65% less in cold-treated corals as

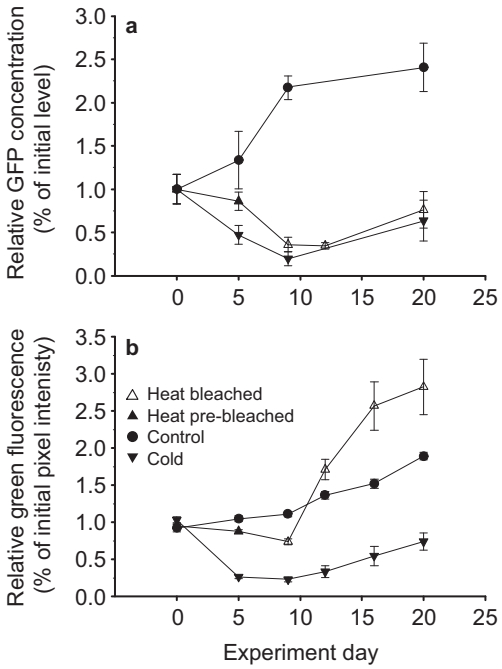

Figure 2 | Effect of temperature change on the green fluorescent protein in corals over time. (a) GFP concentration relative to initial levels (means ± s.e.m.; n = 4–5). (b) Green fluorescence (pixel intensity) relative to initials levels (means ± s.e.m.; n = 10–19 for 0–9 d, n = 4–9 for 12–20 d).

compared to the controls (Fig. 2a). For both temperature treatments, a minimum GFP concentration was reached during 9–12 d, which resulted in a 91% decrease in the cold treatment as compared to the controls. The GFP concentration in both cold and heat treatments recovered slightly by the end of the experiment, yet levels were still lower than for 0 d. The overall pattern of decrease and recovery in GFP concentration followed a similar profile between cold and heat treatments (Fig. 2a). The increase in GFP in the control corals may have resulted from more growing polyps in the sampled region over the course of the experiment, which can be seen in Fig. 1. The two-way ANOVA showed that both the treatment ($F_{2,43} = 34.7$, $p < 0.0001$) and time ($F_{2,43} = 4.1$, $p < 0.05$) were significant main effects, and that there was a significant interaction between treatment x time ($F_{4,43} = 3.2$, $p < 0.05$). The main variability in the experiment was explained by the experimental treatments (55%); time and treatment x time explained significantly less of the variability, 7% and 10% respectively. Post-hoc analyses showed that the response for each treatment was significantly different from one another, with GFP concentration being significantly different only between 9 d and 20 d (5 d and 20 d, and 5 d and 9 d were not statistically distinct). These data indicate that the recovery in GFP concentration at 20 d reached levels similar to the ones initially found at the onset of the treatment (5 d).

Green fluorescence of the corals initially showed a similar pattern of change to GFP concentration (Fig. 1, Fig. 2). Green fluorescence was less intense in both temperature treatments at 5 d as compared to the controls, and the cold treatment caused a more dramatic decrease in fluorescence than the heat treatment (Fig. 2b). At 9 d, the cold-treated corals had a 79% decrease in green fluorescence as compared to the controls, after when the green fluorescence in the cold-treated corals began to steadily recover. The green fluorescence in heat-treated corals rapidly rose after 9 d, surpassing the control corals by 12 d. The green fluorescence in the control corals steadily increased throughout the experiment, which probably reflected optimal growth and acclimation to the control experimental conditions. The two-way ANOVA showed that both treatment ($F_{2,69} = 160.9$, $p < 0.0001$) and time ($F_{4,69} = 74.3$, $p < 0.0001$) were significant main effects, and that there was a significant interaction between treatment x time ($F_{8,69} = 16.9$, $p < 0.0001$). All three components accounted for the variability in the experiment; treatment was the largest contributor (39%) followed by time (36%) and treatment x time (16%). Post-hoc analyses showed that the response for each treatment was significantly different from another, with green fluorescence being significantly different on all days except 5 d and 9 d, which were not significantly different from each other. These data indicate that green fluorescence was different at the end of the experiment from the onset of treatment.

GFP concentration and green fluorescence were positively correlated in both non-bleached ($y = 0.51\times + 5.47$, $F_{1,39} = 79.2$, $p < 0.0001$, $R^2 = 0.67$) and bleached corals ($y = 2.31\times + 10.92$, $F_{1,11} = 12.5$, $p < 0.01$, $R^2 = 0.53$), but with different relationships (Fig. 3a). The slope of GFP concentration and green fluorescence in bleached corals was ~5× the slope of control and pre-bleached corals, thus indicating a greater change in visual fluorescence in bleached corals.

For the duration of the experiment, corals in the cold and control treatments maintained a brownish coloration and did not bleach, which was in contrast to heat-treated corals that eventually bleached (Fig. 1): by 9 d 80% of heat-treated corals bleached, and by 12 d 100% of the heat-treated corals bleached and remained bleached until the end of the experiment. The largest decline in heat-treated coral dinoflagellate density was observed from 9 d to 12 d when a 15× reduction was observed, going from 9.11×10^5 cells cm^{-2} to 6.01×10^4 cells cm^{-2}. No mortality or tissue sloughing was observed in any treatment during the entire experiment. Transparent coral tissue containing green fluorescence was observed in all bleached corals (Fig. 1, Fig. 2, Supplementary video S1). In non-bleached corals, dinoflagellate density and green fluorescence were positively correlated ($r = 0.47$, $df = 40$, $p < 0.01$), but in bleached corals dinoflagellate density and green fluorescence were negatively correlated ($r = -0.82$, $df = 12$, $p < 0.001$) (Fig. 4a).

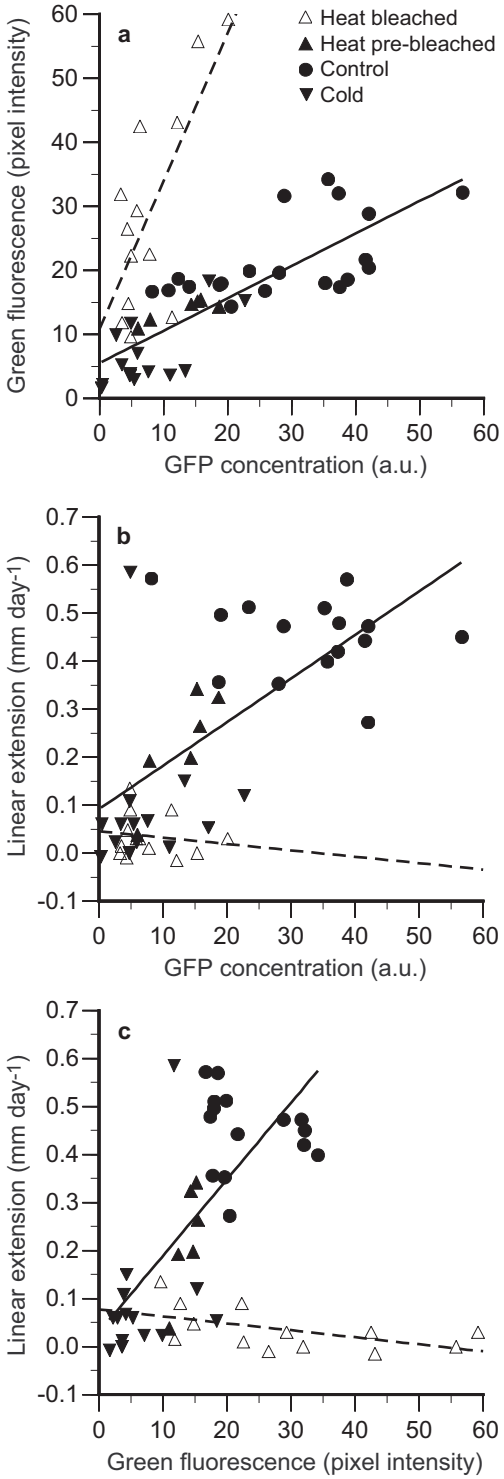

Figure 3 | Relationship between GFP concentration and green fluorescence (a) and correlation between coral growth and GFP (b–c). (a) GFP concentration and green fluorescence was distinctly correlated between non-bleached and bleached corals. Linear extension (coral growth) was significantly correlated with (b) GFP concentration and (c) green fluorescence for non-bleached corals, but not for bleached corals. In all panels, solid lines are for non-bleached corals, dashed lines for bleached corals.

Despite the changes in abundance of fluorescent proteins, green fluorescence (emission peak 516.61 ± 0.04 nm, FWHM 29.58 ± 0.08 nm) was the only color of fluorescence observed in all treatments throughout the experiment, while no other fluorescence was observed under the other excitation settings (see methods for details). The green fluorescence observed was always distributed homogeneously throughout the coral, present in both the coenosarc and polyps (as seen in Fig. 1).

Relationship between coral growth and GFP. Coral growth and GFP concentration as well as coral growth and green fluorescence were positively correlated in non-bleached corals (Fig. 3b–c; respectively, $r = 0.67$, $df = 35$, $p < 0.0001$ and $r = 0.73$, $df = 35$, $p < 0.0001$). In contrast, bleached corals showed no significant correlation between both coral growth and GFP concentration or coral growth and green fluorescence (respectively, $r = -0.15$, $df = 12$, $p = 0.62$ and $r = -0.53$, $df = 12$, $p = 0.06$). Both increasing and decreasing temperatures caused decreases in coral growth; however, cold-treated corals, which did not bleach, continued to grow and even increased growth rates toward the end of the experiment, while heat-treated corals that bleached ceased growing as previously described[13].

Relationship between green fluorescence and dinoflagellate photobiological characteristics. Dinoflagellate photobiological characteristics showed specific significant relationships with green fluorescence (Fig. 4). Effective quantum yield and maximum quantum yield were positively correlated with fluorescence in non-bleached corals ($r = 0.63$, $df = 40$, $p < 0.0001$ and $r = 0.43$, $df = 40$, $p < 0.01$, respectively), and negatively correlated in bleached corals ($r = -0.86$, $df = 6$, $p < 0.05$ and $r = -0.77$, $df = 7$, $p < 0.05$, respectively). In contrast, the pressure over PSII (Q_{max}) had a negative correlation with fluorescence in non-bleached corals ($r = -0.63$, $df = 40$, $p < 0.001$) and a positive correlation in bleached corals ($r = 0.86$, $df = 6$, $p < 0.05$). Changes in temperature decreased effective quantum yield and maximum quantum yield and increased pressure over PSII as previously described[13]. Bleached corals had dramatic declines in effective quantum yield and maximum quantum yield and rapid increases in pressure over PSII until it was no longer possible to obtain measurements due to the low density of dinoflagellates.

Discussion

This study showed that both heat (+5°C) and cold (−5°C) temperature changes strongly affected GFP concentration of corals: GFP concentration declined until ~9 d, and after which GFP concentration slowly started to recover. GFP declined during temperature change faster than the decay rate of FPs (half-life ~20 d[24]) and more rapidly than acclimation to reduced light[28]. FPs are energetically cheap to produce[24], and mature FPs are stable at high temperatures *in vitro*[21]. Therefore, the rapid reduction in GFP suggests that the protein was actively degraded and/or depleted, possibly from antioxidant activities[34,35], as opposed to resulting from a decline in

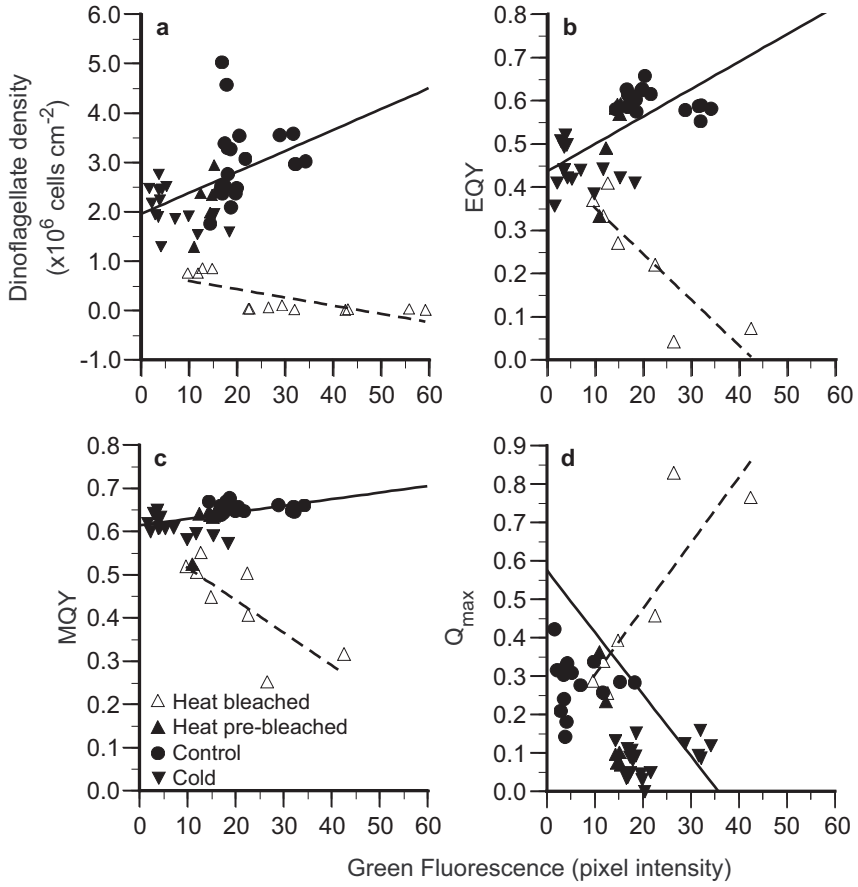

Figure 4 | Correlations between green fluorescence and dinoflagellate population characteristics. Green fluorescence was significantly correlated with (a) dinoflagellate density in non-bleached corals and in bleached corals, (b) effective quantum yield in non-bleached corals and in bleached corals, (c) maximum quantum yield in non-bleached corals and in bleached corals, and (d) pressure over PSII (Q_{max}) in non-bleached corals and bleached corals. Solid lines represent correlations between green fluorescence and non-bleached corals, and dashed lines represent correlations between green fluorescence and bleached corals. Chlorophyll measurements could not be obtained for the heat treatment at 20 d due to the large reduction in dinoflagellate density (see Methods).

production. Similarly, a decrease in GFP-like proteins was observed in naturally bleached sea anemones[47]. A decline in a GFP-homologue (non-fluorescent) during heat stress has also been reported in *Montipora monasteriata*[48]; all together these results support one role of the protein itself as an antioxidant leading to its depletion during stress.

This study incorporated multiple time points (covering short and long term effects) and showed the dynamics of GFP (both in abundance and fluorescence) through temperature change over time. These data may help explain some of the controversy about heat stress being reported in the literature to cause either up- and/or down-regulation of GFP-like homologues gene expression[49,50], probably reflecting differences amongst coral species used, but also time scale of the analyses performed. This has been one of the major controversies (or roadblocks) in using fluorescence as an optical property associated with ecological significance for corals[28,45,46]. It is likely however that the use of GFP fluorescence as an indicator of coral health could be highly reliable when measuring the same corals through time.

The overall pattern of GFP dynamics between heat and cold treatments was similar, showing first fluorescence decreasing with increasing stress and then increasing fluorescence (yet to different levels) with both recovery (for the cold stress) and coral bleaching (for the heat stress). In contrast, we have shown the symbiont response to be clearly distinct between the two treatments[13], and therefore the coral response appears somewhat independent of the symbiont response. Nonetheless it is important to note that the GFP concentration declined more rapidly in the cold than the heat treatment, similar to the changes in photobiological responses the symbionts experienced upon the same temperature changes[13]. Additionally, the trends of GFP concentration in both treatments suggest that temperature was initially more harmful for the coral but ultimately more harmful for the symbiotic dinoflagellate. The immediate reductions in coral growth were dramatic compared to the reductions in photosynthetic efficiency[13]. In trials of extended heat stress we observed corals still alive >20 days after the coral had bleached (M.S.R. and D.D.D. unpublished data). Bleached corals maintained under temperature stress until tissue necrosis contained intense green fluorescence despite being barely detectable in white light (Supplementary video S1).

GFP concentration and green fluorescence was significantly correlated in non-bleached corals as well as in bleached corals. However, the relationship changed when corals bleached. Green fluorescence showed distinct dynamics in cold and heat-treated corals in comparison to GFP concentration, mainly because of the bleaching that occurred in the heat treatment (see next paragraph). Green fluorescence in the cold treatment matched the GFP concentration response, showing a decrease in fluorescence that reached a low at 9 d and then recovering until the end of the experiment. The cold-treated corals appeared to acclimate to the temperature change by adjusting their photosynthetic efficiency and photoprotective pigments resulting in increased growth rates towards the end of the experiment[13], which was also reflected with the observed recovery of GFP concentration and green fluorescence.

Coral bleaching or the loss of endosymbiotic dinoflagellates resulted in an indirect increase in green fluorescence, as a side-effect of less shading from the dinoflagellates within the coral tissue. Indeed, in heat-treated corals, green fluorescence declined until 9 d, at which point the fluorescence increased to surpass the control treatment by 12 d, ending more than 1.5× above the control at 20 d. This timing coincides with 15× reduction in dinoflagellate density rather than an increase in GFP concentration. Large decreases in dinoflagellate density may allow for more excitation light to reach GFP and/or the resulting fluorescence emission of GFP to be less obstructed. Additionally, reduced dinoflagellate density may cause more reflection and scattering by the coral skeleton[51] and amplify the fluorescence output. Therefore, these data suggest that the presence versus absence of symbiotic dinoflagellates and their associated pigments can affect the optical fluorescence of GFP. Accordingly in bleached corals, there was a negative correlation between dinoflagellate density and green fluorescence intensity, which contrasted with the positive relationship in healthy corals. During a natural bleaching event in Australia, bleached corals had lower fluorescence than unbleached corals[22]. Salih and coauthors interpreted these results in terms of corals with more fluorescent proteins having more bleaching resistance. Additionally, the authors also reported large variability in FP pixel intensity in corals with comparable low-level dinoflagellate densities. Considering the results of the present study on the dynamics of bleaching and green fluorescence in a controlled setting would suggest that the corals that Salih and coauthors sampled had only recently bleached, which seems to agree with the timing of when the corals were sampled and analyzed[22].

In a heat stress experiment of *Montastrea faveolata,* the ratio of green to orange fluorescence switched when corals bleached and eventually died, then being mainly orange[52]. However, it was not determined if the source of the orange fluorescence was from coral FPs or phycoerythrin from endosymbiotic cyanobacteria[53]. In the present study, only one GFP was observed and it clearly declined (both in abundance and fluorescence output) with temperature change until bleaching; however, it is possible that other types of FPs may have different responses to temperature stress as well as different functions.

This experiment provides evidence that green fluorescence could be best used as a proxy for coral growth rate in non-bleached and pre-bleached colonies of the coral *A. yongei*. In the field, we have observed that coral growing regions, for example edges and tips, typically have brighter fluorescence (Panama; Fiji; Palmyra Atoll, USA; Hawaii, USA) as well as in our experiments with branching corals (M.S. Roth and D.D. Deheyn, pers. obs.). Recently, visual increases in fluorescence and chromoproteins and higher expression of transcripts of a growth antigen was observed in the growing regions of *Montipora foliosa*[39]. Growth, like reproduction, is often considered a higher-level metabolic/physiological process[54], with a much narrower threshold range than survival. Monitoring the growth of corals will be particularly valuable because sea level rise will require corals to grow faster while ocean acidification may also decrease coral productivity and metabolism[55,56]. However, coral growth rates on a reef are difficult to obtain because they require careful repeated measurements over a long period of time 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. Here, our data suggest that once a correlation between growth rate and green fluorescence is obtained for a specific species of coral, it may be possible to use green fluorescence to infer general growth information of a coral, even in the field.

Green fluorescence also had a strong correlation with endosymbiotic dinoflagellate physiological characteristics including both effective quantum yield and pressure over PSII. Rapid reductions in green fluorescence at the initiation of stress may suggest that green fluorescence could be used as an early indicator of change in health of the coral holobiont prior to conditions leading to bleaching or acclimation. This is an exciting prospect of the *in situ* use of fluorescence for remote coral health assessment; it is clear however that further research with different species of corals and different types of stressors are necessary to determine how general this response can be to the increasing number of environmental changes.

The time scale of decline in green fluorescence is an important component to distinguish from changes of acclimation, which have been previously observed over longer periods only[28,29] and may arise seasonally in the field. In this study, rapid declines in green fluorescence were observed both prior to coral bleaching in heat-treated corals and during degenerating health in cold-treated corals. The

cold-treated corals did not bleach, and their growth and green fluorescence eventually recovered as well. These data suggest that changes in green fluorescence are consistent with declines in coral health that are more subtle and potentially ecologically relevant than when reaching the bleaching process. Here, we present a conceptual model of coral color and GFP fluorescence for a shallow reef-building coral exposed to temperature stress (Fig. 5). We propose that the fluorescence intensity of a healthy coral on a reef has a range of fluctuation through time with seasonal shifts. During an environmental change that causes stress to the coral holobiont, the fluorescence rapidly declines. If the coral can acclimate, the fluorescence will slowly recover to pre-stress levels. However, if the coral continues to decline until reaching a bleaching state, then the fluorescence will dramatically increase to greater values than original ones due to the substantial loss of dinoflagellates that otherwise shade the fluorescence output. Coral color or bleaching seems to provide a delayed indication of coral health as compared to coral fluorescence, but more research and engineering would help develop the instrumentation and metrics for further establishment of this assay in the future.

In the field, coral fluorescence in most corals can be easily observed with a blue flashlight and yellow filter over one's snorkel mask[57], captured with a simple digital camera strobe with appropriate filters or underwater spectrophotometer[30]. Digital images could then be processed to quantify the fluorescence. Currently, scientists are developing underwater coral fluorescence monitoring systems[58]. For consistency between samples at different depths and differences in water turbidity, it will be important for the instrument to supply most if not all of the light to obtain the fluorescence measurement. Additionally, a set distance between the instrument and the coral as well as a fluorescence standard should be used to ensure reliability between measurements.

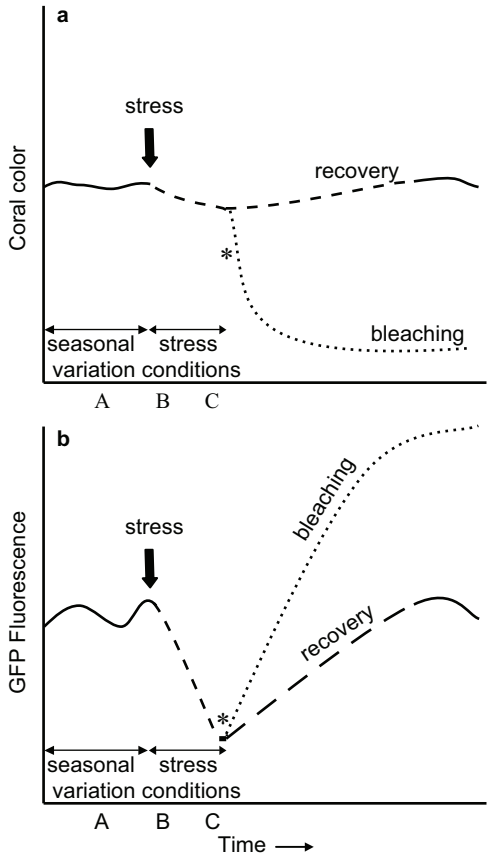

Figure 5 | Conceptual model of (a) coral color and (b) GFP fluorescence during the life of an adult coral colony. Phase (A) represents a healthy coral with seasonal variation (solid line), (B) indicates stress conditions such as an environmental change (dashed line), and (C) represents the trajectory of coral acclimating/recovering (long dashed line) or if the coral continues to decline until bleached (dotted line). The asterisk represents the onset of coral bleaching.

Our study focused on the biological aspects of coral fluorescence and is the first to follow over time coral fluorescence, GFP concentration, and health proxies during exposure to stressors in a well-controlled experimental setting. It establishes correlation between coral fluorescence and conventional coral health proxies currently in use, and provides evidence that the level of fluorescence intensity may be a useful proxy for coral growth in the field. We believe that in the future such study with biological emphasis together with engineering research will allow a digital imaging system to capture the visual nature and the quick responses in abundance of fluorescent proteins and indeed provide a noninvasive approach to assessing coral health prior to bleaching during times of increased environmental challenges.

Methods

Temperature experiment design. To determine the effects of temperature on coral GFP concentration and green fluorescence, parallel heat and cold treatments were applied to the common shallow-water branching coral *Acropora yongei*, which were originally obtained from the Birch Aquarium at the Scripps Institution of Oceanography, and subsequently cultured at the Marine Biology Research Division experimental aquarium at the Scripps Institution of Oceanography. *A. yongei* emits an 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[28]. Prior to the temperature experiment, *A. yongei* fragments (~5 cm) were attached to terracotta tiles with cyanoacrylic adhesive and maintained in individual 1 L glass aquaria (seawater flow rate ~0.7 L min^{-1}) and under 300 μmol quanta m^{-2} s^{-1} 12 : 12 h light:dark photoperiod (T5 Teklight, Sunlight Supply). Corals were maintained in steady-state at 26°C for 16 d during which coral tissue regenerated over the cut region and no coral mortality was observed. The subsequent 20 d experiment included a cold treatment (21°C), a heat treatment (31°C), and a control treatment (26°C). The temperature changes were introduced incrementally over a 5 h period starting 0 d at sunrise. Corals were non-invasively sampled for green fluorescence intensity, spectral analysis, coral growth rate and photosynthetic efficiency on days 0 (n = 15–19), 5 (n = 15–19), 9 (n = 10–14), 12 (n = 5–9), 16 (n = 4–5) and 20 (n = 4–5). A subset (destructive sampling) of each treatment (n = 4–5) was collected for GFP western blots and dinoflagellate abundance on days 0, 5, 9, 12 (heat treatment only), and 20.

GFP quantitative western blots. Quantitative western blots were used to determine the protein abundance of GFP in the corals. A 16 mm long piece of each experimental coral was used. The piece was collected from 8 mm below the tip of the coral to avoid the growth region of the coral, which may have different physiology. The protein extraction and quantitative western blot methodology were conducted as previously described[28]. 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 (based on coral peptide sequences and produced by Open Biosystems and Biosynthesis, Inc.) were used for immunoblotting; immunoblots were visualized with secondary antibodies with a peroxidase label and a chemiluminescence kit, and imaged on a high performance blot scanner (Typhoon 9410, 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 standard derived from healthy corals[28].

Green fluorescence intensity and fluorescence spectral analyses. Green fluorescence intensity was determined on live coral on a 16 mm long piece (8 mm from the tip, the same portion used for the immunochemistry analyses). 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 previously described[28]. An exposure of 0.20 s was used for fluorescence images. Additionally images of the same field of view were taken under white light to use in image processing. In brief, images were processed in MATLAB 7.5 (Mathworks) with which the average green fluorescence intensity was determined by taking the average pixel intensity of the coral (area determined by the white light image) in the green channel of the fluorescence image subtracted by the average background[28]. A photoacclimation experiment using these methods showed that GFP concentration and green fluorescence were positively correlated[28]. The fluorescence emission spectrum was measured with a low-light Echelle Spectrograph (SE200 Digital Spectrograph, Catalina Scientific) and probe using a 0.6 mm optic fiber on the live coral fragment using a micro-manipulator[28]. Emission was measured under ultraviolet (379–401 nm), cyan (426–446 nm), and blue (450–490 nm) excitation

light to screen for any possible change to fluorescence excitation. Because green fluorescence intensity was the only aspect of the fluorescence changing during the experiment, further references to green fluorescence refer to the intensity.

Dinoflagellate density. Dinoflagellate density was determined from a 1.5 cm long section of the coral (2.4 cm from the tip). Dinoflagellates were isolated with an artist's airbrush followed by centrifugation, as previously described[28]. The dinoflagellate density was determined in triplicate using a Neubauer ruled haemocytometer and normalized to the surface area of the coral (calculated by simple cylinder geometry). A bleached coral was defined as a coral with a dinoflagellate density below 1.0×10^6 cells cm^{-2} and verified by its white color (cfr heat coral at day 20; Fig. 1).

Coral growth rate. Coral growth rate was determined by linear extension of the tip of the coral calculated by white light digital imagery captured by the stereoscope (Nikon SMZ1500). The images were taken perpendicular to the growth axis on coral fragments fixed to square tiles to maintain orientation. 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.

Photosynthetic efficiency of photosystem II. Photosynthetic efficiency of photosystem II (PSII) was measured using a pulse amplitude-modulated (PAM) fluorometer (Diving-Pam, Walz). Measurements were taken ~1 cm below the tip of the coral on the same side of the coral as the spectroscopy and optical fluorescence measurements. The dark-acclimated maximum quantum yield (MQY) of PSII (F_v/F_m; F_v, variable fluorescence; F_m, maximum fluorescence) was measured pre-dawn, and the light-acclimated effective quantum yield (EQY) of PSII ($\Delta F/F_m'$) was measured at the corals' midday. The pressure over PSII was determined as: $Q_m = 1 - [(\Delta F/F_m' \text{ at noon})/(F_v/F_m \text{ at pre-dawn})]$[59]. Measurements could not be obtained for heat treatment corals on 20 d because the chlorophyll fluorescence was below the detection limits of the instrument.

Statistical analyses. Statistical analyses were conducted using JMP version 8.0 (SAS Institute, Inc.). Data were tested for assumptions of normality and homoscedasticity. Accordingly, GFP concentration data were log transformed prior to analyses. A two-way analysis of variance (ANOVA) was used to test the effects of temperature on GFP concentration, and a nested two-way ANOVA was used to test the effects of temperature on green fluorescence (nested factor was not significant; data not shown). The variability from the temperature treatment and time was determined by calculating the ratio between the sum of squares associated with the factor and the total sum of squares (all possible factors of variation + residual)[60]. 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. Data represent arithmetic means ± standard errors. A regression (model II) was used to determine the relationship between GFP concentration and green fluorescence. The correlations (co-variation) between GFP concentration/green fluorescence and growth, and green fluorescence and dinoflagellate characteristics were determined using Pearson's correlation coefficient. Statistical differences were all tested for significance at the $\alpha = 0.05$ level.

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Author contributions

M.S.R. and D.D.D. designed the research; M.S.R. performed the research and analyzed the data; and M.S.R. and D.D.D. wrote the manuscript.

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