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## **Cellular Diagnostics and Coral Health: Declining Coral Health in the Florida Keys**

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## **Abstract**

Coral reefs within the Florida Keys are disappearing at an alarming rate. Coral cover in the Florida Keys National Marine Sanctuary declined by 38% from 1996-2000. From a resource management perspective, the primary question is whether coral declines result from global or climatic forces, local stresses, or synergistic effects of both. Traditional coral mapping and monitoring programs have difficulty resolving this question, which leaves the identity of the stressors and the mechanism of morbidity/mortality unknown. In 2000, populations of *Montastraea annularis* at four sites within the Florida Keys National Marine Sanctuary and one reef within Biscayne National Park were sampled on a quarterly basis. Biopsy samples of *M. annularis* were analyzed using Environmental Cellular Diagnostics (ECDS). Corals at Alina's Reef in Biscayne National Park appeared healthy in March, but experienced an acute loss of coral coverage by August. Environmental Cellular Diagnostic analysis indicated that Alina's Reef corals were in distress: they had experienced severe oxidative damaging and protein-denaturing stress that affected mitochondrial function in both the coral polyps and their symbiotic zooxanthellae. This condition was associated with a significant xenobiotic detoxification response in both species, indicating that the stressor was a probable chemical contaminant. These results demonstrate that a simple Environmental Cellular Diagnostic assessment is effective in assessing coral health and can identify the mechanism of pathology, as well as providing prognostic and forecasting biomarkers for coral health.

**Keywords:** Biomarker, Cellular Diagnostics, Coral, Florida Keys, Ecosystem Health

## **1. Introduction**

Coral reefs are experiencing rapid declines from deteriorating environmental conditions across the globe (Dustan and Halas, 1987; Bryant and Burke, 1998; Dustan 1999, Hoegh-Guldberg 1999; Wilkinson 1999). Recent reports indicate that 58–70% of coral reefs worldwide are directly threatened by human-associated activities, while over 80% of the Caribbean reefs have disappeared in the last 30 years (Bryant and Burke, 1998; Wilkinson 1999, Hoegh-Guldberg 1999; Goreau et al., 2000). Coral reef communities experiencing persistent environmental disturbances (e.g., coastal development and land-based pollution) can undergo changes that lead to loss of coral diversity, increased incidence of disease, reduced growth, reduced reproduction, and mass mortality (Richmond, 1993; Hoegh-Guldberg, 1999; Nystrom et al., 2000; Knowlton, 2001; Porter and Tougas, 2001; Patterson et al., 2002; Turgeon, et al., 2002). From 1996-2000, a time span that included a major El Niño event, corals in the Florida Keys National Marine Sanctuary lost a record 38% of their living coral cover (CRMP, 2001; Porter et al., 2001). The causes of these devastating declines, however, remain largely unknown.

The primary question facing resource managers is whether coral declines result from global or climatic forces, local stresses, or synergistic effects of both. If declines are caused solely by global factors, there may be little resource managers can do to immediately ameliorate the problem. However, if local factors contribute to coral declines, resource managers may be able to respond with mitigation efforts. For example, if urban effluent is the principle stressor, these point-source discharges can be managed and regulated. Unfortunately, conventional coral monitoring and mapping protocols have

had limited success in demonstrating the causes of coral reef declines in the Florida Keys and elsewhere (Pennisi, 2002). Most monitoring programs were designed to establish baseline conditions and determine trends, not to identify sources of stress (Risk, 1999). Inability of coral monitoring programs to pinpoint stress causing local reef declines makes it difficult for resource managers to effectively manage coral reefs.

Resource management can be more effective, if a coordinated, interdisciplinary (e.g., biochemistry, biology, chemistry, geology, and toxicology) approach is used to rapidly diagnose and accurately forecast expected outcomes of different mitigation strategies (Risk, 1999). We recently developed a novel biotechnology that employs a suite of diagnostic indicators to assess the cellular-physiological condition of reef-building corals (Downs et al., 2000; Downs et al., 2002, Brown et al., 2002a; Woodley et al., 2002, Fauth et al., 2002). The Environmental Cellular Diagnostic System (ECDS) focuses a comprehensive array of biotechnologies to diagnose population and ecosystem health, and was tailored to determine coral health condition and help identify mechanisms of coral declines (Downs et al., 2000; Downs et al., 2002; Brown et al., 2002a; Woodley et al., 2002; Table 1). The ECDS is used to measure changes in cellular parameters, thus allowing: (1) assessment of cellular-physiological condition of an individual or population, (2) monitoring of cellular stress responses, (3) identifying putative stressors, and (4) forecasting outcomes of environmental problems. The ECDS measures aspects of membrane integrity and composition, anti-oxidant redox status, protein metabolic condition, xenobiotic detoxification pathways, intra-cellular metal regulation, and genomic integrity. The ECDS provides a new approach to environmental health

assessment, based on concepts and methodologies already used in medical diagnostics and epidemiology (Downs et al. 2000).

Previously, we used the ECDS to demonstrate that oxidative stress was a factor in the pathology of coral bleaching (Downs et al., 2000; Downs et al., 2002; Brown et al., 2002a; Brown et al., 2002b). In 1999, we established that climatically-driven oxidative stress in corals was contributing to some small extent to the overall decline in corals at Molasses Reef in the Florida Keys (Downs et al., 2002). Here, we assess the health of corals along the same depth gradient during a non-El Niño/La Niña climatic year (2000) and sample an additional reef within another protected area: Biscayne National Park. Our objectives were to: (1) document baseline, seasonal changes in cellular parameters during a non-elevated sea-surface temperature period, (2) provide an interpretive diagnosis of the health condition of these corals in 2000, and (3) detect and characterize subtle and chronic effects of environmental stressors on coral cellular physiology. This paper is the first of several papers that examines the biological, environmental chemistry, and anthropogenic variables of coral reefs in the upper region of the Florida Keys.



canister and samples transferred to a liquid nitrogen dry shipper. Samples were stored at -80° C until analysis.

### **Sample Preparation, ELISA validation, and ELISA**

Coral samples were ground to a powder using liquid nitrogen-chilled ceramic pestle and mortar. Samples (~10 mg) of frozen tissue were placed in 1.8ml microcentrifuge tubes along with 1,400 µl of a denaturing buffer consisting of 2% SDS, 50 mM Tris-HCl (pH 6.8), 25 mM dithiothreitol, 10 mM EDTA, 0.001 mM sorbitol, 7% polyvinylpolypyrrolidone (wt/vol), 0.1% polyvinylpyrrolidone (wt/vol), 0.01 mM alpha-tocopherol, 0.005 mM salicylic acid, 0.01 mM AEBSF, 0.04 mM Bestatin, 0.001 E-64, 2 mM phenylmethylsulfonyl fluoride, 2 mM benzamide, 0.01 mM apoprotin, 5 µM amino-caproic acid, and 1 µg/100 uL pepstatin A. Samples were heated at 95°C for three minutes, vortexed for 20 seconds, incubated at 95°C for another three minutes, and then incubated at 25°C for five minutes. Samples were centrifuged at 10,000 g for five minutes. Supernatant free of a lipid/glycoprotein mucilage matrix was transferred to a new tube, centrifuged at 10,000 g for five minutes and supernatant free of a lipid/glycoprotein mucilage matrix was again transferred to a new tube and subjected to a protein concentration assay (Ghosh et al., 1988).

To ensure equal sample loading, 40 µg of total soluble protein of samples from one site and time point were loaded onto a 12.5% SDS-PAGE gel (16 cm), the gel run until the bromophenol blue front was near the bottom of the gel, stained with a Coomassie blue solution (BB R-250) overnight, and then destained for 4 hours with multiple washes of



H100-P), algal anti-heat-shock protein 70 (AB-H101-P), algal glutathione-s-transferase (AB-GST-PA), anti-chloroplast small heat-shock protein (AB-H104-C), anti-ubiquitin (AB-U100), cnidarian anti-heat-shock protein 70 (AB-H101-CDN), cnidarian anti-heat-shock protein 60 (AB-H100-IN), cnidarian anti-glutathione-S-transferase (AB-G100-MU), cnidarian anti-manganese superoxide dismutase (AB-S100-MM), cnidarian glutathione peroxidase (AB-GPX-IN), cnidarian glutathione-s-transferase (pi-isoform homologue; AB-GST-INV), invertebrate anti-small heat shock protein (AB-H103), and anti-MXR (ABC family of proteins; P-glycoprotein 140 & 160; AB-MDR-160). Samples were assayed in triplicate with intra-specific variation of less than 6% for the whole plate. An eight-point calibrant curve using a calibrant relevant to each antibody was plated in triplicate for each plate.

### **Diagnostic Strategy and Statistical Analysis**

Planned (*a priori*) and unplanned comparison tests were used, depending on whether the question posed for a specific data set contained fundamental hypotheses, as in the case of a quantitative diagnostic strategy as used by the Environmental Cellular Diagnostic System (Schaeffer, 1996; Panzer et al., 1999). The Environmental Cellular Diagnostic System posits that changes in cellular integrity and homeostatic responses are fundamental end-points in discriminating whether a xenobiotic or environmental agent is affecting the cell, hence the organism. The terms cellular integrity and homeostatic responses can be partially and stipulatively defined by the behavior of the parameters in the following categories: genomic integrity, protein metabolic condition, xenobiotic response, metabolic integrity, and oxidative-stress effects and response. It should be noted that these *definiens* of cellular integrity and homeostatic responses are used because

## MATERIALS AND METHODS

### Reagents

All chemicals for buffered solutions were obtained from Sigma Chemicals Co. (St. Louis, Missouri, U.S.A.) or EnVirtue Biotechnologies, Inc. (Winchester, VA, USA). PVDF membrane was obtained from Millipore Corp. (Bedford, Massachusetts, U.S.A.). Antibodies against all the cellular parameters, as well as calibrant standards, were obtained from EnVirtue Biotechnologies, Inc. (Winchester, VA, USA). Anti-rabbit conjugated horseradish peroxidase antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA).

### Coral Collection

We sampled boulder corals (*Montastraea annularis* species complex) at four sites near Molasses Reef, within the Florida Keys National Marine Sanctuary (FKNMS), Key Largo, FL, USA. We chose these sites because coral bleaching exhibited a bathymetric gradient; in 1999, fewer colonies bleached in shallower (3 and 6 m sites) than in deeper water (9 and 18 m sites; Downs et al., 2002). We also sampled one site at Alina's Reef, within Biscayne National Park (BNP), Homestead, FL, USA. We included it as a northern, shallow (6 m) site, potentially influenced by agricultural and landfill runoff and operations of two pressurized-water nuclear reactors at Turkey Point.

We collected samples (2.5 cm diameter) from five colonies at each site in March, June, August, and November 2000. Samples were biopsied using a modified leather punch, and then placed in film canisters. On deck, water was quickly removed from the film

### **3. Results**

#### **Antibody Validation**

Antibodies against cnidarian cellular parameters did not exhibit significant non-specific cross-reactivity (Fig 1., Panels A-G), hence could be validly used in an ELISA format. Antibodies against dinoflagellate cellular parameters also did not exhibit significant non-specific cross-reactivity (Fig 2, Panels A-G). Because of the evolutionary conservation of ubiquitin and MXR, these antibodies detected proteins from both species in the coral homogenate (Fig 3; ubiquitin data not shown). Therefore, an ELISA measurement using this antibody detects total concentration for these proteins. In MXR, the faster migrating band is the dinoflagellate homologue while the slower migrating band is the cnidarian homologue. This was determined by running cultured *Symbiodinium* protein next to the coral homogenate using an SDS-PAGE/western blotting protocol (data not shown).

#### **ELISA Results**

ELISA results are divided into the following Environmental Cellular Diagnostic System Categories:

- Protein Metabolic Condition which includes dinoflagellate Hsp60, dinoflagellate Hsp70, cnidarian Hsp60, cnidarian Hsp70 and Ubiquitin (Fig. 4),
- Oxidative Stress Response which includes dinoflagellate MnSOD, dinoflagellate Cu/ZnSOD, dinoflagellate GPx, cnidarian MnSOD, cnidarian GPx (Fig. 5),
- Xenobiotic Response which includes GST, cnidarian GST, and MXR (Fig. 6), and
- Metabolic Condition which includes the Chloroplast sHsp and the four major cnidarian sHsp classes (Fig. 7).

destaining solution. Equal loading was determined by visualization and optical density using a Canonscan scanner and analysis performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

One-dimensional SDS-PAGE and western blotting validated the legitimacy of an ELISA on this species of coral using a specific antibody. Five to fifteen micrograms of total soluble protein of coral supernatant was loaded onto a 20-, 16-, or 8-cm SDS-polyacrylamide gel with various concentrations of bis/acrylamide. A TCEP concentration of 0.001M was added to gels loaded with samples to be assayed with antibody to the chloroplast sHsp and the invertebrate sHsp. Gels were blotted onto PVDF membrane using a wet transfer system. Membrane was blocked in 7% non-fat dry milk, and incubated with the primary antibody for one hour. The blots were washed in tris-buffered saline (TBS)-0.05% Tween (v/v) four times, and incubated in a horseradish peroxidase-conjugated secondary antibody solution for one hour. Blots were washed four times in TBS, and developed using a chemiluminescent reporter system.

Once validated, antibodies and samples were optimized for ELISA using an 8 x 6 x 4 factorial design (Crowther, 2001). A Beckman-Coulter Biomek 2000 using 384-well microplates was used to conduct the ELISA assays. Samples were assayed using the following EnVirtue Biotechnologies, Inc. antibodies: algal anti-glutathione peroxidase (AB-G101-P), algal anti-manganese superoxide dismutase (AB-S100-P), algal anti-copper/zinc superoxide dismutase (AB-101-PA), algal anti-heat shock protein 60 (AB-

The most striking biomarker pattern was exhibited by corals from Alina's Reef in March 2000. Parameters of protein metabolic condition suggested that a massive insult was occurring, and that the insult not only resulted in increased protein denaturation, but a considerable level of irrevocable damage to functioning enzymes. Cnidarian and dinoflagellate Hsp70s and Hsp60s at Alina's Reef in March were significantly higher than Sites 1-4 at any time point (one-way ANOVA,  $p < 0.05$ ). Ubiquitin at Alina's Reef in March was considerably higher than almost all the sites at any time (one-way ANOVA,  $p < 0.001$ ). Two conclusions that can be inferred from this data is (1) that the stressor was affecting only Alina's Reef and (2) that the stressor was also adversely affecting both symbionts. Cnidarian and dinoflagellate Hsp70 and cnidarian Hsp60 remained high through November (one-way ANOVA,  $p < 0.01$ ). Levels of dinoflagellate hsp60 were no longer significantly elevated in August (ANOVA,  $p < 0.01$ ), suggesting that at least organellar protein metabolism was returning to basal levels. Increased cnidarian Hsp60 suggests that mitochondrial function in this symbiont was still disturbed through November.

Small heat-shock proteins protect various metabolic pathways and cellular function and usually are only present when these systems are experiencing a level of damage or dysfunction. The chloroplast sHsp is an evolutionarily conserved protein found only in the chloroplast of photosynthetic eukaryotic organisms and protects Photosystem II of the photosynthetic electron-transport chain during stress (Heckathorn et al., 1998; Downs et al., 1999a). Basal levels of this protein through the year in corals from the Upper Keys averaged 0.075 RU/ng TSP. Levels of this protein at Alina's Reef in March were over

they are measurable by the present technology. As the technology evolves, so will the categories and parameters; thus providing a more meaningful explanation for *cellular integrity and homeostatic response*.

Each category is defined by a functional process and the physical components constituting that process. For example, protein metabolic condition can be defined, in part, by the protein, RNA, and enzymatic components (sub-systems) that have a role in protein synthesis, protein chaperoning, and protein degradation. Shifts in these parameters are indicative of a shift in the equilibrium of these sub-systems, as well as the system as a whole. The behavior of each cellular parameter is independently regulated from the behavior of other cellular parameters.

Parametric statistics were used to test whether cellular parameters differed among sites at the same time point and at different time points. If data were normally distributed and homogeneous, a one-way analysis of variance (ANOVA) with  $\alpha=0.05$  was employed. When Bartlett's test for homoscedasticity or graphical assessment of normality showed variates did not meet ANOVA assumptions, we substituted a Welch ANOVA (Sokal and Rohlf, 1995). When significant differences were found among treatment means, we used the Tukey-Kramer Honestly Significant Difference (HSD) method as an exact alpha-level test for all differences between means (Sokal and Rohlf, 1995).

October, though still higher than GPx levels at Sites 1-4 ( $p < 0.001$ ). This increase of the major enzymatic antioxidant defenses indicates that Alina's Reef was responding to an oxidative stress.

Combined results for parameters of Protein Metabolic Condition, small Hsps, and oxidative stress defense argue that Alina's Reef was experiencing a significant stress that was severely damaging proteins and threatening nominal metabolic equilibrium. Assays that examine mitochondrial membrane potential, mitochondrial electron transport, cytoskeletal integrity, and protein, lipid, and DNA oxidation would better refine the mechanism of cellular disruption that occurred at Alina's Reef. Some of these assays could be done in the field on the same day of collection, enhancing the ability of managers/scientists to identify and possibly mitigate the stressor.

The significant effect on both biomarker responses and coral coverage of the unknown stressor on Alina's Reef strongly argues that the stressor is localized, and localized to or around Alina's Reef. Based on the proximity of Alina's Reef to a number of anthropogenic activities, there is a high probability that the stressor may be a chemical xenobiotic. To test this hypothesis, we examined three cellular parameters that would be significantly modulated by an organic contaminant: cnidarian GST, dinoflagellate GST, and the P-glycoprotein 180 homologue from both species. Cnidarian GST levels were significantly elevated in March at Alina's Reef (Welch ANOVA,  $p < 0.001$ ), and continued to be significantly elevated (Welch ANOVA,  $p < 0.01$ ). Dinoflagellate GST was elevated for at Alina's Reef in March, as well as the rest of the year, but not as

#### 4. Discussion

Resource managers and scientists are faced with the issue of determining the source and mechanism of coral reef decline, as well as predicting or forecasting the effects that result between the interaction of a stressor(s) and an organism. Environmental Cellular Diagnostics (ECDS) is a newly developed tool that promotes the concept of the resource manager to the role of a resource or ecological clinician. Employment of the ECDS in an appropriate monitoring and assessment design that couples other assessment methods of biological and environment indices (e.g., contaminant analysis, tissue regeneration, body weight, coral coverage) may provide invaluable information as to the 'health condition' of a population (or localized community), as well as insight to the source of ailments that may be responsible for an affliction. This form of diagnosis gives added value to current coral reef monitoring methods because it ascribes an underlying mechanism when describing higher order biologic effects, such as changes in population density, community structure, or symptomology (e.g., type and incidence of infectious disease).

Within Sites 1 - 4, Sites 3 and 4 had slight decreases in coral cover in the same coral colonies that had been sampled since 1999, according to subjective observation. Site 1 showed no indication of coral coverage loss. At Alina's Reef, coral coverage looked unchanged from December 1999 to June 2000. In August, a significant level of coral coverage loss occurred on the massive *Montastraea* heads at this site. There was significant coverage of brown algae on the dead coral, and we had lost the ability to repeatedly sample one entire coral colony in August, and another one by October.

with contaminant analysis (e.g., endosulfan, atrazine, mercury, etc...), would tremendously increase the power in determining stressor identity from among a number of potential suspects. It should be noted that the cellular biomarker profile of Alina's Reef in March is similar to the cellular biomarker profile of *Montastraea* and other coral species when exposed to xenobiotics such as certain pesticides, antibiotics and heavy metals, but does not match the profile for hyposalinity, high nitrogen exposure (e.g., ammonia, nitrite), or heat/high-light stress (Downs et al., in preparation). Extensive laboratory toxicological studies may provide further corroborating evidence as to the potential stressor, especially since the samples have been archived and can be analyzed with new assays as they are developed.

Generating a prediction or prognosis for the outcome of a population and a probability for that outcome is achievable only with an understanding of the underlying cellular physiological condition. Though a number of cellular diagnostic parameters were elevated in March for Alina's Reef, six months before the observed mortality, only ubiquitin was a useful prognostic marker for coral mortality. By themselves, increases of Hsp70, Hsp60 and MXR were correlated with the mortality event, but none of these biomarkers can be used legitimately to prognosticate morbidity or mortality. This is because these biomarkers can increase significantly in response to non-morbid states. Both chaperones can increase 200-900% as a result of growth, reproductive development, and infection, while MXR can significantly accumulate in response to a xenobiotic exposure not associated with morbidity (Sauna et al., 2001). Good prognostic markers for morbidity/mortality are those that are usually directly associated with cellular

twice this (Welch ANOVA,  $p < 0.001$ ), indicating that PSII was severely stressed, if not damaged. Chloroplast sHsp levels then decreased to basal levels in June and remained there for the remainder of the year. Cnidarian sHsp 22 and 24 (Welch ANOVA,  $p < 0.001$ ) had a similar pattern, cnidarian sHsp levels at Alina's Reef in March were almost double the levels at the other sites through the year, but then decreased significantly in June to levels comparable to seen at the other sites. The sHsp22 is localized to the mitochondria in cnidarians and other invertebrates and thought to protect aspects of respiration specifically oxidative phosphorylation (Morrow et al., 2000). This data suggests stressors affected electron-transport of major metabolic pathways and destabilized cnidarian mitochondrial function for the remainder of the year.

Induction of both sHsp homologues suggests that oxidative damage may be occurring in both symbionts. To determine if anti-oxidant defenses of both species were elevated, we examined superoxide dismutases and glutathione peroxidases. Cnidarian Cu/ZnSOD was not significantly elevated at Alina's Reef in March or any other time point. Cnidarian MnSOD was significantly elevated throughout the year compared to all the other sites and time points, but especially in March (Welch ANOVA,  $p < 0.001$ ). The expression pattern of dinoflagellate MnSOD reflected the pattern of cnidarian MnSOD, but its accumulation was even more pronounced in Alina's Reef, compared to the other sites at any of the time points (Welch ANOVA,  $p < 0.001$ ). Elevation of both MnSODs suggests that the unknown stressor is affecting the mitochondria. Both cnidarian and dinoflagellate GPx levels were significantly increased in March at Alina's Reef (ANOVA,  $p > 0.001$ ), but like the MnSODs, decreased in accumulation in June through

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pronounced at the cnidarian GST (Welch ANOVA,  $p < 0.001$ ). Multixenobiotic resistance protein, MXR, is an ABC cassette protein that lowers the intracellular concentration of toxic compounds below their level of toxicity. Increase of this protein is usually only in response to an organic xenobiotic. Unfortunately, this antibody binds both the cnidarian and dinoflagellate isoforms, hence results are a composite of both MXR expression. MXR levels were significantly elevated in March at Alina's Reef above all other sites at all time points (Welch ANOVA,  $p < 0.001$ ). MXR levels at Alina's Reef decreased in June and were not significantly different than MXR levels Sites 1-4 throughout the rest of the year.

Levels of almost all of the cellular diagnostic markers between Sites 1-4 were not significantly different during any of the sampling time points (ANOVA followed by Tukey-Kramer HSD Test,  $p > 0.05$ ). This significant difference in biomarker levels between Alina's Reef and Sites 1- 4, coupled with field observations, suggests that coral decline in the Upper Keys may be related to local factors. Though we are able to ascertain that the stressor induces an oxidative stress, is disruptive to mitochondrial 'nominal' equilibrium, and elicits a strong xenobiotic defense response, the identification of the stressor remains unknown. This inability to directly identify the stressor could be alleviated by increasing the scope of the cellular biomarkers in corals, and by contaminant/chemistry analysis. For example, assays for cnidarian metallothioneins, cnidarian methyl arsenic reductases, dinoflagellate sulfate permease, dinoflagellate nitrate reductase, and dinoflagellate calcium-dependent alkaline phosphatase could be developed to aid in deducing the identity of the stressor(s). Environmental chemistry analysis, along

- 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.
- Goreau, T.J., McClanahan, T., Hayes, R. and Strong, A.E. 2000. Conservation of coral reefs after the 1998 global bleaching event. *Conserv. Biol.* 14: 5-15.
- Heckathorn, S.A., Downs, C.A., Sharkey, T.D. and Coleman, J.S. (1998) The small methionine-rich chloroplast heat-shock protein protects photosystem II electron transport during heat stress. *Plant Physiol.* 116:439-444.
- Hoegh-Guldberg, O. 1999. Climate change, coral bleaching and the future of the world's coral Reefs. *Mar. Freshwater Res.* 50:839-66.
- Knowlton, N. 2001. The future of coral reefs. *Proc. Nat. Acad. Sci. USA* 98:5419-5425.
- Morrow, G., Inaguma, Y., Kato K, Tanguay RM. 2000. The small heat shock protein Hsp22 of *Drosophila melanogaster* is a mitochondrial protein displaying oligomeric organization. *J. Biol. Chem.* 275: 31204-10.
- Nystrom, M., Folke, C., and Moberg, F. 2000. Coral reef disturbance and resilience in a human-dominated environment. *TREE* 15:413-417.
- Panzer, R.J., Black, E.R. and Griner, P.F. 1999. Interpretation of diagnostic tests and strategies for their use in quantitative decision making. In: *Diagnostic Strategies for common medical problems*. E.R. Black, D.R. Bordley, T.G. Tape, R. J. Panzer, eds. American College of Physicians, Philadelphia, Pennsylvania, pp.18-30.
- Patterson, K. L., J. W. Porter, Ritchie, K. B., Polson, S. W., Mueller, E., Peters, E. C., Santavy, D. L., Smith, G. W. 2002. The etiology of white pox, a lethal disease of the Caribbean elkhorn coral, *Acropora palmata*. *Proc. Natl. Acad. Sci. U S A* 99: 8725-30.
- Pennisi, E. 2002. Survey Confirms Coral Reefs Are in Peril. 297: 1622-1623.
- Porter, J. W. and Tougas, J.I. 2001. Reef Ecosystems: Threats to their biodiversity. Encyclopedia of Biodiversity. S. Levin, Academic Press. 5: 73-95.
- Porter, J.W., Dustan, P., Jaap, W.C., Patterson, K.L., Kosmynin, V., Meier, O.W., Patterson, M.E., Parsons, M. 2001. Patterns of spread of coral disease in the Florida Keys. *Hydrobiologia.* 460: 1-24.
- Richmond, R.H. 1993. Coral reefs: Present problems and future concerns resulting from anthropogenic disturbance. *Am Zool* 33: 524-536.

damage. Protein carbonyl, lipid peroxidation products, and DNA damage products have been demonstrated to be good prognostic indicators for corals, while stress defense proteins and solutes have demonstrated value as indicators of stress susceptibility (Downs et al., 2002; Fauth et al., 2002). Further development of cellular biomarkers in corals and other coral reef species could greatly enhance the ability of resource managers to make ecological forecasts.

In summary, we have provided evidence for the cogent argument that coral decline in the Florida Keys may, in part, be rooted in local stressors. These local stressors, at least for Alina's Reef, are not environmentally (i.e., atmospheric, hydrologic) derived, but are possibly chemical xenobiotics. Integration and further development of environmental cellular diagnostics for coral reef health assessment can be a powerful tool in providing resource managers valuable information as to the health condition of specific coral reefs, as well as evidence as to the identity and locality of the putative stressors, and forecasting the fate of coral reefs in light of a stress event. Developing a sound strategy that integrates biological, chemical, and environmental protocols that can be effectively implemented by resource managers to quickly and confidently assess the condition of coral reefs is paramount in addressing the crisis of coral reef declines. Once an understanding is made concerning the nature and source of the declines, wise mitigation strategies can be developed and enacted.

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## References

- Brown, B.A., Downs, C.A., Dunne, R.P. and Gibb, S.W. 2002a. Exploring the basis of thermotolerance in the reef coral *Goniastrea aspera*. *Mar. Ecol. Prog. Ser.* **242**: 119–129.
- Brown, B.A., Downs, C.A., Dunne, R.P. and Gibb, S.W. 2002b. Preliminary evidence for tissue retraction as a factor in photoprotection of corals incapable of xanthophyll cycling. *J. Exp. Mar. Biol. Ecol.* **277**: 129–144.
- Bryant, D. and Burke, L. 1998. *Reefs at Risk: A map-based indicator of potential threats to the world's coral reefs.* (World Resources Institute, Washington, DC) 56pp.
- Coral/Hardbottom Monitoring Project (CRMP) Executive Summary 1996-2000, Steering Committee Report, August 1, 2001.
- Crowther, J.R. 1999. *The ELISA Guidebook.* Humana Press. Totowa, New Jersey.
- DeZwart, L.L., Meerman, J.H.N., Commandeur, J.N.M., and Vermeulen, N.P.E. 1999. Biomarkers of Free Radical Damage Applications in Experimental Animals and in Humans. *Free Rad. Biol. Med.* **26**: 202-226.
- Derham B.K., Harding J.J. 1999. Alpha-crystallin as a molecular chaperone. *Prog Retin. Eye Res.* **18**:463-509.
- Downs, C.A., Coleman, J.S. and Heckathorn, S.A. (1999) The chloroplast 22-Ku heat-shock protein: a luminal protein that associates with the oxygen evolving complex and protects photosystem II during heat stress. *J. Plant Physiol.* **55**:477-487.
- Downs, C.A., E. Mueller, S. Phillips, J.E. Fauth, and C.M. Woodley. 2000. A molecular biomarker system for assessing the health of coral (*Montastraea faveolata*) during heat stress. *Marine Biotechnology.* **2**: 533-544.
- Downs, C.A., Fauth, J.E., Halas, J.C., Dustan, P., Berniss, J.A., and Woodley, C.M., 2002 *Oxidative Stress and Coral Bleaching.* *Free Rad. Biol. Med.* **33**:533-543.
- Dustan, P. 1999. Coral reefs under stress: sources of mortality in the Florida Keys. *Nat. Res. Forum* **23**:147-55.
- Dustan, P. and Halas, J.C. 1987. Changes in the reef-coral community of Carysfort Reef, Key Largo, Florida: 1974-1982. *Coral Reefs.* **6**:91-106.
- Fauth J. E., C. A. Downs, J. C. Halas, P. Dustan, and C. M. Woodley. 2002. Mid-range prediction of coral bleaching: a molecular diagnostic system approach. In D. Scavia and N. Valette-Silver, eds. *NOAA Technical Report on Ecological Forecasting*, in press.



- Risk, M.J. 1999. Paradise lost: how marine science failed the world's coral reefs. *Mar. Freshwater Res.* 50:831-37.
- Sauna, Z.E., Smith, M.M., Müller, M., Kerr, K.M. and Ambudkar S.V. 2001. The Mechanism of Action of Multidrug-Resistance-Linked-P-Glycoprotein. *J. Bioenerg. Biomem.* 33: 481-491.
- Schaeffer, D.J., Herricks, E.E., and Kerster, H.W. 1988. Ecosystem Health : I. Measuring Ecosystem Health. *Environ Manag* 12: 445-455.
- Sies, H. 1999. Glutathione and its role in cellular functions. *Free Rad. Biol. Med.* 27: 916-921.
- Sokal, R. R. & Rohlf, F. J. 1995. *Biometry*. W. H. Freeman, New York, New York, U.S.A.
- Turgeon, D.D., R.G. Asch, B.D. Causey, R.E. Dodge, W. Jaap, K. Banks, J. Delaney, B. D. Keller, R. Speiler, C.A. Matos, J.R. Garcia, E. Diaz, D. Catanzaro, C.S. Rogers, Z. Hillis-Starr, R. Nemeth, M. Taylor, G.P. Schmahl, M.W. Miller, D.A. Gulko, J.E. Maragos, A.M. Friedlander, C.L. Hunter, R.S. Brainard, P. Craig, R.H. Richmond, G. Davis, J. Starmer, M. Trianni, P. Houk, C.E. Birkeland, A. Edward, Y. Golbuu, J. Gutierrez, N. Idechong, G. Paulay, A. Tafiichig, and N. Vander Velde. 2002. The State of Coral Reef Ecosystems of the United States and Pacific Freely Associated States: 2002. National Oceanic and Atmospheric Administration/National Ocean Service/National Centers for Coastal Ocean Science, Silver Spring, MD. 265pp.
- Wilkinson, C.R. (1999). Global and local threats to coral reef functioning and existence: review and predictions. *Mar. Freshwater Res.* 50: 867-878.
- Woodley, C.M., Downs, C.A., Fauth, J.E., Mueller, E., Halas, J.C., Bemiss, J.A., Ben-Haim, Y., and Rosenberg, E., "A novel molecular biomarker system to assess the physiological status of corals". In M.K. Kasim Moosa, S. Soemodihardjo, A. Nontji, A. Soegiarto, K. Romimohtarto, Sukarno and Suharsono. eds. 2002. *Proceedings of the Ninth International Coral Reef Symposium, Bali, Indonesia, October 23-27 2000*. Published by the Ministry of Environment, the Indonesian Institute of Sciences and the International Society for Reef Studies. 1267-1272 pp. ISBN 979-8105-97-4.