

A NOVEL MOLECULAR BIOMARKER SYSTEM TO ASSESS THE PHYSIOLOGICAL STATUS OF CORALS

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Abstract

We developed a novel molecular biomarker system that assesses the physiological status of corals by assaying specific parameters of cellular physiology indicative of a non-stressed or stressed condition. This technology is known as the Downs Molecular Biomarker System™ (DMBS). It is particularly relevant for corals because multiple environmental stressors (e.g., heat stress, uv, cold stress, pathogens) may contribute to coral bleaching, one etiology for coral mortality worldwide. In laboratory studies, the DMBS distinguished the separate and combined effects of heat and light on a scleractinian coral (*Montastraea faveolata*) and its symbiotic dinoflagellate (zooxanthellae) and provided strong evidence supporting the hypothesis that oxidative stress plays a central role in coral bleaching. In field samples, colonies of *Montastraea annularis* in the Florida Keys showed a strong correlation between DMBS parameters of oxidative stress and bleaching. We also used this new biotechnology to characterize the pathology of coral bleaching by assessing cellular physiological parameters in explants of *Oculina varicosa* exhibiting bacteria-induced bleaching. Our data suggest that bacteria-induced coral bleaching may involve non-oxidative stress mechanisms. Collectively, our data showed that the DMBS can (1) diagnose whether corals were physiologically stressed, (2) can potentially discriminate between multiple mechanisms involved in coral bleaching, (3) predict the occurrence of coral bleaching at least 3-6 months before visual indications of a bleaching event, and (4) provide evidence that the chloroplast small heat-shock protein is a significant adaptation against coral bleaching.

Introduction

We designed an integrated molecular biomarker system that focuses a comprehensive array of diagnostic tools from modern biomedical research on environmental health issues (Downs, et al., 2001). This technology is known as the Downs Molecular Biomarker System™ (DMBS). The unique array of biomarkers in the DMBS is selected based on known biochemical, cellular and physiological processes and can be tailored to individual species and likely sources of environmental stress. The system is designed to have a degree of redundancy; several parameters from differing biochemical pathways characterize a given physiological status, thereby providing concordance among markers for a more reliable diagnosis. These include, but are not limited to cellular parameters such as lipid peroxide levels (LPO), malondialdehyde (MDA), 4-hydroxy-2-trans-nonenol (HNE), total glutathione (GSH), heat-shock protein 60 (Hsp60), heat shock protein 70 (Hsp70), α B-crystallin homologue, chloroplast small heat-shock protein (chlpsHsp), homologues to the plant class I-IV small heat-shock proteins (sHsps), Mn superoxide dismutase (MnSOD), Cu/Zn superoxide dismutase (Cu/ZnSOD), metallothioneins, specific P450 family members, protein carbonyl and ubiquitin. Assaying these parameters provides indicators of the physiological condition of critical components of the cellular superstructure (i.e., enzymes, DNA, membranes and metabolic pathways) and the status of general properties of cellular metabolism (i.e., photosynthetic and oxidative phosphorylation). The DMBS also quantifies responses indicative of oxidative stress and provides evidence for identifying putative stressor(s) (i.e., heat, xenobiotic, heavy metal, pathogens). Taken together these physiological measurements can be used to diagnosis

organismal health by coupling an understanding of the cellular physiological response to an understanding of how those responses are linked to organismal fitness.

One of the most serious environmental health issues today is the extensive degradation of coral reefs worldwide (Dustan, 1999; Stone et al., 1999). Among the dominant etiologies for this global degradation is a syndrome known as coral "bleaching" (Dustan, 1999). Reports of destructive worldwide bleaching events have increased since 1979, (Goreau et al., 1992; Goreau and Hayes, 1994; Brown et al., 1996) with the most devastating mass coral bleaching event to date recorded in 1997-1998 (Hendee, 1999; Hoegh-Guldberg, 1999). Bleaching is a process whereby corals lose their obligate symbiotic algae (zooxanthellae) and/or experience degradation of the symbionts' photosynthetic pigments (Brown, 1997a; Gates et al., 1992). Although elevated seawater temperatures (i.e., heat stress) is emerging as a primary factor in most large scale coral bleaching events (Hoegh-Guldberg and Smith, 1989; Lesser, 1990; Glynn, 1993; Goreau et al., 1992; Goreau and Hayes, 1994; Lesser, 1996; Brown, 1997b; Hoegh-Guldberg, 1999), a number of other environmental factors have been linked to coral bleaching. These include cold stress (Coles and Fadlallah, 1991; Muscatine, et al., 1991), elevated visible light (Hoegh-Guldberg and Smith, 1989), light deprivation (Yonge and Nichols, 1931), ultraviolet light exposure (Kinzie, 1993; Lesser et al., 1990), low salinity (Fang et al., 1995; Goreau, 1964), starvation (Yonge and Nicholls, 1931) and bacterial infection (Kushmaro, et al., 1996; Ben-Haim et al., 1999). Since several stressors may induce coral bleaching, it is essential to determine the mechanisms responsible for this devastating pathology and understand its epidemiology in order to manage or control it.

Research has only recently focused on cellular and molecular aspects of coral bleaching (Black et al., 1995; Fang et al., 1997; Jones et al., 1998; Moshe et al., 1999; Downs et al., 2001).

To systematically investigate this syndrome's pathology, nominal levels for cellular and molecular parameters that reflect coral physiological status must be identified and quantified. In addition, there is a need to differentiate among coral bleaching mechanisms (Brown, 1997a; Ben-Haim et al., 1999). These needs can be addressed using reliable and precise technologies to quickly determine the condition of corals, both in the laboratory and in the field. Meeting these needs will enhance our understanding of the causes and mechanisms of coral bleaching, thereby opening potential avenues for the prevention, rehabilitation, and/or conservation of coral reefs.

In this paper, we present data from a new biotechnology that determine the cellular physiological status of corals, the Downs Molecular Biomarker System (DMBS). In laboratory studies, we tested whether the DMBS could distinguish the separate and combined effects of high temperature and light stress on a scleractinian coral (*Montastraea faveolata*). These two factors are strongly correlated with coral bleaching (Downs et al., 2001). We also investigated whether the cellular parameters measured by the DMBS could determine changes in physiological status and cellular processes of corals (*Montastraea annularis*) from a field site in the Florida Keys (near Molasses Reef) over a growing season that included a bleaching event. We also used the DMBS to determine whether the cellular responses of coral bleaching induced by biotic vs. environmental stresses differed, by evaluating cellular responses in corals (*Oculina varicosa*) infected by a pathogen (*Vibrio shiloi*) compared to those subjected to high temperature stress.

Materials and Methods

All chemicals were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). PVDF and nitrocellulose membranes were obtained from Millipore Corp. (Bedford, Massachusetts, USA).

Dot blot and gel electrophoresis equipment were obtained from Bio-Rad Corp. (San Diego, California, USA). LPO-560 assay kits (Cat. #21025) were obtained from Oxis International, Inc. (Portland, Oregon, USA). Antibodies against ubiquitin (Cat. #SPA200) were obtained from StressGen Biotechnologies, Inc. (Victoria, British Columbia, Canada). Anti-rabbit and anti-mouse conjugated alkaline phosphatase antibodies were obtained from Promega (Madison, Wisconsin, USA). Antibodies to the chloroplast small heat-shock protein were produced as described in Heckathorn et al. (1998) and Downs et al. (1998). Protein standards of ubiquitin were obtained from Sigma-Aldrich. Protein standards for the chlpsHsp were produced as described in Heckathorn et al. (1998) and Downs and Heckathorn (1998).

Sample Collection and Maintenance

Skirt fragments from three colonies of *Montastraea faveolata* were collected near the Looe Key Special Use Zone south of Big Pine Key, FL (depth = 6 m) for use in laboratory heat stress experiments. Coral explants were maintained in aquaria at 27° C under an irradiance of ~350 $\mu\text{moles photons m}^{-2} \text{ s}^{-1}$ (12 hour light: 12 hour dark), and fed *Artemia nauplii* once per week.

Field samples of *Montastraea annularis* were collected in the Key Largo National Marine Sanctuary, Key Largo, FL near Molasses Reef. This site was chosen because it exhibited a putative "stress gradient" with a depth, allowing analysis of at least two possible stressors, temperature and UV light. Coral samples (< 2.5 cm fragments) from five separate colonies were collected at each of four depths (3 m, 6.1 m, 9.1 m, and 18.3 m) with each colony repeatedly sampled monthly from March-November 1999. Samples were wrapped in aluminum foil and placed in whirl-pak bags underwater, the water removed from the bags and the samples

transferred to a liquid nitrogen dry shipper on the boat. Samples were stored frozen at -80° C until analysis.

One colony of *Oculina varicosa* was collected from Caper's Reef (depth = 18 m) near Charleston, SC for use in laboratory infection studies. Coral explants were maintained in aquaria at 26° C under 55 W blue ultra-actinic light (CustomSeaLife, Inc.) on a 12 hr light:12 hr dark cycle and fed *Artemia nauplii* once per week.

Heat Stress Treatment

Coral explants were treated with three environmental conditions (1) optimal growth conditions, (2) high temperature stress in the absence of light, and (3) high temperature stress under nominal photosynthetically active radiation (PAR), as described in Downs et al. (2001). Control samples (three explants from each parent colony) were removed from optimal growth conditions and frozen. Heat-shock experiments were conducted in the light and the dark by ramping the temperature from 27° C to 33° C over a three hour period and maintained at 33° C for another nine hours. As with the controls, three explants from each parent colony (nine total) were used in each experiment, and all samples were frozen at the end of the experimental periods.

Bacterial Infection

A single colony of *Vibrio shiloi* was used to inoculate 25 mL of marine broth containing tryptone (MBT) media and grown at 30°C with orbital shaking (200 rpm) for 19 hours. The inoculum was prepared by centrifuging 10 mL of the culture for 10 min at 9000 rpm and

washing the cell pellet twice in filter sterilized sea water. The final pellet was then resuspended in 1mL of sterile sea water.

Coral explants were acclimated in one liter dosing chambers adjusted to 28.5 - 29°C for one week prior to inoculation. Explants were infected by placing them in a sterile petri dish and applying 20 µL of the bacterial inoculum (1.5×10^8 cells). The explants remained in the inoculating dish for 1 minute after the application and then returned to their dosing chambers. Control coral explants were dosed as above except 20 µL of sterile sea water was applied in place of the bacterial inoculum.

Coral sample preparation

Samples were prepared for DMBS analyses according to Downs *et al.* (2001). Briefly, tissue from frozen samples were either scraped with a spatula (heat stressed and field samples) or ground frozen in a mortar and pestle using liquid nitrogen (bacterial infection samples). The tissue was then suspended in a solution consisting of 10 mM phosphate buffer (pH 7.8), 5 mM butylhydrotoluene, and 0.5% SDS and sonicated on ice until the tissue was homogenized. Samples were centrifuged and the supernatant stored at -80° C until sample analysis for LPO. Protein concentrations were determined by the method of Ghosh *et al.* (1988). Nine hundred microliters of the remaining sample were further denatured by adding 100 µL of a 10X denaturing solution containing 20% SDS, 50 mM Tris-HCl (pH 7.8), 100 mM dithiothreitol, 80 mM EDTA, 3% polyvinyl pyrrolidone (w/v), 20 mM phenylmethylsulfonyl fluoride, 20 mM benzamide, 50 µM α-amino-caproic acid, and 1 µg pepstatin A. Samples were then boiled for three minutes, allowed to sit at 25°C for five minutes, and then centrifuged at 10,000 x g for five

minutes. Supernatants were transferred to a new tube and subjected to another protein concentration assay.

Spectrophotometric assays

Samples were analyzed for LPO content following the manufacturer's instructions. Each assay was done in triplicate.

Immunoassays

DMBS immunoassays were conducted according to Downs et al. (2001). Briefly, 40 µg of protein per sample were assayed using the appropriate ELISA for each of the molecular and cellular biomarkers as detailed in Downs et al. (2001). Briefly these included general indicators of cell integrity and oxidative stress (LPO, ubiquitin, HNE and MDA), and small heat shock proteins (sHsp) produced in response to heat stress (chloroplast sHsp). All samples were done in triplicate. Once developed, blots were scanned into a computer and analyzed using NIH image software (National Institutes of Health, Bethesda, Maryland, U.S.A.). Protein standards were used to determine the concentration for each assay.

Statistical Analyses

We used mixed model analysis of variance (ANOVA) with treatments as fixed effects and coral clones as random effects to test the null hypotheses that mean biomarker levels did not vary among treatments or coral clones. When preliminary analyses showed that clone effects were small (F -statistics near 1.00), their sums of squares were pooled with the residual error to maximize statistical power, as recommended by Sokal and Rohlf (1995). Separate ANOVAs

were performed on each bioassay. We used the Dunn-Sidak method (Sokal and Rohlf, 1995) and set a critical probability of $P' = 0.00512$ for each test to maintain the experimentwise Type I error rate at $\alpha = 0.05$ for all ten ANOVAs combined. When ANOVA identified significant differences 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). These conservative procedures limited the probability of rejecting a true null hypothesis to the desired ($\alpha = 0.05$) level. All statistical analyses were performed using JMP v. 3.2.2 (SAS Institute, Inc., Cary, NC, U.S.A.).

Results

Laboratory Heat Stress

Laboratory challenged corals samples were assayed for at least 10 of the DMBS parameters (Downs et al., 2001). The levels of each parameter differed in corals grown under each of the three environmental conditions. Results from three representative biomarkers are shown in Figures 1-3. Two of the DMBS parameters, lipid peroxide (LPO) and ubiquitin reflect the cumulative responses of the anthozoan and its zooxanthae. Both LPO and ubiquitin levels differed significantly among all three treatments. Mean production of LPO was 4.4 and 5.9 times greater when corals were heat-stressed in the dark and light, respectively, than in controls (Fig. 1B). Similarly, ubiquitin accumulation was 3.3 and 4.7 times greater when corals were heat-stressed in the dark and light, respectively, than in controls (Fig. 2B). Another of the DMBS suite of biomarkers is indicative of only the zooxanthae's physiological responses to stress, the chloroplast small heat shock protein (chlpsHsp). Levels of chlpsHsp (Fig. 3B) were

significantly higher in corals exposed to both heat stress and PAR than in either control or heat stress- no light treatments.

Field Samples

Changes were observed in twelve DMBS parameter levels assayed in corals collected at our field sites which correlated with changes in ocean temperature. Three representative DMBS profiles, LPO (Fig. 4B), ubiquitin (Fig. 4C) and chlpsHsp (Fig 4D) are shown, with corresponding water temperatures (Fig. 4A). In March and May, corals in four depth zones were actively photosynthesizing as determined by coloration and chlorophyll a concentration (data not shown). However, levels of LPO, ubiquitin and chlpsHsp (Fig. 4) already varied significantly with depth; however the sampled coral colonies displayed no visual indication of distress. Levels of LPO and ubiquitin showed significant increases at different depths over time, with the deepest corals having significantly higher levels of both LPO and ubiquitin than those in shallow waters. In contrast, chlpsHsp levels also varied significantly with depth and over time, but displayed an inverse correlation to LPO and ubiquitin, with corals in shallow waters having significantly higher levels than the deepest corals.

Bacterial Infection

DMBS responses were measured in healthy and bleached coral samples, where bleaching was associated with corals inoculated directly with *V. shiloi*. Results of three DMBS parameters, malondialdehyde (MDA), 4-hydroxy-2-trans-nonenol (HNE) and protein carbonyl, are shown in Figure 5. Each of these biomarkers correspond to products resulting from an oxidative stress. HNE (Fig 5A) and MDA (Fig. 5B) represent lipid oxidation products. Levels of these two biomarkers in the bleached sample did not differ significantly from control. Protein carbonyl

(Fig. 5C) levels, corresponding to protein oxidation products, were significantly lower in the bleached sample than in the control.

Discussion

In the laboratory, cellular physiological parameters measured by the DMBS indicated significant differences in physiological status between *M. faveolata* heat-stressed in the dark and those heat-stressed in the light. Further, levels of cellular parameters indicative of oxidative stress were exacerbated when corals were heat-stressed in the light compared to corals heat-stressed in the dark, strongly suggesting a light-dependent generation of active oxygen species. The most likely source of this light-dependent generation of active oxygen species would be Photosystem II and Photosystem I generation of active oxygen species (H_2O_2 and O_2^-) (Havaux, 1992; Lesser, 1996, 1997; Warner et al., 1996, 1999; Downs et al., 2001) (Fig. 3A). This photosynthetic-mediated active oxygen species generation is hypothesized to be one cause of coral bleaching. The hypothesis suggests that bleaching occurs when certain active oxygen species diffuse out of the chloroplast, through the dinoflagellate, and into the coral cell, thus producing an oxidative stress within the coral cell itself (Lesser, 1997; Brown, 1997a; Warner et al., 1999). Above some threshold level, the coral then either expels or destroys the zooxanthellae as a defensive strategy against oxidative stress.

Data generated by the DMBS in this experimental system support this hypothesis. Parameters of oxidative stress were significantly higher in corals heat-stressed in the light compared to corals heat-stressed in the dark. For example, LPO levels were significantly higher in heat-stressed samples in the light than those in the dark (Fig. 1B), indicative of cell membrane

damage (Fig. 1A) (Duthie, 1993; Girotti, 1998) and suggesting that perhaps the anti-oxidant defenses were overwhelmed. The DMBS also provided an index for many aspects of cell structure integrity. For example, LPO levels (an indicator of decreasing membrane integrity) were much higher in corals heat-stressed in the light than in the dark (Halliwell and Gutteridge, 1999). In addition, increased LPO levels following or during a stress event also indicate a decline in functional lipid levels (Glynn et al., 1985). Ubiquitin levels were also significantly higher in corals heat-stressed in the light than in the dark (Fig. 2B). Ubiquitin is a 76-residue polypeptide that marks proteins for degradation and is an indicator of protein turnover (Fig. 2A) (Goff et al., 1988; Hershko and Ciechanover, 1998; Iwai, 1999). These two parameters alone suggest a major insult to cell structure integrity occurred during heat stress, and was exacerbated under light conditions.

Chloroplast sHsp levels were significantly higher in samples heat-stressed in the light compared to both controls, and to coral samples heat-stressed in the dark (Fig 3B). The function of the chlpsHsp (Fig. 3A) is to protect Photosystem II activity during oxidative stress. These data and what is known about Photosystem II stress-lability, active oxygen species generation, and the function of the chlpsHsp (Downs et al., 1999a; 1999b) support a mechanism for oxidative stress/Photosystem II-associated coral bleaching.

The laboratory baseline data for *M. faveolata* quantified the nominal levels of a suite of molecular biomarkers (Downs et al., 2001) needed to field test the DMBS technology. We sampled a sibling species (*M. annularis*) monthly throughout the 1999 growing season, which included a bleaching event. The DMBS was used to establish whether we could determine the physiological status of corals at different depths, and thus experiencing different temperature and light levels, and whether oxidative stress was correlated with coral bleaching. In March and May,

when water temperatures were just warming, corals at all depths were actively photosynthesizing, and levels of LPO, ubiquitin and chlpsHsp already varied significantly with depth. Increased LPO and ubiquitin levels with depth suggested that the deepest corals were distressed. Levels of LPO continued increasing throughout the summer, suggesting oxidative stress increased with increased ocean water temperatures. Ubiquitin levels, indicate increased protein turnover, also increased with increasing water temperature. Together these two biomarkers indicate that oxidative stress disrupted the overall cellular physiology, which we hypothesize leads to bleaching. Indeed, by September photosynthetic rates in the deep-water corals were much lower than in May because they had bleached. In September, mean LPO and ubiquitin levels of deep-water corals were similar to those in shallow-water that had not bleached, suggesting alleviated oxidative stress.

Chloroplast sHsps play a role in protecting photosynthesis against heat stress by associating with the oxygen evolving complex and protecting photosystem II during oxidative stress (Downs, et al. 1999a; 1999b). Levels of this biomarker showed significant differences across all sites from May through October. In contrast to LPO and ubiquitin, however, the levels of chlpsHsp were inversely correlated with depth, suggesting shallow corals were more protected against oxidative stress than deeper corals. Indeed, by the September bleaching event, shallow water corals, which had higher chlpsHsp levels did not bleach, while deep water corals, with lower levels of chlpsHsp, did bleach. Our data indicates that these and other markers (e.g., protein carbonyl) can predict coral bleaching 4-5 months before of any visual indication of bleaching. In addition, the inverse relationship between chlpsHsp accumulation and bleaching strongly suggests that a major cause of coral bleaching may be the inability to accumulate

enough chlpHsp to protect against photoinhibitory production of oxidative stress (Downs et al., 1999b; Downs et al., 2001).

Although this study and others provide strong evidence for heat-induced coral bleaching, not all bleaching is correlated with abnormal sea surface temperatures. For example, Oliver (1985) and Fisk and Done (1985) showed extensive bleaching on the Great Barrier Reef with no associated increase in sea surface temperature. Kushmaro et al. (1996; 1998) and Rosenberg et al. (1999) reported that bleaching of the Mediterranean coral, *Oculina patagonica*, results from bacterial infection. The causative agent, *Vibrio shiloi*, was obtained in pure culture and shown to cause bleaching in controlled laboratory experiments. Furthermore, infection and subsequent bleaching were temperature dependent (Kushmaro et al., 1998). We examined the cellular physiology of the temperate coral, *Oculina varicosa*, inoculated with *V. shiloi*, using three biomarkers from the DMBS suite, malondialdehyde (MDA), 4-hydroxy-2-trans-nonenal (HNE) and protein carbonyl. These particular biomarkers are used to determine whether cells are undergoing oxidative stress. Malondialdehyde and HNE are produced during lipid peroxidation (Halliwell and Gutteridge, 1999), while protein carbonyls form as a result of oxidative protein damage (Robinson et al., 1999; Halliwell and Gutteridge, 1999). None of these three oxidative stress markers were elevated in a bacterial-bleached coral compared to controls, suggesting that a non-oxidative stress mechanism occurs during bacteria-associated coral bleaching.

Investigations are underway to determine the differences between pathogen-associated coral bleaching and environmental bleaching (i.e., heat stress). We also are investigating additional parameters that can possibly distinguish between non-pathogenic and pathogenic states of the bacteria.

We have introduced a newly developed environmental biotechnology, the DMBS, and shown it can provide “environmental diagnostics” analogous to those used in human biomedical tests to assess the physiological status of corals. We demonstrated that the DMBS allows evaluation of the effects of single and compound stressors, and provided evidence that oxidative stress is a major mechanism in environmental coral bleaching. Certain biomarkers among the DMBS suite could predict coral bleaching 4-6 months before visual indications of the pathology, and through the use of the DMBS we showed that pathogen-associated coral bleaching may not be a result of oxidative stress. Through continued use of the DMBS and other newly developed molecular biomarkers, we will be able to provide scientists and resource managers with the means to identify, control and possibly alleviate hidden stresses before they become environmental crises.

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Figure Legends

Figure 1.

Lipid Peroxidation (LPO). (A) Schematic of the effect of lipid peroxidation on cell membranes. (B) Mean (± 1 SE) LPO levels in corals raised under optimal growing conditions (control), or heat-stressed in either the dark or light.

Figure 2.

Ubiquitin. (A) Schematic of ubiquitin pathway used to mark damaged proteins for degradation. (B) Mean (± 1 SE) levels of ubiquitin in coral explants raised under optimal conditions (control), or heat-stressed in the dark or light.

Figure 3.

Chloroplast small heat shock protein (chlpsHsp). (A) Schematic of the induction and function of the chlpsHsp during heat stress (adapted from Heckathorn et al., 1999) (B) Mean (± 1 SE) levels of chlpsHsp in coral explants raised under optimal conditions (control), or heat-stressed in the dark or light.

Figure 4.

Coral Field Samples. (A) Water temperature at depth. Mean (± 1 SE) biomarker levels in corals field-collected from four depth zones near Molasses Reef, FL in 1999. (B) LPO, (C) ubiquitin, (D) chlpsHsp.

Figure 5.

Bacteria-Associated Coral Bleaching. Biomarker levels in healthy or bacteria-induced bleached coral. (A) 4-hydroxy-2-trans-nonenol (HNE), (B) malondialdehyde (MDA), (C) protein carbonyl.

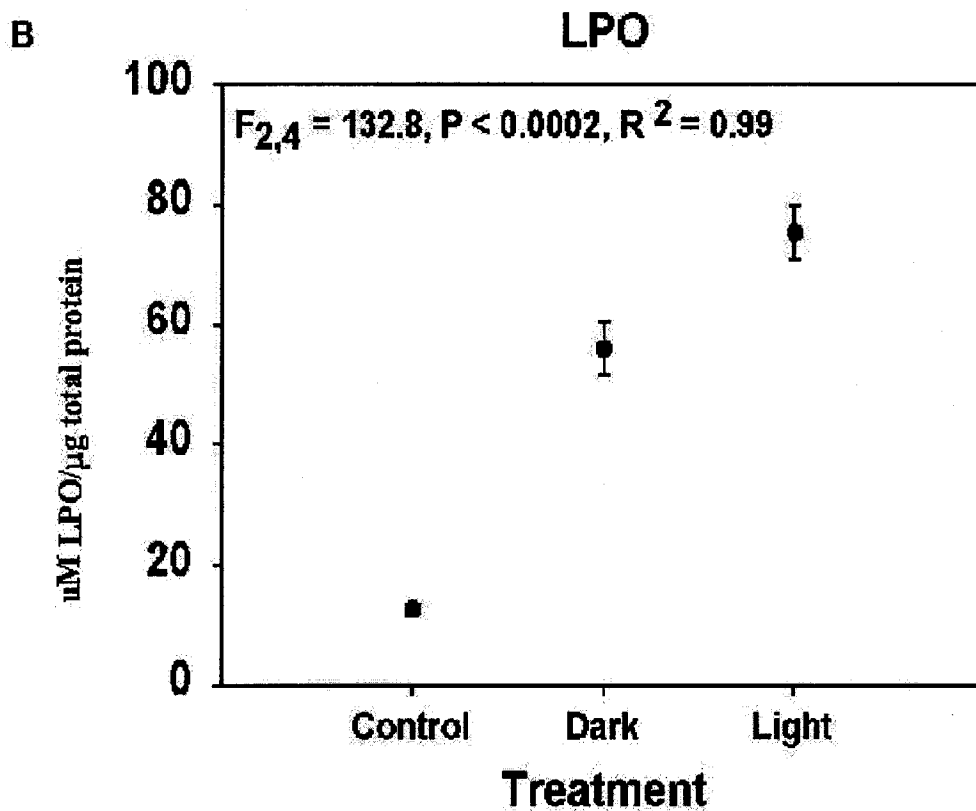
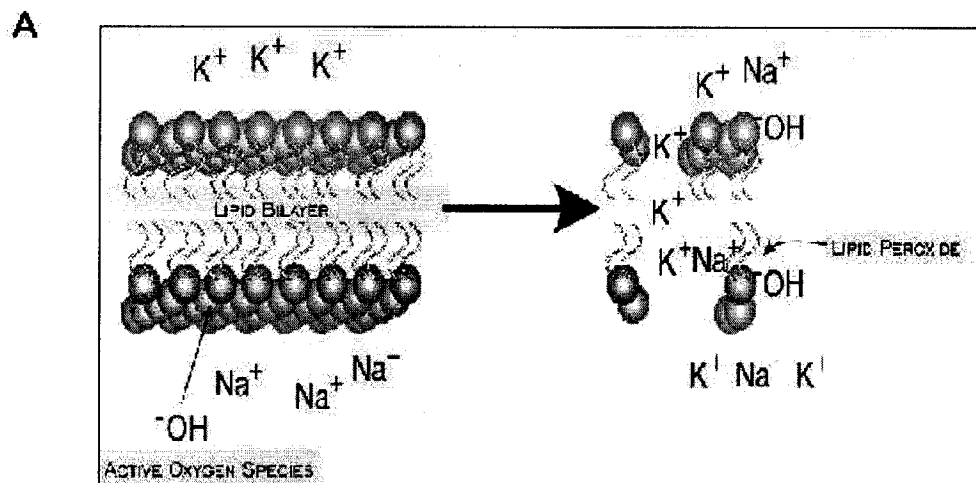
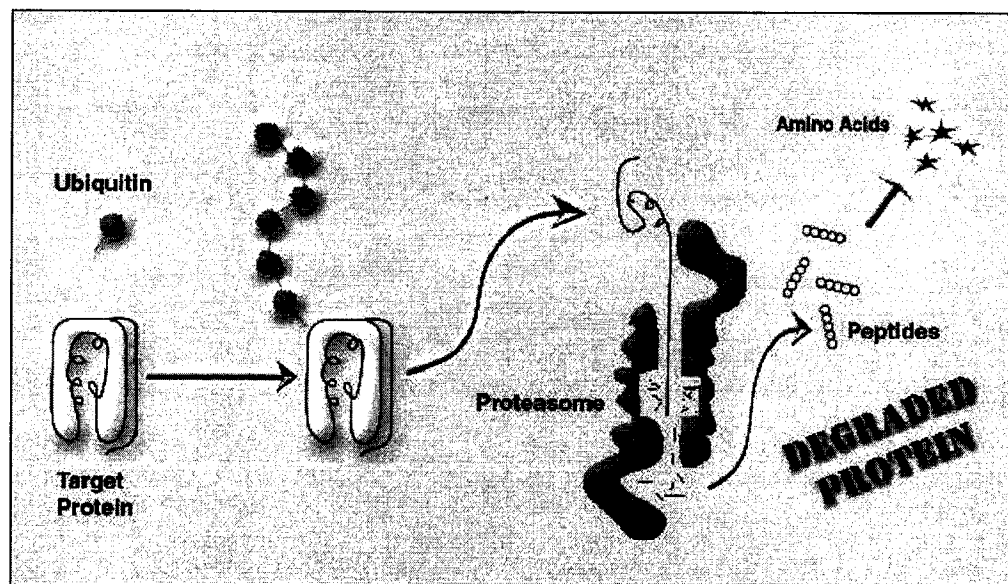


Figure 1.

A



B

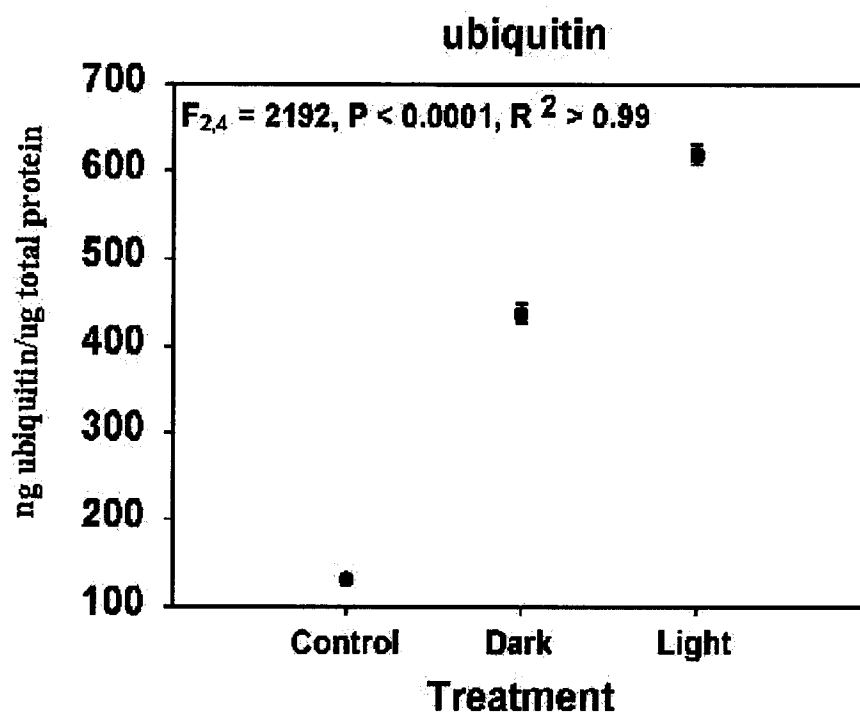


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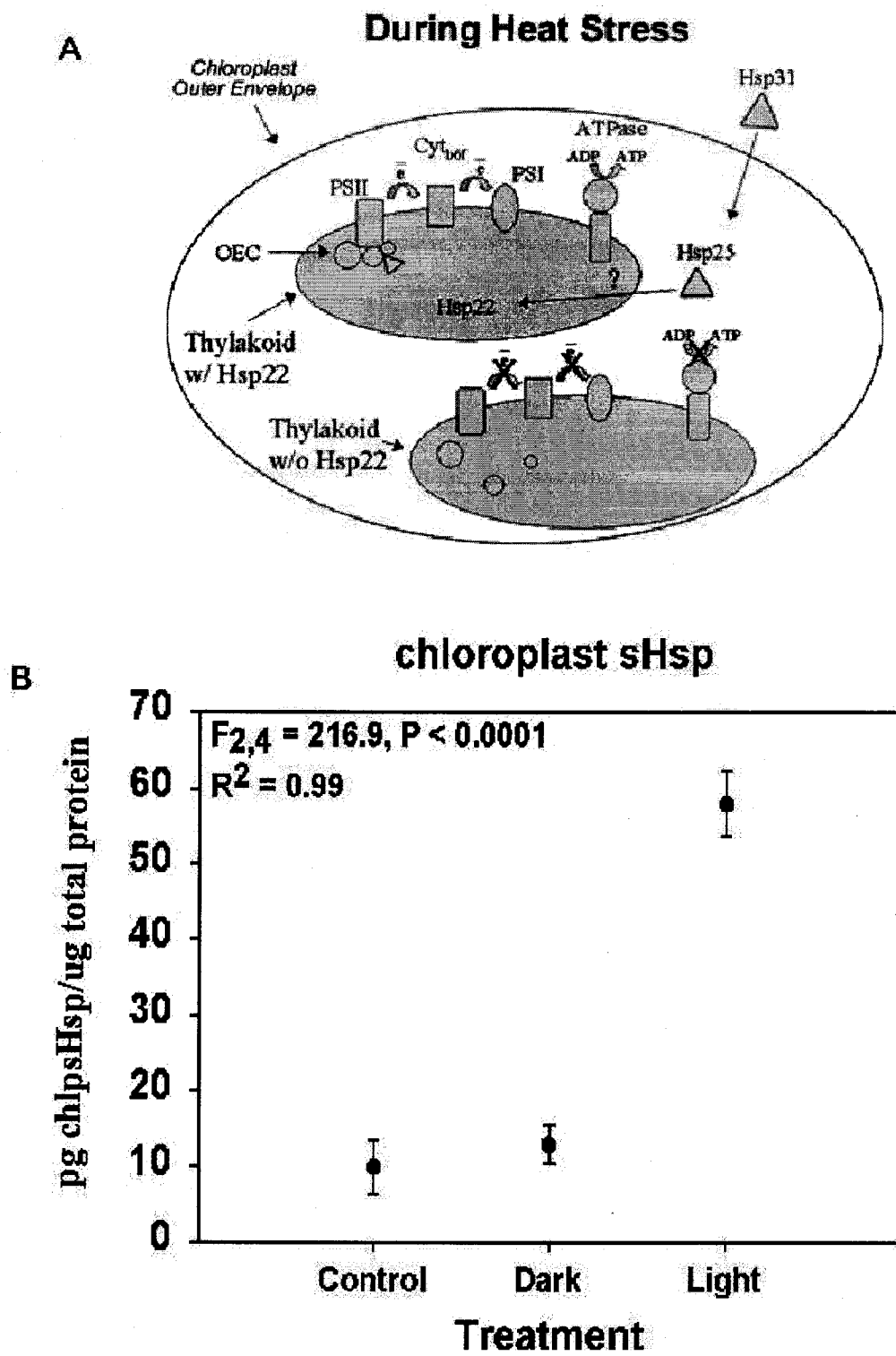


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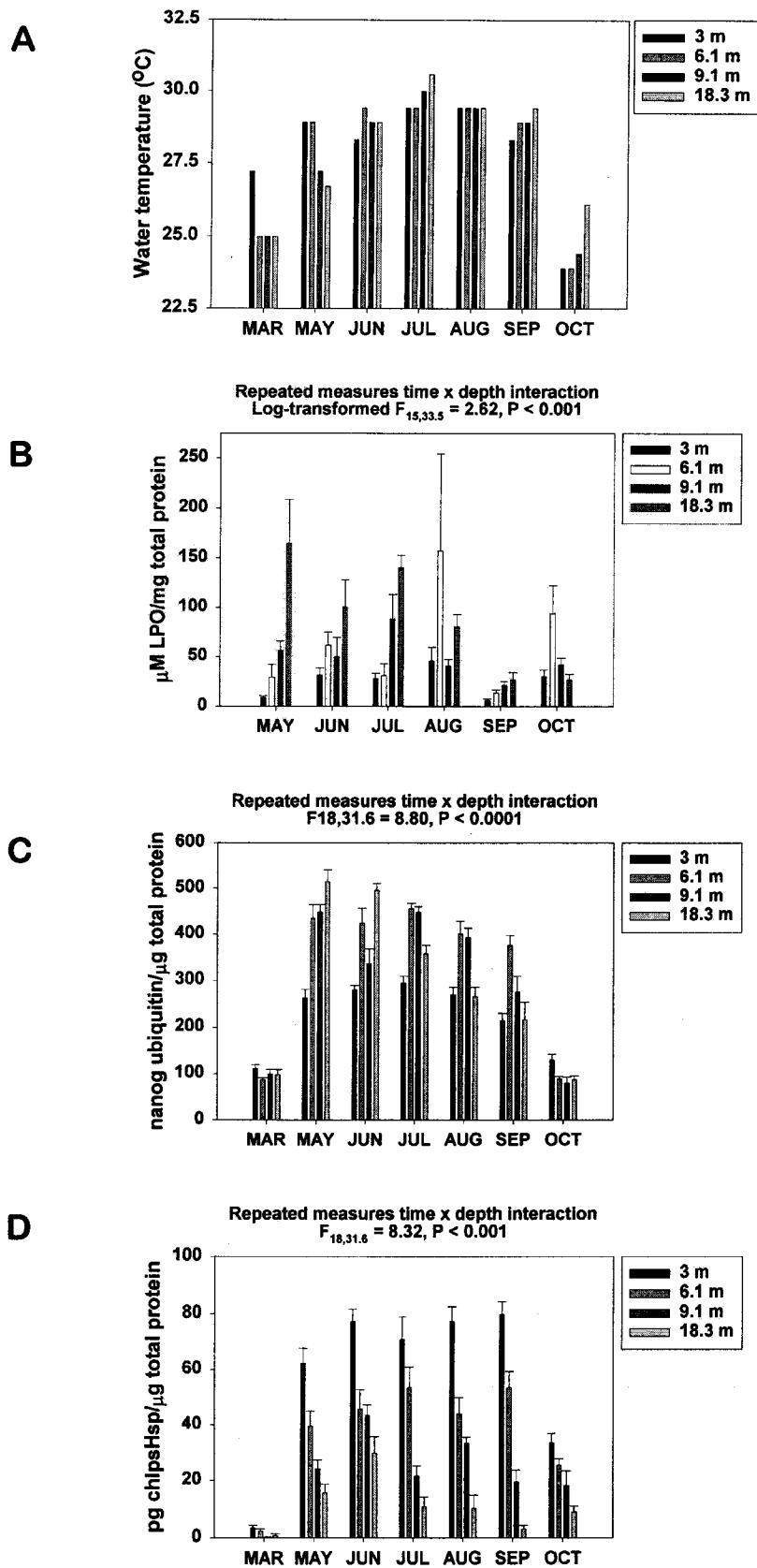


Figure 4.

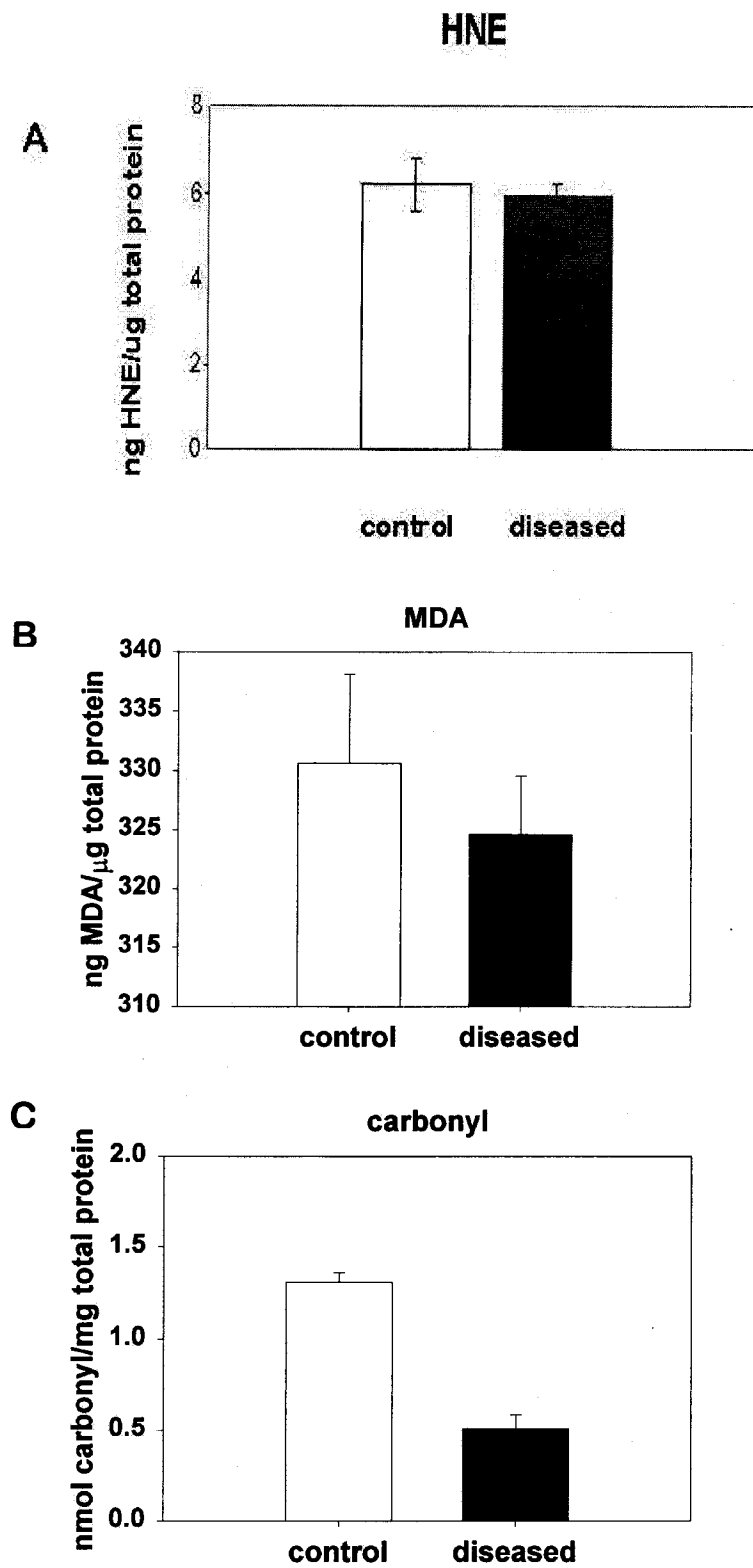


Figure 5.