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Report

Coral Bleaching Independent of Photosynthetic Activity

Dimitri Tolleter,[1,4] François O. Seneca,[2,4] Jan C. DeNofrio,[3] Cory J. Krediet,[3] Stephen R. Palumbi,[2] John R. Pringle,[3] and Arthur R. Grossman[1,*]

[1]Department of Plant Biology, Carnegie Institution for Science, Stanford, CA 94305, USA

[2]Department of Biology, Hopkins Marine Station, Stanford University, Pacific Grove, CA 93950, USA

[3]Department of Genetics, Stanford University School of Medicine, Stanford, CA 94305, USA

Summary

The global decline of reef-building corals is due in part to the loss of algal symbionts, or "bleaching," during the increasingly frequent periods of high seawater temperatures [1, 2]. During bleaching, endosymbiotic dinoflagellate algae (*Symbiodinium* spp.) either are lost from the animal tissue or lose their photosynthetic pigments, resulting in host mortality if the *Symbiodinium* populations fail to recover [3]. The >1,000 studies of the causes of heat-induced bleaching have focused overwhelmingly on the consequences of damage to algal photosynthetic processes [4–6], and the prevailing model for bleaching invokes a light-dependent generation of toxic reactive oxygen species (ROS) by heat-damaged chloroplasts as the primary trigger [6–8]. However, the precise mechanisms of bleaching remain unknown, and there is evidence for involvement of multiple cellular processes [9, 10]. In this study, we asked the simple question of whether bleaching can be triggered by heat in the dark, in the absence of photosynthetically derived ROS. We used both the sea anemone model system *Aiptasia* [11, 12] and several species of reef-building corals to demonstrate that symbiont loss can occur rapidly during heat stress in complete darkness. Furthermore, we observed damage to the photosynthetic apparatus under these conditions in both *Aiptasia* endosymbionts and cultured *Symbiodinium*. These results do not directly contradict the view that light-stimulated ROS production is important in bleaching, but they do show that there must be another pathway leading to bleaching. Elucidation of this pathway should help to clarify bleaching mechanisms under the more usual conditions of heat stress in the light.

Results

In this study, we used both the emerging sea anemone model system *Aiptasia* [11, 12] and aquarium-grown and field-collected corals to explore the effects of heat stress in the dark. Two strains of *Aiptasia* were used. Strain H2 is a clonal population derived from a single animal collected in Hawaii (species *A. pulchella*); it contains a homogeneous population of endogenous symbionts in *Symbiodinium* clade B [11]. In contrast, the holobiont CC7-SSB01 was created in the laboratory by infecting fully bleached animals of clonal strain CC7 (species *A. pallida*) [13] with the clonal, axenic *Symbiodinium* strain SSB01, which was isolated from *Aiptasia* strain H2 [11]. During incubation in the dark for several days at 27°C, the constructed strain, but not the natural one, showed a modest but significant loss of algae (Figure 1A). In contrast, both strains bleached extensively when incubated in the dark at the nonlethal temperature of 34°C (Figure 1A), and there was no detectable enhancement of bleaching at this temperature by light at the relatively low levels used here (~20 μmol photons/m^2/s) (Figure 1A). Although this bleaching largely reflected a loss of algal cells from the host, there also appeared to be some modification to the photosynthetic apparatus as evidenced by a small decrease in the average intensity of chlorophyll *a* fluorescence of the remaining algal endosymbionts (Figure 1B; see also below). Bleaching continued for several days after the anemones were returned to 27°C, but the algal populations in the host tissue eventually recovered to the prestress levels (Figure 1C; see also Figure S1 available online).

We next examined the effects of heat stress on nine reef-building corals in the genus *Acropora*. Seven of these corals were from back-reef locations at Ofu Island, US National Park of American Samoa [14, 15], whereas two other Pacific corals had been maintained for years in the Monterey Bay Aquarium (Monterey, California, USA). The experimental regimen mimicked natural conditions experienced by Ofu corals during hot summer days, when logged temperatures in back-reef pools commonly reach or exceed 34°C. Thus, a 3 hr heat ramp from 27°C or 29°C to 34°C was followed by 3 hr at 34°C and a gradual return to 27°C or 29°C; a constant flow of fresh seawater was maintained throughout (see Supplemental Experimental Procedures for all methods). Despite these relatively mild conditions, the results showed clearly that for four of the nine species, there was rapid bleaching both in the light and in the dark (25%–80% loss of chlorophyll relative to controls; Figures 2A and 2B). Three other species also bleached both in the light and in the dark under these conditions (12%–20% chlorophyll loss in the dark; Figure S2), although the results were less clear, at least in part because of the variation in chlorophyll content among field-collected replicate colonies. The remaining two species were resistant to this particular temperature treatment either in the light or in the dark (Figure 2C, left), or in the dark but not in the light (Figure 2C, right). It remains to be determined whether a stronger temperature stress would produce bleaching in the dark in these latter species. Moreover, further studies in which both temperature and light intensity are varied will be needed to characterize the presumed interplay of heat and light stress on the rate, extent, and specific mechanisms of bleaching in various species [16].

The bleaching of the coral samples appeared to be due almost entirely to the loss of algal cells from the host tissue, since the chlorophyll *a* content of the algae remaining in the corals appeared little changed when judged either by fluorescence levels in the flow cytometer (Figure 3A) or by quantitative chlorophyll determinations (Figure 3B). Moreover, under the conditions and short duration of stress used in these

[4]These authors contributed equally to this work

*Correspondence: arthurg@stanford.edu

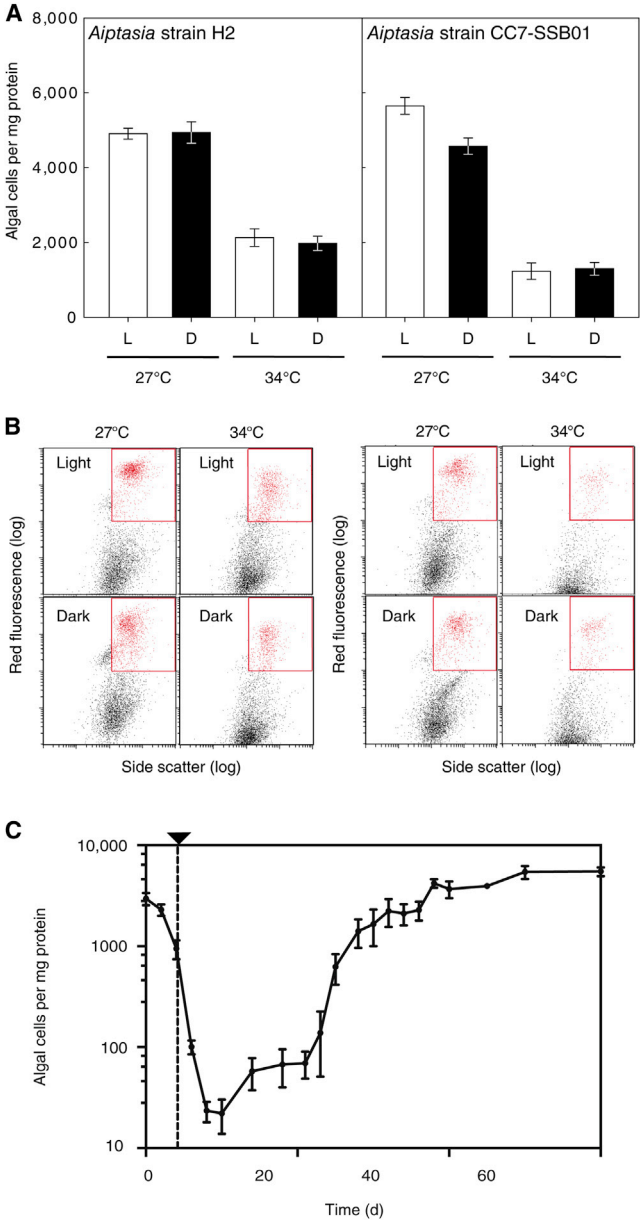

Figure 1. Rapid Loss of Algae by Symbiotic Anemones during Heat Stress in the Light or Dark, and Recovery after Return to the Control Temperature

(A) *Aiptasia* strains H2 (left) and CC7-SSB01 (right) were exposed to control (27°C) or elevated (34°C) temperatures for 3 days in the dark (D) or light (L; a 12:12 L:D cycle), as indicated; the anemones were not fed for at least 2 days before or during the heat stress. Algal cell counts by Guava flow cytometer were normalized to total protein for each homogenate of an individual anemone (see Supplemental Experimental Procedures). Shown are means ± SEMs ($n \geq 20$ for each population).

(B) Guava plots for one representative anemone for each strain and condition shown in (A). Fluorescence and side scatter (related to size) profiles of particles in the homogenates are shown. Red boxes gate the algal cells.

(C) Strain H2 anemones were exposed to 34°C for 4 days in the light as in (A) and then returned to 27°C, indicated by the arrowhead at the top of the graph, with a concomitant water change. They were then maintained at 27°C with feeding every 5 days and a water change on the day after each feeding. Algal cell counts and normalization were as in (A). Shown are means ± SEMs (n = 8 for each sample).

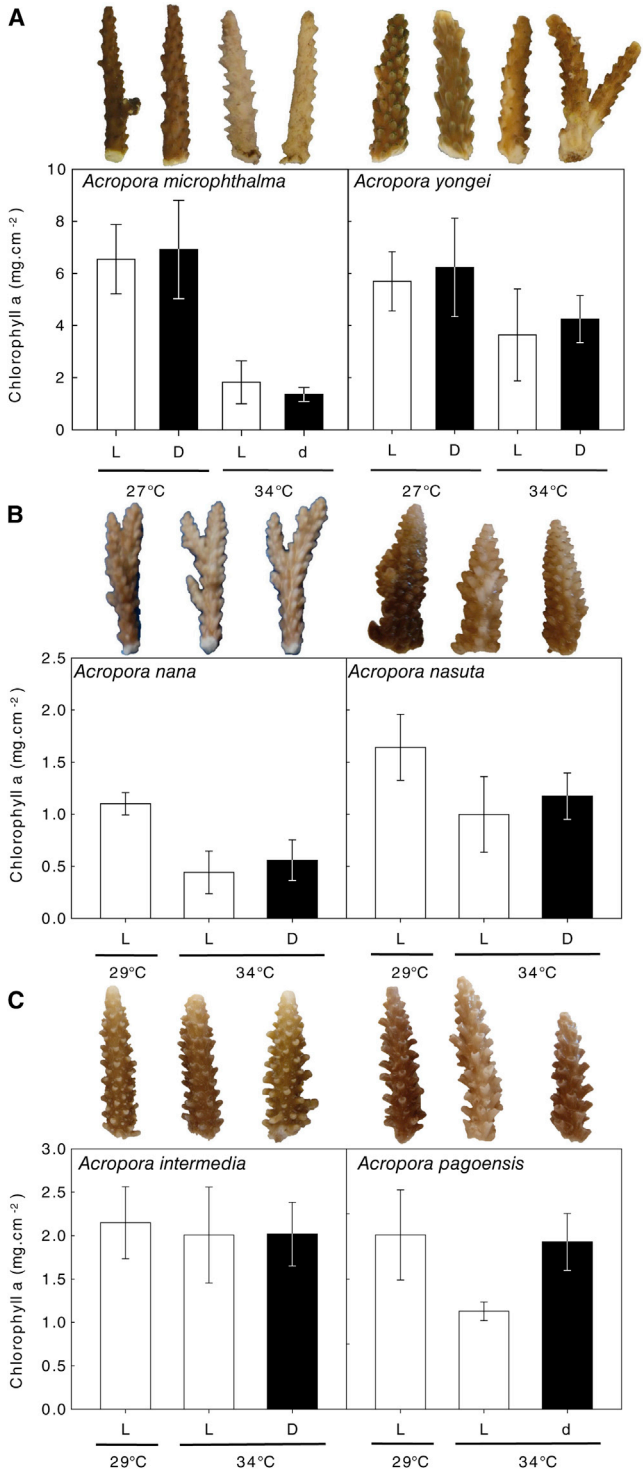

Figure 2. Rapid Bleaching of Coral Nubbins during Heat Stress in the Light or Dark

Nubbins of aquarium-raised (A) or field-collected (B and C) corals of the indicated species were held for 6 hr in the light or dark at a control (27°C or 29°C) or elevated (34°C) temperature, as indicated, and allowed to recover for ~6 hr in the dark at the control temperature before determining algal chlorophyll *a* per unit area of the nubbins (see Supplemental Experimental Procedures). Shown are means ± SDs (n = 3 or 4 nubbins for each treatment).

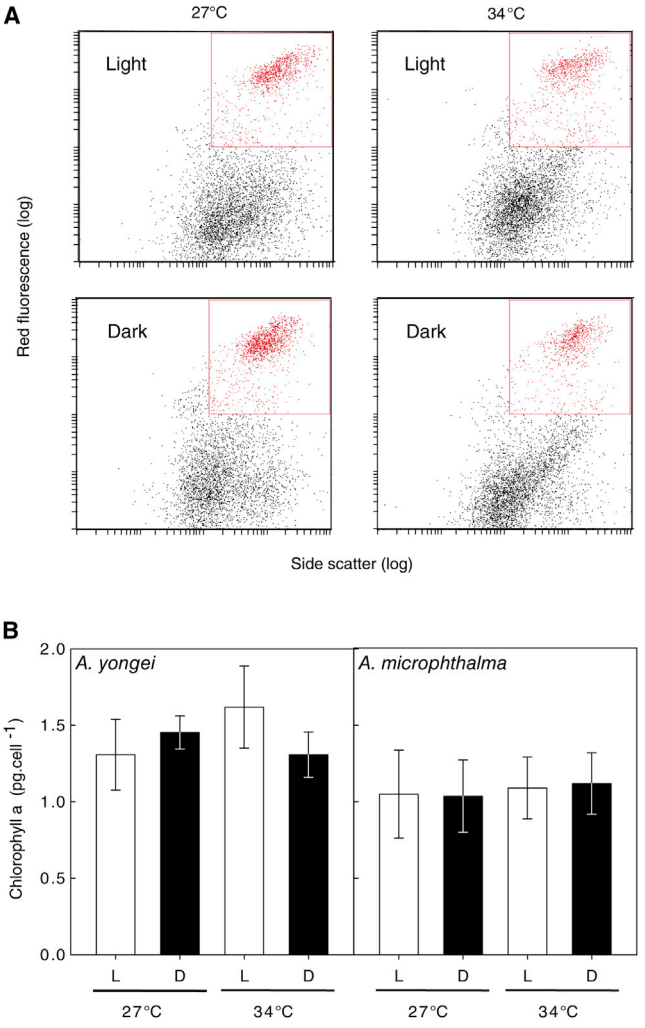

Figure 3. Maintenance of Chlorophyll *a* Concentrations by Algae Remaining in the Coral during Heat Stress

Nubbins of *A. yongei* (A and B, left) and *A. microphthalma* (B, right) were exposed to control or elevated temperatures in the light or dark and allowed to recover as in Figure 2. Homogenates of coral tissue were then analyzed by Guava flow cytometry (A, as in Figure 1B) or by extraction and quantification of chlorophyll *a* (B) as described in Supplemental Experimental Procedures. For (B), chlorophyll *a* amounts were normalized to algal cell numbers as determined by Guava flow cytometry. Shown are means ± SDs (n = 3 nubbins for each treatment).

experiments, visual inspection revealed no significant death or sloughing of coral tissue (Figure S3).

The occurrence of rapid bleaching in the dark demonstrates that photosynthetically derived ROS are not always essential for bleaching but leaves open the question of whether chloroplast damage also occurs in endosymbiotic algae during heat stress in the dark. Heat damage to the photosynthetic apparatus has been observed previously in cultured *Symbiodinium* [4, 16, 17], freshly isolated algae [18], and algae in hospite [16, 17], but it has been thought to be dependent on light [4, 7, 17]. To investigate this question further, we monitored the integrity of the algal photosynthetic apparatus during 4 days of heat stress on either symbiotic *Aiptasia* or cultured algae in either light or dark. For the algae in hospite, we observed that the loss of algae from the host (Figure 4A) was accompanied by disruption of photosynthetic function in the remaining algae, as indicated by a decline in the maximum quantum yield (F_v/F_m) of photosystem (PS) II (Figure 4B). For the same strain of algae in culture, although the number of cells per unit volume showed only a small change during the 4-day heat treatment (Figure 4A′), the cells in either the light or dark showed a marked decline in F_v/F_m (Figure 4B′). As noted previously [19], cultured *Symbiodinium* exhibit PS II donor-side damage after short-term heat stress in the dark and acceptor-side damage after short-term heat stress in the light. Although the decline in photosynthetic activity for the cultured algae appeared slightly more severe in the light, as expected if photosynthetically produced ROS contribute to chloroplast damage, the reverse was observed for the algae in hospite (Figure 4B).

Heat stress in either light or dark also caused a disconnection of light-harvesting complexes from photosynthetic reaction centers. The dissociation appears to be between the soluble peripheral peridinin chlorophyll *a* proteins (PCPs) and PS II reaction center cores [17, 20], as there is a progressive increase in 77 K fluorescence emission at 672 nm (Figures 4C–4D′), a signal specifically associated with the PCP complex [21]. We have not yet been able to measure this parameter accurately in our coral samples because of a high level of interfering autofluorescence from the animal tissue.

Finally, we observed a marked increase in ROS ($^1O_2$) production by photosynthetic electron transport when anemones that had been heat stressed for 3 days were challenged with high light (Figure 4E, right). Importantly, we observed essentially identical increases in ROS regardless of whether the heat treatment had occurred in the light or in the dark, demonstrating that heat, not light, induced the damage. The absence of a substantial accumulation of $^1O_2$ in heat-stressed anemones exposed to low light levels (Figure 4E, left) suggests that the bleaching occurring under these conditions was not accompanied by a marked increase in cellular levels of ROS, but a more comprehensive analysis of the levels of various types of ROS during bleaching would be necessary to elucidate this point.

Discussion

Coral reefs have enormous ecological, economic, and aesthetic importance and support about 25% of the total biodiversity of the oceans [22]. Unfortunately, reefs are threatened by global climate change and other anthropogenic factors; healthy reefs have declined by 22%–70% around the world [23]. The urgency for government action to reduce climate change and halt the continued decline of the reefs was recently endorsed by thousands of scientists in a consensus statement at the 12th International Coral Reef Symposium (http://www.centerforoceansolutions.org/initiatives/climate-change-coral-reefs). Efforts to save the reefs would be aided by a deeper understanding of the molecular and cellular biology of the *Symbiodinium*-coral symbiosis and its breakdown during stress-induced bleaching [8, 24].

It has long been known that cnidarians can lose their dinoflagellate symbionts during prolonged incubation (>10 days) in constant darkness [1, 25], and there is a single report of data suggesting that such dark bleaching can be slightly accelerated by heat stress [26]. Otherwise, the extensive literature on coral bleaching has focused exclusively on the effects of elevated temperatures in the light. However, we have shown here that incubation in complete darkness at an elevated but nonlethal temperature can result in a rapid loss of algal cells on timescales at which there is little or no loss of algae by

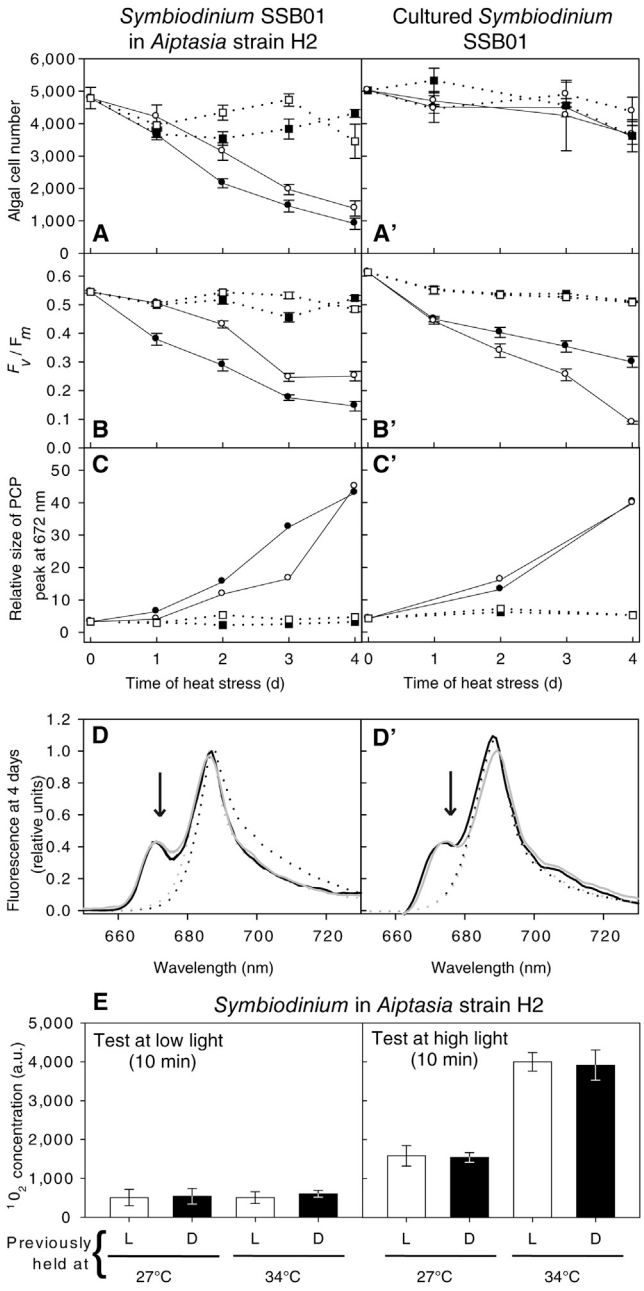

Figure 4. Damage to the Algal Photosynthetic Apparatus during Heat Stress in the Light or Dark

(A–D′) Strain H2 anemones containing their endogenous SSB01 algae (left column) and algal strain SSB01 in culture (right column) were exposed to control (27°C, dotted lines) or stressful (34°C, solid lines) temperatures for 4 days in the dark (black symbols) or light (white symbols; a 12:12 L:D cycle).

(A and A′) Algal cell counts by Guava flow cytometer were normalized to total protein (A, as in Figure 1A) or to an arbitrary unit volume of culture (A′).

(B and B′) Maximum quantum yields of photosystem (PS) II as measured by F_v/F_m (see Supplemental Experimental Procedures).

(C and C′) Relative sizes of peridinin chlorophyll *a* protein (PCP) fluorescence emission peaks at 672 nm (see D and D′, arrows) as a function of the time of exposure to the various conditions.

(D and D′) Low-temperature (77 K) fluorescence-emission spectra (see Supplemental Experimental Procedures) after 4 days of elevated temperature.

(E) *Aiptasia* strain H2 anemones were held for 3 days at 27°C or 34°C in the light (L; a 12:12 L:D cycle) or dark (D), as indicated, and then irradiated for 10 min at either 20 (left) or 1,700 (right) μmol photons/m^2/s before measuring $^1O_2$ levels (see Supplemental Experimental Procedures). a.u., arbitrary units. Data are shown in (A), (A′), (B), (B′), and (E) as means ± SD (n = 6 animals or culture samples for each treatment except for the 1-day time points in A–B′, where n = 3).

animals incubated in the dark at control temperatures. The loss of algal cells from the anemone host during heat stress was paralleled by the accumulation of damage to the photosynthetic apparatus of the remaining algae, as revealed by a decrease in F_v/F_m, a disconnection of the PCP from PS II reaction centers, and the production of large amounts of $^1O_2$ in response to a short but intense illumination. The decline in PS II quantum yield may represent either donor-side or acceptor-side damage [19]. Similar damage probably occurs in corals that are heat stressed in the dark, although we have so far been unable to test this hypothesis. The 77 K fluorescence resulting from PCP disconnection may ultimately become a useful signature of incipient stress that, unlike the commonly used F_v/F_m, is independent of the potentially variable redox state of the cells.

Our anemone experiments were conducted at relatively low light levels, perhaps accounting for the failure to see any exacerbation of the dark bleaching by light. The coral experiments were conducted at higher light levels, and in at least one case (*A. pagoensis*), light appeared to exacerbate bleaching. However, the rapid dark bleaching seen in many of the corals suggests that similar mechanisms operate in corals and in anemones.

It is generally believed that the primary trigger for cnidarian bleaching during heat stress is the ROS produced as a result of photosynthetic activity by heat- and light-damaged algal chloroplasts, and considerable evidence supports this model [5, 27, 28]. Our results do not directly contradict this model, but the rapid bleaching that occurs in the dark shows that there must be at least one bleaching signal that is not associated with photosynthetically produced ROS. The nature of this signal, the detailed molecular events that it triggers, and its relationship to the signal (or signals) that trigger bleaching during heat stress in the light all await elucidation, but the dark-bleaching signal might involve nonphotosynthetically produced ROS, nitric oxide production [10, 29], and/or immune reactions involving tumor necrosis factor receptors [15].

It is unlikely that the dark-bleaching mechanisms operate frequently by themselves, because heat stress is generally associated with sunlight in the natural environment, and the mechanisms that produce bleaching in the dark and in the light may thus operate synergistically. However, dark bleaching itself might sometimes be a significant factor in the field during warm-water events. For example, reefs in Palau sustained bleaching temperatures even at night during the intense 1998 bleaching event [30]. Therefore, warm sea surface temperatures at night, which have been largely ignored, should be considered when evaluating the impact of temperature on coral reef ecosystems. Furthermore, under conditions of marked temperature elevation, the bleaching that occurs during the day will be neither alleviated at night nor wholly prevented by remediation strategies that include physically shading the reefs [31]. Finally, there seems likely to be a direct benefit to the hosts of triggering bleaching during high-temperature exposure in the dark: expulsion of algae during the night would reduce the number of endosymbionts resident in the host tissue at daybreak, when photosynthetically produced ROS (or another light-dependent mechanism) would probably augment the damaging effects of elevated

temperature. Thus, the pathway that triggers bleaching in the dark may represent a previously unsuspected coral adaptation to life in intimate symbiosis with a partner that can become a liability under stress conditions.

Supplemental Information

Supplemental Information includes three figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.07.041.

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