Free Radical Biology & Medicine, Vol. 33, No. 4, pp. 533–543, 2002
Copyright © 2002 Elsevier Science Inc.
Printed in the USA. All rights reserved
0891-5849/02/$–see front matter

ELSEVIER

PII S0891-5849(02)00907-3

Original Contribution

OXIDATIVE STRESS AND SEASONAL CORAL BLEACHING

C. A. Downs,*† John E. Fauth,‡ John C. Halas,§ Phillip Dustan,§|| John Bemiss,† and Cheryl M. Woodley†

*EnVirtue Biotechnologies, Inc., Walnut Creek, CA, USA; †Marine Biotechnology Program, U.S. National Oceanic and Atmospheric Administration, Charleston, SC, USA; ‡Department of Biology, College of Charleston, Charleston, SC, USA; §Florida Keys National Marine Sanctuary, U.S. National Oceanic and Atmospheric Administration, Key Largo, FL, USA; and ||The Cousteau Society, Chesapeake, VA, USA

(*Received* 9 *April* 2002; *Revised* 30 *April* 2002; *Accepted* 2 *May* 2002)

Abstract—During the past two decades, coral reefs have experienced extensive degradation worldwide. One etiology for this global degradation is a syndrome known as coral bleaching. Mass coral bleaching events are correlated with increased sea-surface temperatures, however, the cellular mechanism underlying this phenomenon is uncertain. To determine if oxidative stress plays a mechanistic role in the process of sea-surface temperature-related coral bleaching, we examined corals along a depth transect in the Florida Keys over a single season that was characterized by unusually high sea-surface temperatures. We observed strong positive correlations between accumulation of oxidative damage products and bleaching in corals over a year of sampling. High levels of antioxidant enzymes and small heat-shock proteins were negatively correlated with levels of oxidative damage products. Corals that experienced oxidative stress had higher chaperonin levels and protein turnover activity. Our results indicate that coral bleaching is tightly coupled to the antioxidant and cellular stress capacity of the symbiotic coral, supporting the mechanistic model that coral bleaching (zooxanthellae loss) may be a final strategy to defend corals from oxidative stress. © 2002 Elsevier Science Inc.

Keywords—Coral bleaching, Coral reef, Oxidative stress, Oxidative damage, Stress protein response, Free radicals

INTRODUCTION

Coral reefs constitute some of the largest and most diverse ecological communities on earth and result from interactions between symbiotic organisms composed of photosynthetic dinoflagellate algae and cnidarian corals [1,2]. Over the past 30 years, coral reefs have experienced extensive degradation worldwide [2]. One etiology for this global degradation is a syndrome known as coral bleaching, a process whereby corals lose their algal symbiont or the symbiont's photosynthetic pigments degrade [3,4]. Coral bleaching can be induced by diverse factors, including high temperature, cold temperature, supersaturating light, and bacterial infection. Few models of coral bleaching that incorporate cellular and molecular mechanisms have been proposed or even tested [2–4].

Many environmental factors associated with mass bleaching events can concomitantly induce increased production of reactive oxygen species (ROS), an antioxidant response, and significant oxidative damage to symbiotic coral [3–5]. Early studies conducted in symbiotic anemone (*Anthopleura elegantissima*) demonstrated an increased antioxidant enzymatic capacity in response to hyperbaric oxygen conditions as a result of increased oxygen production from its endosymbiotic algae [6]. Additional experiments led several workers to conclude that an increase in ROS production in *A. elegantissima* was mainly derived from light-dependent processes, such as photosynthesis [7]. Recent work from our laboratory demonstrated that there is a significant increase in oxidative damage products in coral as a result of a high-temperature and light interaction, further corroborating the conclusions of several groups [5,7–9].

One proposed model concerning a possible mechanism of coral bleaching is based on the responses to oxidative stress by both components of the symbiotic relationship [5]. Stressors such as heat stress, osmolarity, high light, and ultraviolet radiation can destabilize the

Address correspondence to: C. A. Downs, EnVirtue Biotechnologies, Inc., 1866 East Market Street, Suite 164, Harrisonburg, VA 22801, USA; Tel: (925) 944-9630; Fax: (925) 944-9661; E-Mail: cdowns@envirtue.com.

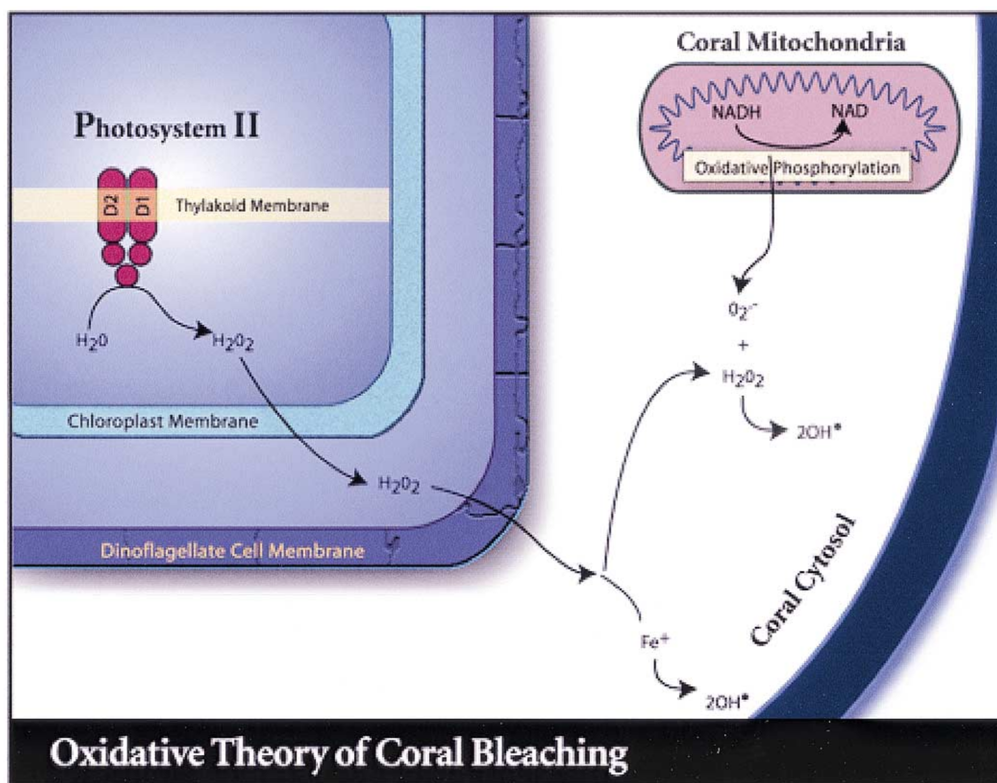

Fig. 1. Schematic representation of the proposed model in corals for the production of reactive oxygen species, their diffusion from one cellular location to another, and some of the predominant products from hydrogen peroxide as a result of increased sea-surface temperatures.

photosynthetic electron-transport chain resulting in increased production rates of reactive oxygen species (ROS) [10–13]. Production of ROS occurs in the chloroplast by several mechanisms associated with Photosystem I- and Photosystem II-catalyzed electron transfer, the most notable being the Mehler reaction and generation of hydrogen peroxide (H_2O_2) by the Oxygen-Evolving Complex [12–15]. It is proposed that algal-generated H_2O_2 can diffuse from the algal symbiont into the coral cytoplasm (Fig. 1). Once inside the coral cytosol, H_2O_2 can be either 'neutralized' by enzymatic and nonenzymatic antioxidant pathways or it can converted into a more noxious ROS, the hydroxyl radical, either by Fenton or Haber-Weiss chemistry [10]. Below some ROS concentration threshold, the antioxidant defenses of the coral can compensate and ameliorate the destructive capacity of these ROS. Above this threshold, ROS will cause oxidative damage. At some intensity of oxidative damage, corals will eradicate the dominant source of ROS production by expelling their endosymbiotic algae. Thus, the *Oxidative Theory of Coral Bleaching* proposes that bleaching is a coral's final defense against oxidative stress (Fig. 1).

Observed correlations between global bleaching events and increased sea-surface temperatures (SST) suggest that high temperatures may trigger oxidative-stress-driven bleaching in coral reef systems [5–9,16]. However, it is unknown if increased SST events cause coral bleaching by an oxidative-stress-induced mechanism. The primary objective of this study was to determine if levels of oxidative damage products, antioxidant enzymes, and specific components of cellular structural integrity in the star coral (*Montastraea annularis* species complex) were correlated with coral bleaching, seasonal and increased SST, and water depth in order to test the validity of certain aspects of this theory.

MATERIALS AND METHODS

Materials

All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Polyvinylidene difluoride (PVDF) membrane was obtained from Millipore Corp. (Bedford, MA, USA). Dot blot and gel electrophoresis equipment were obtained from Bio-Rad Corp. (San Diego, CA, USA). GSH-420 glutathione (Cat. No. 21023) and LPO-560-assay (Cat. No. 21023) kits and cnidarian Mn superoxide dismutase (Cat. No. 24327) were obtained from Oxis International, Inc. (Portland, OR, USA). Antibodies

to αB-crystallin (Cat. No. SPA224), cnidarian heat-shock protein (Hsp)70 (Cat. No. SPA-812), cnidarian Hsp60 (Cat. No. SPA-805), cnidarian Cu/Zn superoxide dismutase (Cat. No. SOD100) were obtained from Stressgen Biotechnologies (Victoria, BC, Canada). Antibodies against algal mitochondrial small heat-shock protein (Cat. No. AB-H106), chloroplast small heat-shock protein (AB-H104), and protein carbonyl (Cat. No. X1) were obtained from EnVirtue Biotechnologies, Inc. (Harrisonburg, VA, USA). Antibody to ubiquitin and protein standards of Hsp70, Hsp60, αB-crystallin, Cu/ZnSOD, MnSOD, and ubiquitin were obtained from Sigma-Aldrich. Protein standards for the chloroplast and mitochondrial small heat-shock proteins (sHsps) were obtained from Envirtue Biotechnologies, Inc. Secondary anti-rabbit and anti-mouse alkaline phosphatase conjugated antibodies were obtained from Promega (Madison, WI, USA).

Coral sampling

Sampling was conducted within the Florida Keys National Marine Sanctuary, Key Largo, FL, USA, near Molasses Reef. This site was chosen because it exhibited a putative bleaching gradient with depth. Coral samples (<2.5 cm fragments) from five separate colonies were collected at each of four depths (ca. 3 m, 6 m, 9 m, and 18 m) with each colony sampled monthly, March–October 1999. Samples were kept in the dark by wrapping them in aluminum foil and placed in whirl-pak bags underwater. On the deck, water was removed from bags and samples immediately transferred to a liquid nitrogen dry shipper. Samples were stored at −80°C until analysis.

Assays

Each sample consisted of about eight polyps. Samples of frozen coral were ground frozen in a mortar and pestle with liquid nitrogen and weighed. Approximately 1 g of ground, frozen tissue was suspended in acetone for chlorophyll analysis as described in [17]. Roughly 4 g of ground, frozen tissue was suspended in a solution of 10 mM phosphate buffer (pH 7.8), 5 mM butylhydrotoluene, and 0.5% sodium dodecyl sulfate. Samples on ice were sonicated until coral tissue was homogenized. Samples were then centrifuged, and 200 ul of supernatant was aliquoted into two new tubes and stored at −80°C until sample analysis. One tube of supernatant was analyzed for protein concentration, LPO and total GSH content as previously described [5]. To the other tube of supernatant, a 20 μl solution containing 20% sodium dodecyl sulfate, 50 mM Tris-HCl (pH 6.8), 200 mM dithiothreitol, 100 mM ethylenediaminetetraacetic acid, 10 mM sorbitol, 15% polyvinylpolypyrrolidone (wt/vol), 3% polyvinylpyrrolodone (wt/vol), 20 mM phenylmethylsulfonyl fluoride, 20 mM benzamide, 50 μM a-aminocaproic acid, and 1 μg/100 ul pepstatin A was added to the sample tube. Samples were boiled for 3 min, allowed to sit at 25°C for 5 min, and then centrifuged at 10,000 × *g* for 5 min. Supernatant absent of a lipid/glycoprotein mucilage matrix was transferred to a new tube and subjected to another protein concentration assay [18]. Samples were then assayed by ELISA for αB-crystallin homologue, chloroplast small heat-shock protein, cnidarian Hsp70, cnidarian Hsp60, cnidarian Cu/Zn SOD, cnidarian MnSOD, total algal small heat-shock protein content, and total ubiquitin as described in [5]. Because of the extensive protein glycosylation and polysaccharide content found in corals, protein carbonyl content was assayed using a modified method of Robinson et al., which takes into account carbonyl formation as a result of technique artifact and nonprotein carbonyls, such as those found in DNA, RNA, and polysaccharides [19].

Statistical analysis

We used repeated measures multivariate analysis of variance (MANOVA) to test whether mean biomarker levels differed over time, among depths, and with the time × depth interaction. We used univariate tests to interpret significant multivariate responses, and the Tukey-Kramer Honestly Significant Difference (HSD) method to compare univariate means. Levels of GSH, LPO, and Hsp 70 were log-transformed to meet analysis assumptions. To avoid temporal pseudoreplication, we used effective sample sizes (n_e) to determine the significance of correlations between time series [20]. Effective sample sizes adjust for first-order autocorrelations; if either series has zero first-order autocorrelation, no reduction is required in the original sample size. All statistical analysis were conducted using JMP V. 3.2 (SAS Institute, Inc., Cary, NC, USA) or higher, with $\alpha = 0.05$ for all tests.

RESULTS AND DISCUSSION

Oxidative stress is suggested to play a role in coral bleaching, though substantial evidence is lacking for a relationship between coral bleaching, oxidative stress, and environmental factors that are relevant to phenomena occurring in the field. More importantly, there is an absence of cellular-based mechanistic models concerning oxidative stress and coral bleaching that could be tested in both the laboratory and in the field. Based on early work in anemones and laboratory work on corals,

we propose a model of oxidative-stress driven coral bleaching [5]. High-temperature destabilization of Photosystem II occurs, in part, by the impairment of the oxygen-evolving complex (OEC) [21–23]. One mechanism of OEC impairment is associated with the release of Mn^{2+} and Cl^{-} from OEC and the dissociation of proteins OEC18, OEC24, and Photosystem II-associated heme catalase [24–27]. As a result, the rate of hydrogen peroxide generation increases by orders of magnitude [25]. Heat stress can further exacerbate the potential for hydrogen peroxide-mediated oxidative stress through the deactivation of a number of hydrogen peroxide-neutralizing pathways, allowing for concentrations to increase [28–30]. Diffusion of hydrogen peroxide out of the chloroplast and into other dinoflagellate organelles and the cnidarian cytoplasm can result in oxidative damage through the entire symbiotic organism, especially in organelles that contain high iron content or are associated with production of superoxide (e.g., mitochondria) [10]. In response to this oxidative stress, the coral expels the dinoflagellate, resulting in the phenomenon observed as coral bleaching.

This model for the oxidative theory of coral bleaching also posits that variation of the occurrence of bleaching should be related to specific molecular mechanisms that prevent the production of ROS or protect against the damaging effects of ROS. For example, increase in the levels of Cu/Zn SOD should be associated with an increase in the threshold at which oxidative damage occurs, because of the reduction of available substrates for Haber-Weiss chemistry [31]. Other protective mechanisms include antioxidant pathways, such as the Asada-Halliwell pathway and xanthophylls cycling, as well as 'preventive' mechanisms such as the production of small heat-shock proteins, changes in lipid composition, and production of stress-stable components of labile enzymatic complexes of pathways associated with electron transport [10,32–34].

The validity of a mechanistic model should be reflected in the accuracy at which it can predict the occurrence of phenomena at various hierarchical levels [35]. For example, if oxidative damage is an initiating event in the expulsion of a coral's zooxanthallae (its algal symbiont), a punctuated increase in levels of oxidative damage products should be detected before bleaching is observed. Furthermore, higher levels of antioxidant components (e.g., ascorbate peroxidase, glutathione peroxidase) in one individual should be associated with a different threshold or occurrence for bleaching compared to an individual that has lower levels of these antioxidant components [10]. This analysis can also help determine the relative contribution and role of different antioxidant and protective pathways (e.g., heat-shock protein response) in the process of coral bleaching [36].

Sea temperatures, at depth, beside each group of five coral heads, recorded at the time of sample collection, varied significantly among months (Fig. 2a). Temperatures at all sites exceeded 28°C from June–September, and peaked in July, when temperatures at the two deepest sites reached or exceeded 30°C. By September, all colonies at the 18 m site exhibited extensive bleaching, and colonies at the 9 m site either were partially bleached or had moderate paling. In contrast, corals at the 3 m site did not exhibit any visual symptoms of bleaching (Fig. 2b). The bleached or nonbleached appearance of colonies was related to July ocean temperature (logistic regression, $\chi^2 = 21.0$, $p < .0001$; $r^2 = 0.76$). The extent of bleaching was quantified using chlorophyll *a* concentration, which was negatively correlated with ocean temperature ($r = -0.53$, $n_e = 23.6$, $p < .01$). Temperatures at all sites remained above 28°C until October, when water temperature decreased over a 2 week period to less than 25°C in the 3–9 m sites, and below 27°C at the 18 m site. By October, colonies at all depths darkened in color and increased in chlorophyll *a* content indicating recovery from bleaching (Fig. 2c). Hence, coral bleaching was not a universal occurrence at high sea-surface temperatures, but was positively associated with depth.

Oxidative stress is defined as an imbalance in the pro-oxidant/antioxidant ratio, which favors increased pro-oxidants and results in oxidative damage [10]. Previous work from our laboratories demonstrated that heat stress causes oxidative damage in corals and that damage is exacerbated by exposure to light [5]. To test whether oxidative damage is associated with and precedes coral bleaching we examined levels of protein carbonyl and lipid peroxidation (LPO), both of which reflect aspects of cellular oxidative damage. Mean carbonyl concentration differed significantly with the exact combination of sampling date and depth (Fig. 3a), and was positively correlated with ocean temperature ($r = +0.47$, $n_e = 23.9$, $p < .05$). Lipid peroxide (LPO) showed a similar pattern (Fig. 3b). As water temperatures increased, so did levels of oxidative damage products. A specific pattern emerged: corals at the greater depths had higher levels of oxidative damage, especially protein carbonyl. There was a statistically significant negative correlation between carbonyl and chlorophyll a content ($r = -0.22$, $n_e = 102.0$, $p \ll .01$), indicating that increased oxidative damage levels were associated with bleaching. Furthermore, oxidative damage products increased as water temperature increased and preceded coral bleaching.

Cellular damage, such as that induced by oxidative stress, often increases protein chaperone and protein turnover activity [5,10,37,38]. To determine if increasing oxidative damage products were associated with decreasing scleractinian cellular integrity (i.e., to determine if corals were experiencing an oxidative stress), we exam-

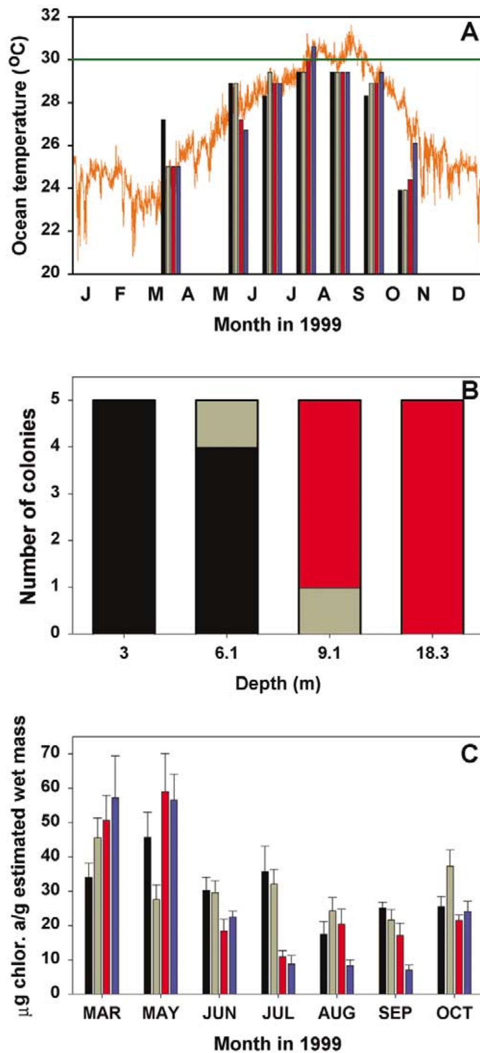

Fig. 2. (A) Seasonal variation in ocean temperatures recorded beside coral (*Montastraea annularis*) colonies at four depths. Bars show depth of colonies: black = 3 m, gray = 6 m, red = 9 m, blue = 18 m. Temperature in summer months (June–September) differed significantly from all others except May [two-way ANOVA: month effect: $F(6,18) = 23.3$, $p < .0001$; depth effect $F(3,18) = 0.19$, $p > 0.90$; Model $R^2 = 0.89$]. Yellow line is data from the C-Man Buoy at Molasses Reef, within 1–3 miles from sampling sites. Green line indicates the temperature threshold at which bleaching often is observed (1–4, 6, 7). (B) Colony condition in September, 1999 varied significantly among depths (log likelihood ratio test, $G = 27.95$, 6 *df*, $p < .0001$; $r^2 = 0.74$). Healthy colonies, black bars; paling colonies, gray bars; bleached colonies, red bars. (C) Chlorophyll *a* levels reflect photosynthetic activity and density of symbiotic zooxanthellae inhabiting coral tissue, providing a quantitative measure of bleaching. Bars show mean (± 1 SE) concentration of chlorophyll *a* in five coral colonies repeatedly sampled at each depth: black = 3 m, gray = 6 m, red = 9 m, blue = 18 m. Differences among months and the month × depth interaction were significant [repeated measures MANOVA: month effect $F(6,11) = 14.68$, $p < .0001$; month × depth interaction, $F(18,31.6) = 2.53$, $p < .011$].

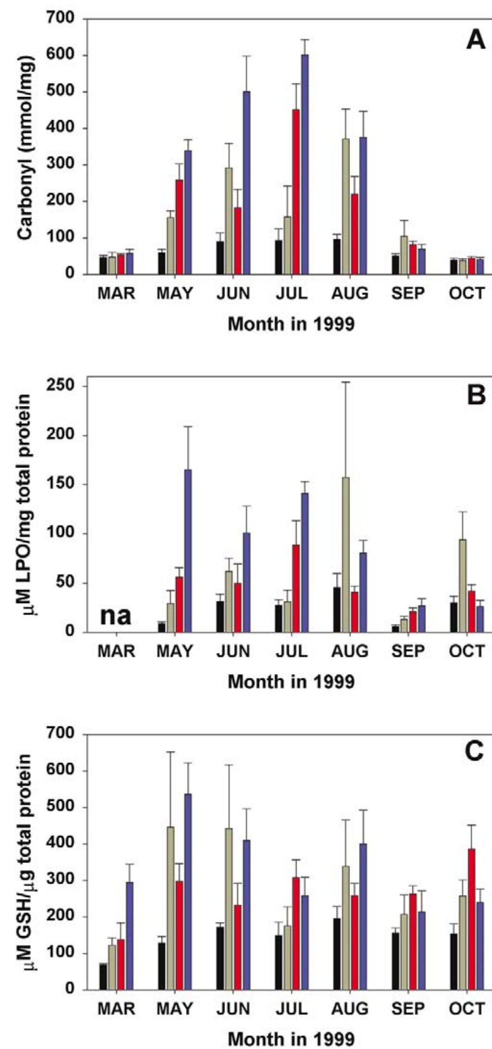

Fig. 3. Oxidative damage. (A) Protein carbonyl concentrations reflect protein oxidative damage, which varied significantly with depth, month, and the depth × month interaction (repeated measures MANOVA: all $F > 3.96$, $p < .0004$). (B) Lipid peroxidation reflects damage to cell and organelle membranes, and $\log_{10}$-transformed means varied significantly with depth, month, and the depth × month interaction (repeated measures MANOVA: all $F > 2.47$, $p < .015$). Lipid peroxide was not assayed (na) in March. (C) GSH reflects cell redox status and is an antioxidant; its $\log_{10}$-transformed means varied significantly with depth, month, and the depth × month interaction (repeated measures MANOVA: all $F > 2.54$, $p < .035$). Bars show untransformed mean (± 1 SE) biomarker concentrations at each depth: black = 3 m, gray = 6 m, red = 9 m, blue = 18 m.

ined levels of scleractinian Hsp60, scleractinian Hsp70, and ubiquitin. Chaperonins Hsp60 and Hsp70 increased significantly at all sites in May compared to March (ANOVA, both $F > 32.1$, $p < .0001$; Figs. 5A and 5B). From May–September, Hsp60 levels were significantly lower in corals at the 3 m site compared to corals at all other sites, except the 6 m site in July–August, which was equivalent [one-way ANOVA followed by Tukey-Kramer HSD Test: all $F(3,16) < 5.93$, $p < .01$]. Levels of Hsp70 showed a similar pattern from March–July,

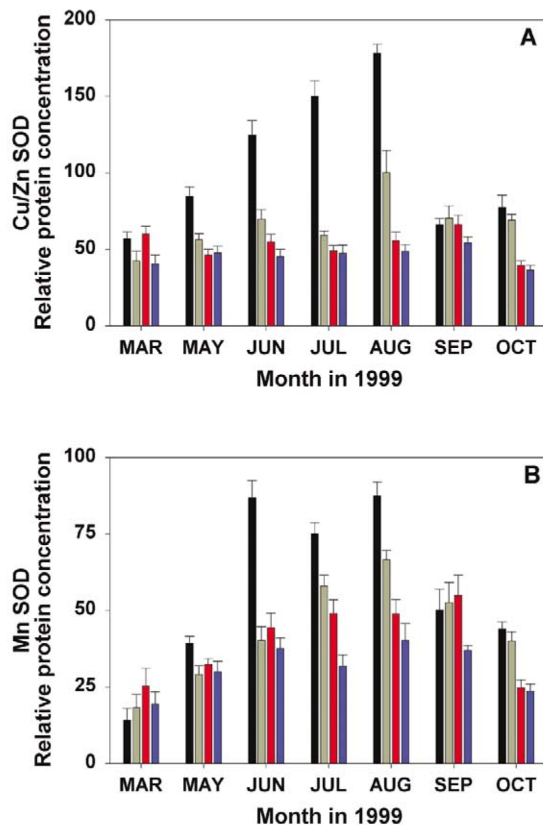

Fig. 4. Levels of cnidarian superoxide dismutases. (A) Cu/Zn superoxide dismutase protein levels reflects overall cnidarian responses, which varied significantly with depth, month, and the depth × month interaction (repeated measures MANOVA: all $F > 6.84$, $p < .0001$). (B) Mn superoxide dismutase protein levels reflects only responses of cnidarian mitochondria, which varied significantly with depth, month, and the depth × month interaction (repeated measures MANOVA: all $F > 6.95$, $p < .0001$). Symbols are as in Fig. 3.

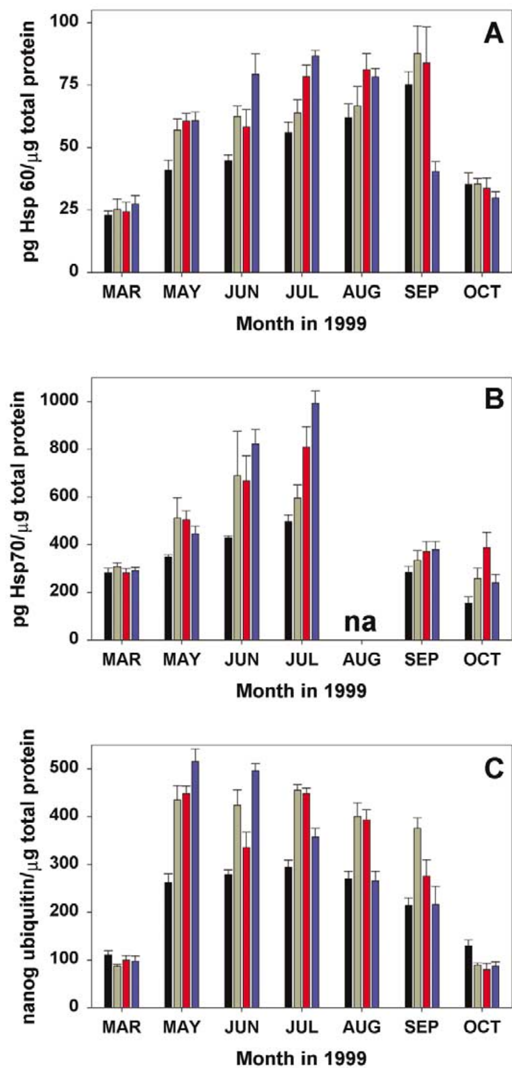

Fig. 5. Levels of chaperonins. (A) Hsp60 and (B) Hsp70 reflect chaperonin levels in the cnidarian and not of the dinoflagellate; mean concentrations of both varied significantly with depth, month, and the depth × month interaction (repeated measures MANOVAs: all $F > 2.56$, $p < .02$; Hsp70 $\log_{10}$-transformed prior to analysis). Hsp70 was not assayed (na) in August. (C) Ubiquitin levels reflect the rate of protein degradation, which varied significantly with depth, month, and the depth × month interaction (repeated measures MANOVA: all $F > 8.80$, $p < .0001$). Symbols are as in Fig. 3.

indicating increased chaperone activity during these months (Fig. 5B). However, elevated chaperone levels do not always indicate cellular damage [39]. To differentiate between increased chaperone activities resulting from cellular damage versus cellular growth, we examined changes in ubiquitin levels [37]. In May, ubiquitin levels were 2.5 to 5.0 times higher than in March [Welch ANOVA, $F(1,20.3)$, $p < .0001$; Fig. 4C]. Furthermore, there was a positive correlation between ubiquitin and protein carbonyl levels ($r = +0.61$, $n_e = 122.5$, $p < .0001$), indicating increased ubiquitin production and accumulation was associated with increased protein damage. Positive correlations between Hsp70 and carbonyl content during the whole sampling season ($r = 0.62$, $n_e = 96.1$, $p < .01$), and especially from June through September ($r = 0.55$, $n_e = 48.1$, $p < .01$) corroborated this interpretation. Further corroboration for this interpretation was found with positive correlations between Hsp60 and carbonyl content, both during the whole sampling season ($r = 0.55$, $n_e = 70.6$, $p \lll .01$) and between June and September ($r = 0.35$, $n_e = 70.6$, $p < .01$).

Total glutathione content could be used, in part, as an index of antioxidant capacity of an organism. Changes in glutathione concentration could also be used as a proxy for a response to specific stressors (e.g., electrophilic xenobiotic or ROS) [5,10,40]. For example, increases in glutathione are observed after a stress event and have been suggested as a means of physiologically acclimating to the stressor [10,40]. We measured total glutathione

concentrations to determine if corals increased their antioxidant capacity either in response to sea-surface temperatures or oxidative damage products. Mean GSH content differed significantly with the exact combination of sampling date and depth (Fig. 3C), with corals at the 3 m site always having lower mean GSH concentrations than those at deeper sites. Mean GSH content was independent of ocean temperature ($r = +0.16$, $n_e = 25.4$, $p > .25$), but significantly correlated with accumulations of the oxidative damage products carbonyl and LPO ($r = +0.42$ and $+0.49$, respectively; both $n_e > 109$ and $p < .0001$). One interpretation of the GSH analyses is that a coral will increase its antioxidant capacity in response to increasing oxidative damage.

To corroborate this interpretation, we examined other aspects of coral antioxidant status, including copper/zinc (Cu/Zn) and manganese (Mn) superoxide dismutase accumulation (SOD). Both SOD proteins reflected only the cnidarian response, not those of the dinoflagellate zooxanthellae [5]. Copper/Zinc SOD concentrations varied significantly with the exact combination of sampling date and depth (Fig. 4A). From May–August, Cu/Zn SOD concentrations were significantly higher at the 3 m site than at all deeper sites [one-way ANOVA followed by Tukey-Kramer HSD Test: all $F(3,16) < 14.9$, $p < 0.0001$]. Manganese SOD, an enzyme found only in mitochondria, showed a similar accumulation pattern (Fig. 4b). Levels of Mn SOD and Cu/Zn SOD were significantly positively correlated ($r = 0.75$, $n_e = 77.6$, $p < .0001$). From March to October, mean accumulations of Mn SOD and Cu/Zn SOD were negatively correlated with *variances* in carbonyl ($-0.70 \leq r \leq -0.52$; all $p < .02$, $n = 20$). Furthermore, between June and September levels of both Cu/Zn SOD and Mn SOD were positively correlated with chlorophyll a content ($r = 0.33$, $n_e = 50.9$, $p \leqslant .05$; $r = 0.38$, $n_e = 97.2$, $p \leqslant .05$, respectively). Both of these analyses strongly suggest that superoxide dismutases are significant components of the antioxidant capacity of corals and that these two proteins may play an important role in preventing the process of coral bleaching.

sHsps protect various metabolic pathways and cellular functions from heat and oxidative damage [32]. Specific sHsps have been demonstrated to protect glycolytic and electron-transport enzymes against oxidative damage, such as those in photosynthesis and respiration [41–43]. Measurement of specific sHsps can help determine whether (i) specific metabolic processes are being stressed, and (ii) gauge the capacity to which these metabolic processes can endure a stress [5,32,40]. We examined three species of sHsps in the symbiotic coral: chloroplast sHsp of the dinoflagellate, dinoflagellate mitochondrial sHsp, and t scleractinian αB-crystallin immunohomologue.

The chloroplast sHsp is evolutionarily conserved and usually is only present in response to a stressed condition [44]. This protein specifically associates with the oxygen-evolving complex of photosystem II and protects this enzyme's function during heat stress, high-light stress, ultraviolet-light exposure, and oxidative stress, most likely via a recycling antioxidant mechanism [43, 45]. Measurement of the chloroplast sHsp is relevant for two reasons: (i) photo-oxidative damage to photosystem II within the zooxanthellae has been implicated in heat-stress-mediated bleaching and (ii) photosystem II is a primary generator of hydrogen peroxide as a result of a degenerating efficiency of the oxygen-evolving complex in hydrolyzing water to diatomic oxygen [15,28,30,46, 47]. Chloroplast sHsps were barely detectable in corals at all depths in March, but increased dramatically in May [Welch ANOVA, $F(1,19.3) = 54.6$, $p < .001$; Fig. 6A]. Levels of this protein were greatest in corals at 3 m and decreased with depth, a pattern that remained consistent throughout the year. Chloroplast sHsp levels were negatively correlated with protein carbonyl ($r = -0.55$, $n_e = 72.6$, $p < .0001$) and LPO ($r = -0.38$, $n_e = 113.4$, $p < .001$) levels during May–September, when ocean waters were warmest. Hence, during heat stress, increased chloroplast sHsp concentrations may have protected zooxanthellae by preventing increased production of ROS. More importantly, the negative correlation between accumulation of chloroplast sHsp and oxidative damage products suggests that Photosystem II may be the principal component responsible for generating the oxidative stress associated with bleaching.

In vascular plants, the mitochondrial sHsp specifically associates with and protects the NADH:ubiquinone oxidoreductase complex of oxidative phosphorylation during heat and oxidative stress [41,48]. Less is known concerning the specific function and localization of the mitochondrial sHsp in dinoflagellates, although in vitro experiments indicate its expression is stress inducible (C. Downs, unpublished data). Mitochondrial sHsps levels showed a pattern of expression similar to that of the chloroplast sHsp in June to August, but mitochondrial sHsp levels did not differ significantly among the 6 m, 9 m, and 18 m sites in September and October (Fig. 6B). Mitochondrial sHsp levels were negatively correlated with protein carbonyl levels during May–September ($r = -0.34$, $n_e = 46.2$, $p < .05$; Fig. 6B), again suggesting a possible protective effect with higher expression of this protein. Furthermore, this data suggests dinoflagellate mitochondria were experiencing and responding to stress, whether in response to increased SST, the ROS generated by the chloroplast, or both.

In both vertebrates and invertebrates, homologues of αB-crystallin are usually found at some basal level and hyper-accumulate in response to a stressed condition

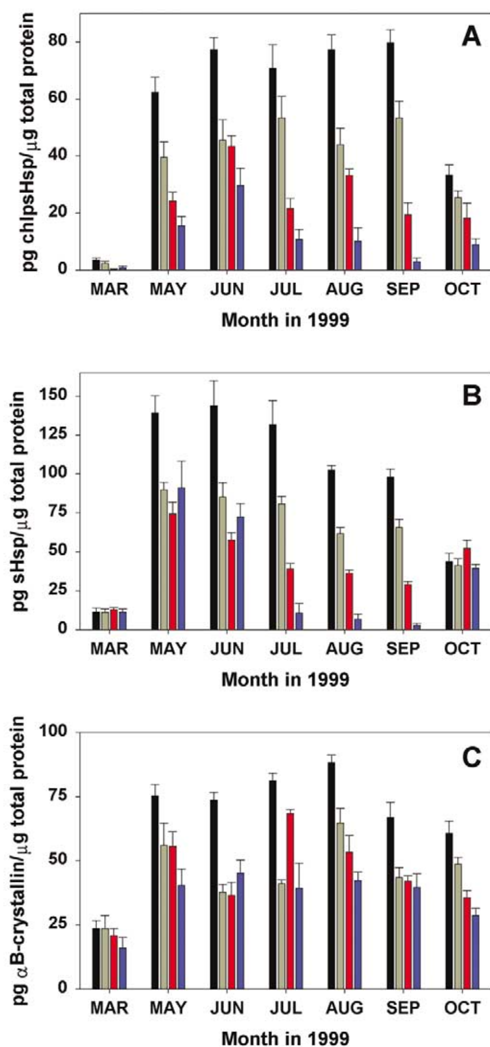

Fig. 6. Levels of small heat shock proteins. (A) Chloroplast sHsp levels varied significantly with depth, month, and the depth × month interaction (repeated measures MANOVA: all $F > 8.32$, $p < .0001$). (B) Dinoflagellate mitochondrial sHsp levels varied significantly with depth, month, and the depth × month interaction (repeated measures MANOVA: all $F > 7.28$, $p < .0001$). (C) In the cnidarian, levels of the immuno-homologue of αB-crystallin varied significantly with depth, month, and the depth × month interaction (repeated measures MANOVA: all $F > 3.46$, $p < .01$). Symbols are as in Fig. 3.

[5,49]. Evidence suggests that this protein species acts as a molecular chaperone, suppressing the unfolding and aggregation of proteins by trapping early unfolding intermediates, thereby increasing the amount of energy required to attain structural transition states [50]. Evidence from vertebrate research indicates that αB-crystallin interacts and stabilizes cytoskeletal elements during stress [51,52]. In corals, a 19 kDa immuno-homologue of this protein exists with an unknown function [5]. Accumulations of the immuno-homologue to αB-crystallin specific to the scleractinian showed a complicated pattern (Fig. 6C). This protein increased one- to two-fold from March to May, and maintained these levels throughout the year. Like the other two sHsp homologues, levels of αB-crystallin were negatively correlated with carbonyl levels during May–September ($r = -0.24$, $n_e = 91.5$, $p < .025$).

In summary, the data presented here support specific aspects of the oxidative stress model of coral bleaching. We demonstrate (i) that the concentration of specific oxidative damage products were highly correlated with coral bleaching, (ii) severe oxidative damage preceded loss of chlorophyll and coral bleaching, (iii) that oxidative damage was associated with increased damage to aspects of cellular integrity, (iv) that increased energy expenditure was required to maintain homeostasis during conditions associated with bleaching, (v) components of cellular defenses (e.g., antioxidant defense and stress-protein response) played a role in protecting against bleaching, and (vi) evidence that Photosystem II is a primary source for ROS production, hence a principal element in oxidative stress and temperature-associated coral bleaching.

Although mass coral bleaching events are often associated with increased sea-surface temperatures, spatial variation in the frequency and intensity of bleaching suggests both corals and their zooxanthellae vary in their individual responses to heat and light stress [3]. For example, different species of coral have different temperature thresholds for coral bleaching [53,54]. Furthermore, there is significant variation among individuals of the same species in their threshold of bleaching. This variation is correlated with components of cellular defense against stress, indicating that these molecular/cellular processes could be the focal point for such variation. The argument that bleaching susceptibility has a genetic component is further supported by studies demonstrating strong correlations between specific genotypic clades of dinoflagellates and incidence of bleaching [55].

Our results also emphasize the importance of further investigations of both cnidarian and dinoflagellate cellular-stress physiology. For example, we only measured levels of Cu/Zn SOD and Mn SOD in the cnidarian. Concentrations and activity of these enzymes in the dinoflagellate, as well as FeSOD, need to be investigated. Oxidative defensive mechanisms that specifically protect photosynthesis, such as aspects of the Photosystem II-associated heme catalase and the xanthophyll cycle, should also be examined and characterized. Mechanisms and pathways other than those of the classical antioxidant defenses should be examined to determine if these are significant processes that mitigate coral bleaching. For example, there are several ORFs in the dinoflagellate chloroplast genome that show significant homology of the *psbA* gene of the D1 protein of photosystem II [56].

In *Synechocystis*, differential induction of the *psbA* genes are associated with tolerance against conditions that induce photoinhibition and production of ROS [36,57,58]. Preliminary evidence from a heat-stressed subtractive library of dinoflagellates obtained from the coral *Montastraea annularis* indicates differential induction of *psbA* transcript homologues, suggesting that a similar mechanism of protection may exist in coral dinoflagellates (Downs, C., Markus, E., Vetter, L, unpublished results). Other adaptations to stress, such as changes in lipid composition of key organellar membranes, induction of scleractinian sHsps, and shifts in metabolic pathways, need to be investigated so that their relative contribution and role in coral stress tolerance can be determined.

Coral bleaching does not always result in coral mortality. There are a number of observations both from the laboratory and the field documenting recovery from a bleaching event [2,3]. One hypothesis for this phenomenon, especially given the data supporting the *Oxidative Theory of Coral Bleaching*, is that the outcome for either mortality or recovery is determined by the extent of oxidative damage experienced by the coral. Irreparable damage to essential cellular structures, such as chromosomes and mitochondria, by ROS can initiate the development of apoptotic or necrotic processes [10]. Minor damage can be repaired and the coral recover, as suggested by the increase in chaperone and protein turnover activity (Figs. 3 and 4). Such a hypothesis can be easily tested, both in the laboratory and in the field, using many of the methodologies and techniques first developed in mammalian studies of ROS-toxicity and pathologies [10].

Finally, chronic or repeated exposures to conditions leading to oxidative stress in corals could have significant impacts to coral ecology that are not readily observable. Many corals, especially the reef building coral species, are long-lived (ca. 200–800 years old). Climatic/oceanic events associated with mass-coral bleachings are argued to have increased in frequency and intensity over the past 40 years in contrast to what has occurred in the last 450–6000 years [2,59]. Some of these events, especially El Niño phenomenon, result in increased sea-surface temperatures that are oftentimes at or above coral bleaching thresholds [3]. By extrapolating some of the tenets of the *Free Radical Theory of Aging* to coral biology, it can be hypothesized that chronic or repeated exposures to conditions conducive to oxidative stress may decrease the overall condition of coral [60]. For example, a decrease in the fitness of the individual or its offspring may result from a decrease in the overall genomic integrity that is usually associated with oxidative stress [10,60]. Such a decline could be further detected as an increase in mutation rate, decline in the stress-protein response, or a decline in enzymatic activity necessary for homeostasis [61]. It has been suggested that many cnidarian species are 'immortal' or lack the deteriorative process associated with senescence [62]. In light of the findings presented here and in the literature, increased significance must be given to the question: are oxidants a determinant of the maximum life span and condition of corals, thereby influencing coral reef ecology?

Acknowledgements — We thank Judy Halas and William Fitt for discussion and support of this work; Mike Brill, Karen Bareford, Darren Wray, Claire Chandler, Hank Becker, and Amy Massey for establishing sites, sample collection, and sample preparation; and Charles E. Robinson, Arnold Huang, and Bruce N. Ames for discussion of oxidative stress, assay development and critical comments on this report. C. Downs thanks Bruce N. Ames for his encouragement during the writing of this manuscript at the University of California, Berkeley. Statistical analyses and manuscript preparation were supported by National Science Foundation grant DEB9727039 and Environment Protection Agency grant R825795 to J. E. Fauth. Corals were collected under permit FKNMS-99-042. This is Envirtue Biotechnologies, Inc. manuscript RD002.

REFERENCES

[1] Dustan, P. Coral reefs under stress: sources of mortality in the Florida Keys. *Nat. Res. Forum* **23:**147–155; 1999.

[2] Stone, L.; Huppert, A.; Rajagopalan, B.; Bhasin, H.; Loya, Y. Mass coral reef bleaching: a recent outcome of increased El Nino activity? *Ecol. Lett.* **2:**325–330; 1999.

[3] Brown, B. E. Adaptations of reef corals to physical environmental stress. *Adv. Mar. Biol.* **31:**222–299; 1997.

[4] Brown, B. E. Coral bleaching: causes and consequences. *Coral Reefs* **16:**S129–S138; 1997.

[5] Downs, C. A.; Mueller, E.; Phillips, S.; Fauth, J.; Woodley, C. M. A molecular biomarker system for assessing the health of coral (*Montastrea faveolata*) during heat stress. *Mar. Biotechnol.* **2:**533–544; 2000.

[6] Dykens, J. A.; Shick, J. M.; Benoit, C.; Buettner, G. R.; Winston, G. W. Oxygen radical production in the sea anemone *Anthopleura elegantissima* and its endosymbiotic algae. *J. Exp. Biol.* **168:**219–241; 1992.

[7] Lesser, M. P.; Stochaj, W. R.; Tapley, D. W.; Shick, J. M. Bleaching in coral reef anthozoans: effects of irradiance, ultraviolet radiation, and temperature on the activities of protective enzymes against active oxygen. *Coral Reefs* **8:**225–232; 1990.

[8] Lesser, M. P. Elevated temperatures and ultraviolet radiation cause oxidative stress and inhibit photosynthesis in symbiotic dinoflagellates. *Limnol. Oceanogr.* **41:**271–283; 1996.

[9] Lesser, M. P. Oxidative stress causes coral bleaching during exposure to elevated temperatures. *Coral Reefs* **16:**187–192; 1997.

[10] Halliwell, B.; Gutteridge, J. M. C. *Free radicals in biology and medicine.* New York: Oxford University Press, Inc.; 1999.

[11] Giardi, M. T.; Masojidek, J.; Godde, D. Effects of abiotic stresses on the turnover of the D1 reaction center II protein. *Physiol. Planta.* **101:**635–642; 1997.

[12] Badger, M. R. Photosynthetic oxygen exchange. *Ann. Rev. Plant Physiol.* **36:**27–53; 1985.

[13] Richter, M.; Ruhle, W.; Wild, A. Studies on the mechanism of photosystem II photoinhibition: II. The involvement of toxic oxygen species. *Photosyn. Res.* **24:**237–243; 1990.

[14] Tjus, S. E.; Scheller, H. V.; Andersson, B.; Møller, B. L. Active oxygen species produced during selective excitation of photosystem I is damaging not only to photosystem I, but also to photosystem II. *Plant Physiol.* **125:**2007–2015; 2001.

[15] Mehler, A. H. Studies on reactions of illuminated chloroplasts. I. Mechanism of the reduction of oxygen and other Hill reagents. *Arch. Biochem. Biophys.* **33:**65–77; 1951.
[16] Goreau, T. J.; Hayes, R. L. Coral bleaching and ocean 'hot spots'. *AMBIO* **23:**176–180; 1994.
[17] Heckathorn, S. A.; Downs, C. A.; Sharkey, T. D.; Coleman, J. S. The small methionine-rich chloroplast heat-shock protein protects photosystem II electron transport during heat stress. *Plant Physiol.* **116:**439–444; 1998.
[18] Ghosh, S.; Gepstein, S.; Heikkila, J. J.; Dumbroff, B. G. Use of a scanning densitometer or an ELISA plate reader for measurement of nanogram amounts of protein in crude extracts from biological tissue. *Anal. Biochem.* **169:**227–233; 1988.
[19] Robinson, C. E.; Keshavarzian, A.; Pasco, D. S.; Frommel, T. O.; Winship, D. H.; Holmes, E. W. Determination of protein carbonyl groups by immunoblotting. *Anal. Biochem.* **266:**48–57; 1999.
[20] Sokal, R. R.; Rohlf, F. J. *Biometry*. New York: W.H. Freeman; 1995.
[21] Havaux, M. Stress tolerance of photosystem II in vivo. *Plant Physiol.* **100:**424–432; 1992.
[22] Feierabend, J.; Schaan, C.; Hertwig, B. Photoinactivation of catalase occurs under both high- and low-temperature stress conditions and accompanies photoinhibition of photosystem II. *Plant Physiol.* **100:**1554–1561; 1992.
[23] Berry, J.; Björkman, O. Photosynthetic responses and adaptation to temperature in higher plants. *Annu. Rev. Plant Physiol.* **31:** 491–543; 1980.
[24] Krieger, A.; Rutherford, A. W.; Vass, I.; Gideh, E. Relationship between activity, D1 loss, and Mn binding in photoinhibition of photosystem II. *Biochemistry* **37:**16262–16269; 1998.
[25] Fine, P. L.; Frasch, W. D. The oxygen-evolving complex required chloride to prevent hydrogen peroxide formation. *Biochemistry* **31:**12204–12210; 1992.
[26] Brudvig, G. W.; Sheptovitsky, Y. G. Catalase-free photosystem II: the oxygen-evolving complex does not dismutate hydrogen peroxide. *Biochemistry* **37:**5052–5059; 1998.
[27] Nash, D.; Miyao, M.; Murata, N. Heat inactivation of oxygen evolution in photosystem II particles and its acceleration by chloride depletion and exogenous manganese. *Biochim. Biophys. Acta* **807:**127–133; 1985.
[28] Miyao, M. Involvement of active oxygen species in degradation of the D1 protein under strong illumination in isolated subcomplexes of photosystem II. *Biochemistry* **33:**9722–9730; 1994.
[29] Miyao, M.; Ikeuchi, M.; Yamamoto, N.; Ono, T. Specific degradation of the D1 protein of photosystem II by treatment with hydrogen peroxide in darkness: implications for the mechanism of degradation of the D1 protein under illumination. *Biochemistry* **34:**10019–10026; 1995.
[30] Mishra, N. P.; Mishra, R. K.; Singhal, G. S. Involvement of active oxygen species in photoinhibition II: protection of photosynthetic efficiency and inhibition of lipid peroxidation by superoxide dismutase and catalase. *J. Photochem. Photobiol. B Biol.* **19:**19–24; 1993.
[31] Haber, F.; Weiss, J. J. The catalytic decomposition of hydrogen peroxide by iron salts. *Proc. R. Soc. Lond. (Biol.)* **A147:**332–351; 1984.
[32] Heckathorn, S. A.; Downs, C. A.; Coleman, J. S. Small heat-shock proteins protect electron transport in chloroplasts and mitochondria during stress. *Am. Zool.* **39:**865–876; 1999.
[33] Asada, K. Radical production and scavenging in chloroplasts. In: Baker, N. R., ed. *Photosynthesis and the environment.* Dordrecht: Kluwer Academic Publishers; 1996:123–150.
[34] Campbell, D.; Eriksson, M.; Oquist, G.; Gustafsson, P.; Clarke, A. K. The cyanobacterium *Synechococcus* resists UV-B by exchanging photosystem II reaction-center D1 proteins. *Proc. Natl. Acad. Sci. USA* **95:**364–369; 1998.
[35] Allen, T. F. H.; Starr, T. *Hierarchy: perspectives for ecological complexity.* Chicago: University of Chicago Press; 1982.
[36] Coleman, J. S.; Heckathorn, S. A.; Hallberg, R. L. Heat-shock proteins and thermotolerance: linking molecular and ecological perspectives. *Trends Ecol. Evol.* **10:**305–306; 1995.
[37] Goff, S. A.; Voellmy, R.; Goldberg, A. L. Protein breakdown and the heat-shock response. In: Rechsteiner, M., ed. *Ubiquin.* New York: Plenum Press; 1988:207–238.
[38] Hartl, F. U. Molecular chaperones in cellular protein folding. *Nature* **381:**571–579; 1996.
[39] Iwai, K. Roles of the ubiquitin system in stress response. *Tanpakushitsu Kakusan Kuso* **44:**759–765; 1999.
[40] Downs, C. A.; Fauth, J. E.; Woodley, C. M. Assessing the health of grass shrimp (*Paleomonetes pugio*) exposed to natural and anthropogenic stressors: a molecular biomarker system. *Mar. Biotechnol.* **3:**380–397; 2001.
[41] Downs, C. A.; Heckathorn, S. A. The mitochondrial small heat shock protein protects NADH:ubiquinone oxidoreductase of the electron transport chain during heat stress in plants. *FEBS Lett.* **430:**246–250; 1998.
[42] Ganea, E.; Harding, J. J. Alpha-crystallin protects glucose 6-phosphate dehydrogenase against inactivation by malondialdehyde. *Biochim. Biophys. Acta* **1500:**49–58; 2000.
[43] Downs, C. A.; Ryan, S. L.; Heckathorn, S. A. The chloroplast small heat-shock protein: evidence for a general role in protecting photosystem II against oxidative stress and photoinhibition. *J. Plant Physiol.* **155:**488–496; 1999.
[44] Downs, C. A.; Heckathorn, S. A.; Bryan, J. K.; Coleman, J. S. The methionine-rich low-molecular-weight chloroplast small heat-shock protein: evolutionary conservation and accumulation in relation to thermotolerance. *Am. J. Bot.* **85:**175–183; 1998.
[45] Downs, C. A.; Coleman, J. S.; Heckathorn, S. A. The chloroplast 22-kDa heat-shock protein: a lumenal protein that associates with the oxygen evolving complex and protects photosystem II during heat stress. *J. Plant Physiol.* **155:**477–487; 1999.
[46] Jakob, B.; Heber, U. Photoproduction and detoxification of hydroxyl radicals in chloroplasts and leaves and relation to photoinactivation of photosystems I and II. *Plant Cell Physiol.* **37:**629–635; 1996.
[47] Warner, M. E.; Fitt, W. K.; Schmidt, G. W. Damage to photosystem II in symbiotic dinoflagellates: a determinant of coral bleaching. *Proc. Natl. Acad. Sci. USA* **96:**8007–8012; 1999.
[48] Hamilton, E. W. III; Heckathorn, S. A. Mitochondrial adaptations to NaCl. Complex I is protected by anti-oxidants and small heat-shock proteins, whereas complex II is protected by proline and betaine. *Plant Physiol.* **126:**1266–1274; 2001.
[49] Downs, C. A.; Dillon, R. T. Jr.; Fauth, J. E.; Woodley, C. M. A molecular biomarker system for assessing the health of gastropods (*Ilyanassa obsoleta*) exposed to natural and anthropogenic stressors. *J. Exp. Mar. Biol. Ecol.* **259:**189–214; 2001.
[50] Dalle-Donne, I.; Rossi, R.; Milzani, A.; Di Simplicio, P.; Colombo, R. The actin cytoskeleton response to oxidants: from small heat-shock protein phosphorylation to changes in the redox state of actin itself. *Free Radic. Biol. Med.* **31:**1624–1632; 2001.
[51] Djabali, K.; Nechaud, B.; Landon, F.; Portier, M. αB-crystallin interacts with the intermediate filaments in response to stress. *J. Cell. Sci.* **110:**2759–2769; 1997.
[52] Muchowski, P. J.; Valdez, M. M.; Clark, J. I. Alpha-B-crystallin selectively targets intermediate filament proteins during heat stress. *Invest. Ophthalmol. Vis. Sci.* **40:**951–958; 1999.
[53] Hueerkamp, C.; Glynn, P. W.; D'Croz, L.; Mate, J. L.; Colley, S. B. Bleaching and recovery of five eastern pacific corals in an El Niño-related temperature experiment. *Bull. Mar. Sci.* **69:**215–236; 2001.
[54] Glynn, P. W.; Mate, J. L.; Baker, A. C.; Calderon, M. O. Coral bleaching and mortality in Panama and Ecuador during the 1997–1998 El Niño-Southern Oscillation event: spatial/temporal patterns and comparisons with the 1982–1983 event. *Bull. Mar. Sci.* **69:**79–109; 2001.
[55] Rowan, R.; Knowlton, N. Intraspecific diversity and ecological zonation in coral-algal symbionts. *Proc. Natl. Acad. Sci. USA* **92:**2850–2853; 1995.
[56] Zhang, Z.; Green, B. R.; Cavalier-Smith, T. Single gene circles in dinoflagellate chloroplast genomes. *Nature* **400:**155–159; 1999.
[57] Mate, Z.; Sass, L.; Szekeres, M.; Vass, I.; Nagy, F. UV-B-induced differential transcription of psbA genes encoding the D1 protein

of photosystem II in the cyanobacterium *Synechocystis* 6803. *J. Biol. Chem.* **273:**17439–17444; 1998.

[58] Giardi, M. T.; Masojidek, J.; Godde, D. Effects of abiotic stresses on the turnover of the D1 reaction center II protein. *Physiol. Planta.* **101:**635–642; 1997.

[59] Quinn, W. H.; Neal, V. T.; Antunes de Mayolo, S. F. El Niño occurrences over the past four and a half centuries. *J. Geophys. Res.* **92:**14449–14461; 1987.

[60] Beckman, K. B.; Ames, B. N. The free radical theory of aging matures. *Physiol. Rev.* **78:**547–581; 1998.

[61] Hall, D. M.; Sattler, G. L.; Sattler, C. A.; Zhang, H. J.; Oberely, L. W.; Pitot, H. C.; Kregel, K. C. Aging lowers steady-state antioxidant enzyme and stress protein expression in primary hepatocytes. *J. Gerontol.* **56A:**B259–B267; 2001.

[62] Martinez, D. E. Mortality pattern suggest lack of senescence in hydra. *Exp. Gerontol.* **33:**217–225; 1998.