Environmental Forensics, 7:15–32, 2006
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ISSN: 1527–5922 print / 1527–5930 online
DOI: 10.1080/15275920500351775

Investigating Coral Reef Degradation at Alina's Reef in the Florida Keys: Cellular Physiology of White Grunt (*Haemulon plumieri*) as a Biological Indicator

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Coral reefs in the Florida Keys are severely degraded with a reported 38% loss between 1996 and 2000, yet the causes of these devastating declines remain largely unknown. Our long-term studies in the Upper Keys and Biscayne National Park indicate acute stress events affecting physiological condition in species representing different trophic levels in the reef community, one of which was white grunt (*Haemulon plumieri*; Lacépède, 1801). We initiated a preliminary investigation describing cellular physiological stress effects and the possible causes of these stress events using cellular diagnostic profiling coupled with a cursory body-load contaminant chemistry analysis. The cellular biomarker profiles from fish taken from Alina's Reef indicated a toxic response profile that was suggestive of a suicide reaction of the cytochrome P450 2-class as a result of an interaction with a xenobiotic that adversely affects heme metabolism. Elevated levels of damaged porphyrin products were also found in fish from Alina's Reef. Liver loads of anthropogenic contaminants (e.g., pesticides, PCBs) were measured and provided further evidence for possible causative agents. Evaluation and synthesis of each type of data were used to establish a biological effect, develop a mechanism of pathogenicity, and build a profile for possible causative agent(s).

Keywords: biomarker, cellular diagnostics, coral reefs, Florida Keys, white grunt

Introduction

Coral reefs are declining worldwide as a result of increased sea temperature anomalies (e.g., El Niño events), infectious disease, destructive fishing practices, sedimentation, pollution, and unidentified stressors (Dustan, 1999; Hoegh-Guldberg, 1999; Wilkinson, 1999). Coral reef degradation is a persistent occurrence in the Florida Keys (Dustan, 1999). From 1996 to 2000, a time span that included a major El Niño event, the Florida Keys National Marine Sanctuary lost a record 38% of their living coral cover (CRMP, 2001; Porter et al., 2001). Several recent studies examining the health of coral reefs in the Upper Keys region of the Florida Keys and in Biscayne National Park (BNP) demonstrate instances of acute stress with associated morbidity and mortality (Downs et al., 2005a). One such site, Alina's Reef (BNP), is thought to have lost between 15 and 25% coral cover of the dominant coral species, *Montastraea annularis*, from March to August in 2000 (Downs et al., 2005a). The causes for these devastating declines on these coral reef natural resources, however, remain largely unknown and have not been thoroughly investigated (Pelley, 2004).

The first step in any environmental investigation of a natural-resource damage event is to describe the damage scene, which must include a description of the biological effects in species from various loci in the community structure (Sullivan et al., 2000; Vasseur and Cossu-Leguille, 2003). Traditional methods of evaluating coral reef condition, such as the Atlantic and Gulf Rapid Reef Assessment (AGRRA) protocol, include monitoring the species composition, diversity, abundance, and size of

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fish populations (Lang, 2003, and references therein). Fish are considered good indicators of coral reef health because of their high trophic position and tendency to bioaccumulate pollutants (Lawrence and Hemingway, 2003). Though it is important in documenting the ultimate effect of an event, fish population data has limited value to an environmental forensic investigation because 1) a significant length of time may transpire between when the damage event was initiated and when the first effects at this level are perceived, and 2) these types of data give no clues as to *cause* or nature of the population change (Vasseur and Cossu-Leguille, 2003; Downs, 2005; Downs et al., 2005b).

Environmental agents that incite natural resource damage events, such as abnormally high sea temperatures, pesticide and herbicide run-off, siltation, and human waste effluent, often do not *directly* affect changes in population density and community structure (Downs et al., 2001a, 2001b; Vasseur and Cossu-Leguille, 2003). Instead, these agents directly affect molecular- and cellular-level processes, which in turn can affect higher-order processes (e.g., population density and community structure) in the biologic hierarchy (Allen and Starr, 1982). Hence, examination of molecular- and cellular-level behavior is crucial in perceiving the relationship between causal factors and their biological effects (Downs, 2005; Handy et al., 2003; Moore, 2003).

One means of perceiving and understanding the changes in molecular and cellular behavior is the use of biomarker methodologies (Downs, 2005; Moore, 1991, 2003). Biomarkers are physical components or rates of reaction (parameters) that indicate the condition or status of a biological property. Biomarkers are analogous to the concept of "parameter" in mathematics; a single biomarker is a term or element in a functional (biological) process that determines the specific form of the function, but not its general nature. For example, the rate of activity of an enzyme can provide information as to its working efficiency, but does not describe a physiological condition in and of itself. To determine the condition of a physiological process, the behavior of other elements or parameters in that function must be known. One methodology that capitalizes on this concept is cellular diagnostics: a systematic approach to defining and integrating biomarkers of exposure, effect, and susceptibility based on their functionality within a cell and how alterations in the behavior of a set of cellular parameters (biomarkers) may reflect overall cellular operation or performance (Downs, 2005). In terms of a coral reef fish, molecular and cellular biomarkers provide a means of assessing both qualitative and quantitative responses of a fish population to a variety of stressors individually and collectively.

In our investigation of a coral reef (Alina's Reef) in Biscayne National Park that has had a recent history of coral degradation and depressed fish densities and biomass, we examined several coral reef species that occupy different trophic positions in the coral reef community structure (Downs et al., 2005a; Fisher et al., 2005). Corals at Alina's Reef exhibited a punctuated coverage decline in 2000, and displayed a severe disability to regenerate lesions, as well as possessing a cellular biomarker profile indicative of a stressed condition (Downs et al., 2005; Fisher et al., 2005). White grunts (*Haemulon plumieri*; Lacepede, 1801) are predatory fish that possess a measure of site fidelity as adults and are abundant in off-shore reefs in the Florida Keys, making this species a good candidate as a sentinel representative of this trophic level within this community. White grunt fisheries studies have documented a continuing trend in population decline in the South Florida area, and more specifically in Biscayne National Park (Ault et al., 2001). We examined the cellular biomarker profiles from the liver of white grunts taken from Alina's Reef and two other reference sites to determine whether the cellular physiological condition of the fish was perturbed and, if so, the nature of the physiological perturbation. Cellular biomarker profiles of white grunt from Alina's Reef indicated a toxic-response profile that is suggestive of a suicide reaction of the cytochrome P450 2-class with a xenobiotic that ultimately adversely affects heme metabolism. Liver loads of anthropogenic contaminants (e.g., pesticides, PCBs) were measured to establish if a possible link exists between white grunt cellular health and their accumulation of biocides and contaminants that are indigenous to areas near these reefs.

Materials and Methods

Study Sites

We sampled white grunts in two marine protected areas within the northern portion of Florida's reef tract (Figure 1): Biscayne National Park (BNP) and Florida Keys National Marine Sanctuary (FKNMS). All three study sites are small, patch, fringing reefs along a 6 m depth contour. Within BNP, white grunts were sampled at Alina's Reef (GPS: 25 23.185′ N; 80 09.775′ W, 18.8 km ESE of Turkey Point, a nuclear power plant, 24.6 km SE from the South Dade County Landfill, which is near Gould's Canal 200–300 m from BNP's west land boundary). Within FKNMS we sampled white grunts at White Banks Dry Rocks (GPS: 25 02.232′ N; 80 22.496′ W) and Molasses Reef (GPS: 25 01.092′ N; 80 23.844′ W).

White Banks Dry Rocks possesses habitat features very similar to Alina's Reef, except that the documented rate of decline for White Banks is much lower compared to other reefs within the Upper Keys region (CRMP, 2001). Molasses Reef is a nearshore patch-reef habitat and is a very different coral reef habitat from that seen at both Alina's Reef and White Banks, with inherently less hard coral coverage, as well as much smaller reef area (CRMP, 2001). Since incorporating this site into our long-term monitoring program, active coral decline was observed relatively recently (e.g., 2002 and after; Downs et al., 2002, 2005a). Alina's Reef has a closer proximity to a nuclear power plant, a major landfill site, intensive agriculture, intensive suburban building development, and the city of Miami, compared to both of the Upper Keys sites.

Collecting Methods and Partial Fish Necropsy

Five white grunts were captured using folding traps baited with frozen squid or ballyhoo and tended by scuba divers. Because of

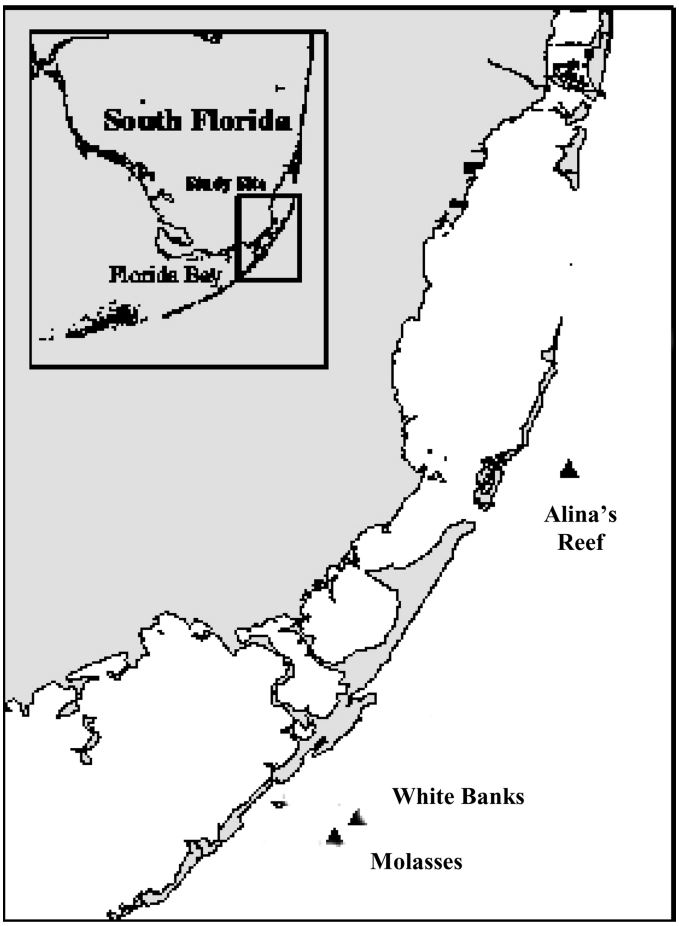

Figure 1. Location of sampling sites. White grunts were collected from the Upper Keys area of the Florida Keys and in Biscayne National Park. The tow refernce sites are Molasses Reef and White Banks, while the suspected site of a natural resource injury is Alina's Reef.

Florida State Regulations, fish traps were set for only 30 minutes and were attended by divers for the entire time. Once on deck, we measured (standard length) and weighed the fish before placing them into labeled muslin bags; they were flash-frozen in a liquid nitrogen cryo-shipper. In the laboratory, samples were stored in a $-80°$C freezer.

Once the fish were in the laboratory, a partial necropsy was conducted on the partially thawed fish to examine gut contents, gender identification, and inspection of gross abnormalities.

Reagents

All chemicals and reagents were obtained from EM Science (Gibbstown, NJ) and CalBiochem (San Diego, CA).

Enzyme Linked Immuno-Sorbent Assay (ELISA) Method

Livers were excised from partially thawed fish and cryogenically ground to a powder using an automated Freezer/Mill® (SPEX CertiPrep, Inc., NJ). Samples (~2–4 mg) of frozen, ground tissue were placed in 1.8-mL microcentrifuge tubes along with 1,400 μL of a denaturing buffer consisting of 2% SDS, 50 mM Tris-HCl (pH 7.8), 10 mM dithiothreitol, 10 mM EDTA, 0.75 mM desferoximine methylate, 0.005 mM salicylic acid, 0.01 mM AEBSF, 0.04 mM bestatin, 0.001 E-64, 2 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 0.01 mM apoprotin, 5 μM α-amino-caproic acid, and 1 μg/100 μL pepstatin A. Samples were heated at 93°C for 3 minutes, vortexed for 20 seconds, incubated at 93°C for another 3 minutes, and then incubated at 25°C for 5 minutes. Samples were centrifuged at $10,000 \times g$ for 10 minutes. Supernatant free of a lipid/glycoprotein mucilage matrix was transferred to a new tube and subjected to a protein concentration assay (Ghosh et al., 1988).

To ensure equal sample loading, 10 μg of total soluble protein from five randomly chosen samples from all three sites were loaded onto a 12.5% SDS-PAGE gel (8 cm), the gel was 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 destaining solution (Downs, 2005). 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 (http://rsb.info.nih.gov/nih-image/).

One-dimensional SDS-PAGE and Western blotting were used to validate antibody specificity and sample integrity for enzyme linked immunosorbent assay (ELISA) analysis of white grunt liver tissue (Downs, 2005). All antibodies were monospecific polyclonal antibodies that were raised against an 8–15 amino-acid polypeptide derived from the target protein sequence conjugated to keyhole limpet hemocyanin. All antibodies used in this study were immuno-purified with a Pierce SulfoLink Kit (cat.# 44895) using the original unconjugated peptide as the affinity binding agent. Five to 15 μg of total soluble protein of liver supernatant was loaded onto an 8-cm SDS-polyacrylamide gel with various concentrations of bis/acrylamide. A TCEP concentration of 1 mM was added to gels loaded with samples to be assayed with antibody against small Hsps and metallothionein. Gels were blotted onto PVDF membrane using a wet transfer system. The 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) 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 and the level of precision for each ELISA was determined using an $8 \times 6 \times 4$ factorial design (Crowther, 2001). A Beckman-Coulter Biomek 2000 using 384-well microplates was used to conduct the ELISA assays. Samples were assayed with the following EnVirtue Biotechnologies, Inc., antibodies (generated in rabbits) that were specific to: anti-ubiquitin (catalog# AB-U100), anti-heat-shock protein 70 (lot ab0452-5), anti-heat-shock protein 60 (lot ab0452-4b), anti-heat-shock

protein 90a (lot ab0452-6c), anti-GRP75 (lot ab03822-c), anti-ferrochelatase (lot ab0175-1c), anti-heme oxygenase 1 (lot pab928c), anti-metallothionein 1 (lot ab0175-7a), anti-fish small heat-shock protein family (ab9928c), anti-CYP P450 2-class (lot 1987), anti-CYP P450 3-class (lot 1985b), and anti-MDR (ABC family of proteins; P-glycoprotein 140 & 160; AB-MDR-160). All antibodies are mono-specific polyclonal antibodies made against a synthetic 8–10 amino-acid residue polypeptide that reflects a specific and unique region of the target protein. Samples were assayed in triplicate with intra-specific variation of less than 7.5% for all samples combined for each assay. An eight-point calibrant curve using a calibrant relevant to each antibody was plated in sextiplicate for each plate.

Porphyrin Concentration Determination

One-hundred and fifty micrograms of total soluble protein from prepared sample supernatants were diluted in a solution containing 1% SDS, Tris-HCl (pH 7.8), 5 mM EDTA into a volume of 300 μL. One-hundred-microliter aliquots of each sample were dispensed in triplicate to a clear-bottom Corning 96-well microtiter plate. Protoporphyrin standards were obtained from Porphyrin Products (Logan, UT) and diluted using an 8-point calibrant standard curve from 0 pmol of protoporphyrin to 1000 pmol of protoporphyrin. Standard curves were dispensed on the microtiter plate in triplicate. Fluorescence signal was detected using a Biotek FL800 series fluorescent/luminescent microplate reader with the excitation filter set for 405 nm and the emission filter set for 610 nm.

N-Alkylprotoporphyrin IX Purification and Concentration Determination

Liver samples were cryogenically ground to a powder using an automated Freezer/Mill® (SPEX CertiPrep, Inc., NJ). One-hundred milligrams (wet weight) from each of the five individuals from each population were pooled. The 500 mg pooled sample was homogenized in chilled phosphate-buffered saline solution (2 mL; pH 7.4) using a vortex. One milliliter of ice-cold H_2SO_4:methanol (1:1 v/v) was added to each sample and incubated in the dark for 28 hours at $-4°C$. Each pooled sample was filtered using a Whatman 0.45 μm filter and diluted with 2 mL of de-ionized water and extracted with 20 mL of dichloromethane in a separatory funnel. The funnel was washed with 100 mL of a 5% sodium bicarbonate solution, and then with 100 mL of de-ionized water. The dichloromethane was dried in a 2 mL beaker containing 5 g of anhydrous sodium sulfate for 48 hours at 32°C. A zinc acetate solution (100 μmol of Zn acetate in 2 mL of methanol) was added to the desulfonated solution to form Zn-N-alkylprotoporhyrin dimethyl ester, mixed in a reaction vessel for 5 hours in the dark at 27°C. The solution was then evaporated to dryness at 32°C over a 24-hour time period. The residue was dissolved in 6.8 mL of dichloromethane and applied to a Whatman Silica gel high-performance thin-layer chromatography plates and developed in a 13:2 solution of dichloromethane:methanol for 30 minutes. Several green bands were observed on the plate $R_f = 0.77$–0.85) that fluoresced red under ultraviolet A/B light. The bands were cut from the plate, and the alkylprotoporphyrins extracted from the silica with acetone, and then evaporated to dryness. The residue was dissolved in 1.0 mL dichloromethane and the electronic absorption spectrum determined using a Milford Spectronic dual-beam UV-visible diode spectrophotometer. The concentration of each of the three pooled samples was estimated using the molar extinction coefficient for zinc N-ethylprotoporphyrin dimethyl ester ($\varepsilon > 128,000\ m^{-1}\ cm^{-1}$ at 432 nm; Ortiz de Montellano et al., 1981; Wong and Marks, 1999). It is assumed that the molar extinction coefficients of the three samples are similar to that of N-ethylprotoporphyrin dimethyl ester since the N-alkylPP dimethyl esters are not available, and the individual molar extinction coefficient for the alkylated protoporphyrins cannot be determined.

Chemistry Analysis

Approximately 0.5–3.4 g of pooled homogenized liver tissue was extracted using a modified NOAA Status and Trends protocol (NOAA, 1998). Appropriate pesticide and PCB internal standards were added to pooled homogenates. The extracts were purified by silica gel-alumina column chromatography. Lipid content was gravimetrically determined.

Pesticides and PCBs were analyzed by gas chromatography using dual-column electron capture detectors (GC-ECD) with select samples analyzed by gas chromatography-mass spectrometry (GC-MS) for verification when possible. Analysis was performed using a Varian Instruments (Sugar Land, TX) 3800 GC-ECD system equipped with 30-m glass capillary DB-5 and DB-608 columns, coupled with a Turbochrome chromatography data processing system. The GC-MS analyses were analyzed with a Thermofinnigan DSQ quadrapole mass selective detector equipped with a 30-m, 0.25-mm glass capillary DB-5 column.

Statistical Analyses

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 (Downs, 2005; Panzer et al., 1999; Schaeffer, 1996). Data were tested for normality using the Kolmogorov-Smirnov test (with Lilliefors' correction) and for equal variance using the Levene Median test. If the data were normally distributed and homogeneous, a one-way analysis of variance (ANOVA) was employed. When data did not meet the homogeneity of variances requirement for one-way ANOVA, we instead used Kruskal-Wallis one-way analysis of variance on ranks. When significant differences were found among treatment means, we used the Tukey-Kramer honestly significant difference (HSD) method, the Dunn's post hoc test, or the Holm-Sidak test as an exact alpha-level test to determine difference between each of the populations (Sokal and Rohlf, 1995).

We used canonical correlation analysis (CCA) as a heuristic tool to illustrate how biomarkers could be used to discriminate between populations. CCA is an eigen-analysis method that reveals the basic relationships between two matrices (Gauch, 1985), in our case those of the three populations and biomarker data. The CCA provided an objective statistical tool for 1) determining if populations are different from another using sets of cellular biomarkers that are indicative of a cellular process (e.g., protein metabolic condition, xenobiotic response), and 2) which biomarkers contributed to those differences. This analysis required combining data from all three populations into one matrix, which we did by expressing biomarker responses in a given population as a proportion of their mean levels. Two assumptions of CCA, that stressor gradients were independent and linear, were constraints of the experimental design.

Results

ELISA Validation

Antibodies against liver cellular parameters did not exhibit significant non-specific cross-reactivity (Figures 2–4), and hence could be validly used in an ELISA format.

ELISA

ELISA results are divided into the following cellular diagnostic categories:

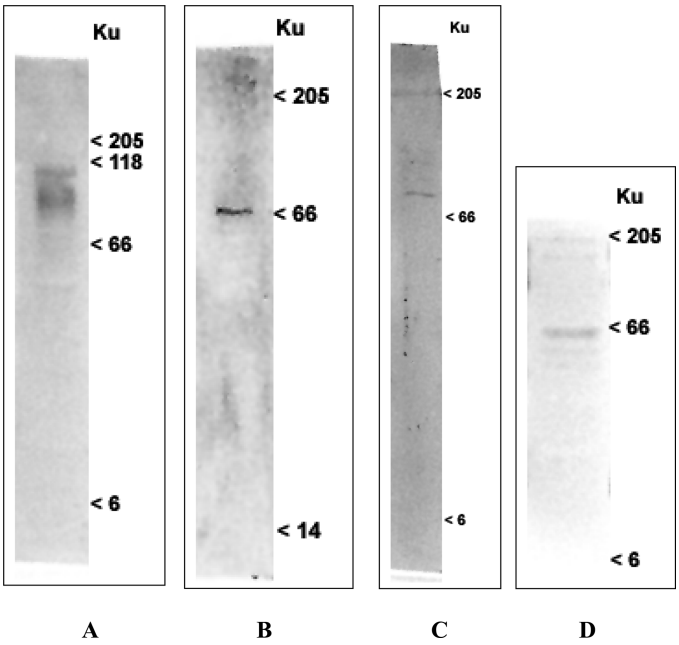

Figure 2. Western blots for protein metabolic condition biomarkers. Fish livers were homogenized and subjected to SDS-PAGE (5 μg total soluble protein per lane), Western blotting, and assayed with affinity purified polyclonal antibody against the following parameters: A = Hsp90; B = Hsp70; C = Hrp75; D = Hsp60.

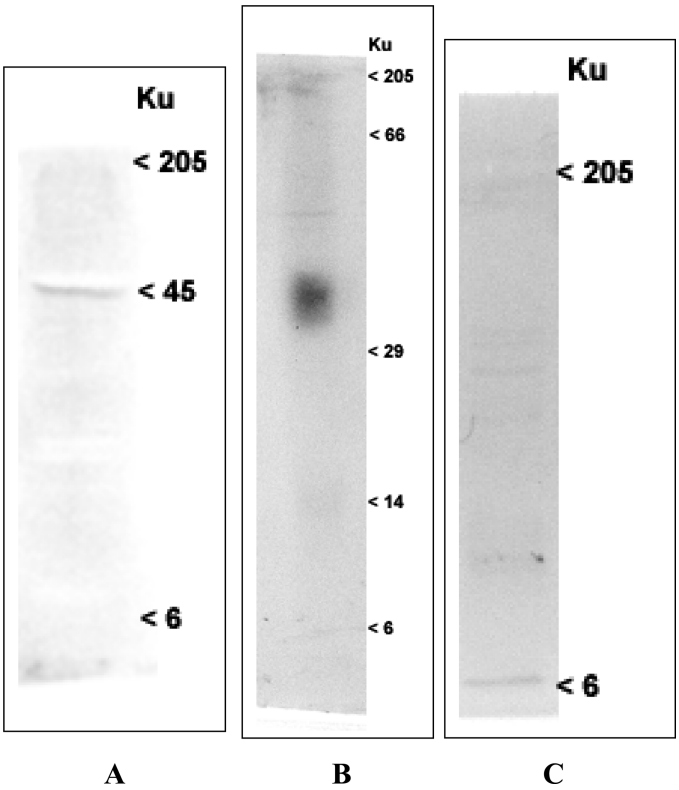

Figure 3. Western blots for metabolic condition biomarkers. Fish livers were homogenized and subjected to SDS-PAGE (5 μg total soluble protein per lane), Western blotting, and assayed with affinity purified polyclonal antibody against the following parameters: A = ferrochelatase, B = heme oxygenase, type I; C = metallothionein, Type I.

- *Protein Metabolic Condition*, which includes heat-shock protein 90 (Hsp90), heat-shock protein 70 (Hsp70), mitochondrial localized Hsp70 homologue of Ssc1 (Grp75), heat-shock protein 60 (Hsp60), and total ubiquitin.
- *Metabolic Condition*, which includes ferrochelatase, metallothionein, heme oxygenase (Type 1), the small heat-shock proteins in fish.
- *Xenobiotic Response*, which includes cytochrome P450 2-class, cytochrome P450 3-class, and MDR1.

Heat-shock protein 70, Hsp90, and ubiquitin were significantly higher in grunts caught at Alina's Reef than in grunts from the other two reefs (Figure 5). Hsp60 also was, on average slightly higher at Alina's Reef, though the difference was not significant. In contrast, Grp75 was significantly lower in grunts from Alina's reef. There were no significant differences in protein metabolic condition between fish caught at White Banks and Molasses Reef.

All of the metabolic condition biomarkers measured were significantly higher in fish caught at Alina's Reef than at the other two sites (Figure 6). There were no significant differences in any of the metabolic condition biomarkers between fish caught at White Banks and Molasses Reef.

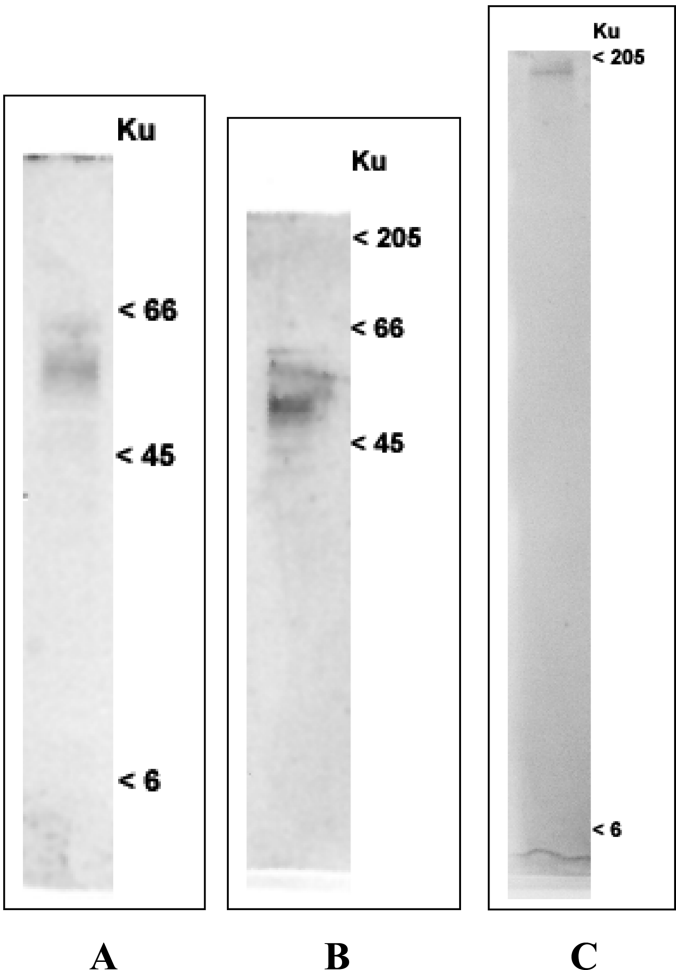

Figure 4. Western blots for xenobiotic response biomarkers. Fish livers were homogenized and subjected to SDS-PAGE (5 μg total soluble protein per lane), Western blotting, and assayed with affinity purified polyclonal antibody against the following parameters: A = CyP P450 2-class, B = CYP P450 3-class; C = MDR, type 1.

Bioindicators of xenobiotic response were more mixed (Figure 7). Cyp P450 Class 2 in grunts from Alina's Reef averaged more than double the concentrations found in grunts from the other two sites. In contrast, Cyp P450 Class 3 concentrations in grunts from Alina's reef were, on average, lower than those found at the other sites; however, the variability at the other sites was much greater. Relative concentrations of MDR1 protein did not differ significantly among the sites.

Analyses from Pooled Fish Liver Samples

Concentration of N-alkylprotoporphyrins in grunt livers from Alina's Reef were much higher (14.4 nmol/g liver) as compared with fish from White Banks (0.48 nmol/g liver) and Molasses Reef (1.9 nmol/g liver). Concentrations of porphyrin mixture in fish liver (Figure 8) were roughly two orders of magnitude higher in the fish from Alina's Reef.

Concentrations of 31 pesticides or pesticide residues were assessed in grunt liver samples, pooled by site (Table 1). It is interesting to note that 14 (45%) of the pesticides were at least twice as concentrated in livers from Molasses Reef than in fish from either of the other two sites. Only four pesticides, hexachlorobenzene, endrin, PCB105, and mirex, were found at higher concentrations in grunt livers from Alina's Reef than in fish from the other two sites.

Liver Coloration

During necropsy, it was apparent that some fish had grayish green livers. Three of the livers taken from Alina's Reef had this grayish/white-green coloration, while livers from both White Banks and Molasses Reef had red livers.

Discussion

Two principal criteria for an investigation of a natural resource damage event are 1) documentation of the biological response and 2) documentation of the putative stressor (Boehm et al., 1995a, 1995b; Downs et al., 2005b). A principal goal of an environmental investigation is to determine if there is an association between a biological response and a putative stressor, and the nature of that association (Boehm et al., 1995a, 1995b; EPA, 2000). This is not a simple task and is usually a multistep endeavor. The first step in a strategy to investigate a natural resource damage event in which the identity of the causative agent(s) is unknown or unconfirmed is to document the behavior of a biological response in a natural resource damage event, which includes an assessment of biotic integrity (i.e., population, community, and ecosystem health; USEPA, 2000). It is not enough to only document the changes in population-level and community-level behavior. Evidence for a *mechanism* between the putative stressor and the biological effect is required to establish a causal link between the biological response and the putative stressor (Boehm et al., 1995a, 1995b; USEPA, 2000). This mechanism of pathology (effect) is embedded at a certain level in the biological hierarchy (Allen and Starr, 1982), which in most cases occurs at the molecular and cellular level. The mode of action for most anthropogenic-generated stressors (e.g., pesticides, sewer effluent) occurs by altering metabolic pathways (e.g., enzyme inhibition) or perturbing cellular structures such as membranes, the cytoskeleton, and genomic integrity (Moore, 2003). Changes at the molecular and cellular level may then result in changes at the tissue, organismal, population, and community level (Downs et al., 2001a).

Biomarkers are end points that reflect an aspect of biological function and integrity (Downs, 2005). The purpose of employing any type of cellular biomarker is to facilitate an understanding of the mechanistic basis of the pathology exhibited by the organism/population, as well as documentation of the biological response. Like assembling a puzzle, the aim is to piece together enough facts so that the investigator can cognize the mechanistic mode of action that underlies the biological response. An understanding of the altered cellular and physiological behavior, coupled with the knowledge of the changes that occurred in

Table 1. *Various Pesticide concentrations in fish livers from Molasses Reef, White Banks/Dry Rocks, and Alina's Reef. Composite samples. Concentrations rounded*

Compound	Molasses Reef (site 2) ng/g wet weight (ng/g lipid)	White Banks (site 5) ng/g wet weight (ng/g lipid)	Alina's Reef (site 7) ng/g wet weight (ng/g lipid)
alpha-BHC	BDL (BDL)	ND (ND)	BDL (BDL)
HCB	BDL (BDL)	BDL (BDL)	1.0 (95)
B-BHC	BDL (BDL)	BDL (BDL)	BDL (BDL)
gamma-BHC	BDL (BDL)	BDL (BDL)	BDL (BDL)
delta-BHC	8.4 (8366)	0.7 (75)	1.3 (123)
malaoxon	ND (ND)	12.4 (1383)	9.4 (853)
heptachlor	23.6 (23580)	8.7 (970)	12.9 (1169)
aldrin	7.0 (7034)	1.3 (142)	3.4 (311)
malathion	ND (ND)	ND (ND)	ND (ND)
chlorpyrifos	6.1 (6055)	ND (ND)	0.9 (78)
dacthal	ND (ND)	ND (ND)	ND (ND)
trans-chlordane	9.7 (9659)	4.5 (504)	3.6 (328)
4-4′-DDMU	ND (ND)	ND (ND)	ND (ND)
o,p′-DDE	ND (ND)	2.1 (235)	ND (ND)
endosulfan I	8.5 (8537)	ND (ND)	ND (ND)
cis-nonachlor	1.9 (1913)	BDL (BDL)	0.9 (84)
trans-nonachlor	7.3 (7252)	0.6 (64)	6.7 (613)
dieldrin	ND (ND)	ND (ND)	ND (ND)
p,p′-DDE	6.2 (6166)	1.8 (203)	ND (ND)
o.p′-DDD	ND (ND)	4.1 (453)	3.5 (319)
oxadiazon	29.4 (29446)	1.9 (210)	3.9 (352)
ND (ND)endrin	ND (ND)	ND (ND)	4.7 (424)
endosulfan II	9.3 (9254)	ND (ND)	ND (ND)
p,p′-DDD + o,p′-DDT +cis-nonachlor	15.8 (15776)	4.8 (533)	5.3 (486)
pcb 105	BDL (BDL)	0.9 (104)	1.4 (126)
endosulfan sulfate	10.9 (10906)	4.8 (535)	4.2 (380)
p,p′-DDT	106.6	15.5 (1724)	25.1 (2281)
methoxychlor	14.2 (14203)	ND (ND)	6.6 (600)
mirex	ND (ND)	12.9 (1429)	30.4 (2767)
oxychlordane	ND (ND)	ND (ND)	BDL (BDL)
heptachlor epoxide	ND (ND)	ND (ND)	ND (ND)

Note. BDL = Below detection limit. ND = Not detected (Site 2 sample wet weight = 0.47 g, Site 5 sample wet weight = 3.35 g, and Site 7 sample wet weight = 2.73 g).

the population and community structures, should then provide clues as to the potential causative factor. These clues and their inferences should subsequently be used to generate a practical investigative design to document the presence of the causative agent.

Undoubtedly, the investigator will discover a "line-up" of potential causative suspects found in both the environment and in the organism. Fortunately, many of these suspects can be excluded from the investigation based on the cellular biomarker profiling and referrals to the toxicokinetics database concerning the xenobiotic for that organism or a sibling species. Detection of an adducted metabolite of the causative suspect, such as benzo[*a*]pyrene adducted to DNA, RNA, or protein, decreases the ability of refuting the argument that a particular causative suspect (in this case, benzo[*a*]pyrene and perhaps other polycyclic aromatic hydrocarbons) is not interacting with the organism/population (Downs et al., 2002). In fact, such data are corroborative evidence for the mechanism of toxicity for that suspect (USEPA, 2000). A dose-response laboratory experimental design using the causative suspect and the subject species can establish a model of toxicity for that stressor in the subject species (Downs et al., 2001a; USEPA, 2000). Though such a study cannot logically confirm that the mechanism of toxicity and the causative suspect is the actual mechanism responsible for the natural resource damage event, it can provide a measure of confidence for the cause-and-effect argument that is proposed for the natural resource damage event. What is crucial is that the evidence is appropriately used in a valid series of arguments that presents a persuasive case linking the exposure event with adverse biological consequences.

The purpose of this study is to determine whether a natural resource damage event has occurred by documenting biological responses of the resource, establishing the presence of putative stressor(s), and determining the nature of their association. The goals of this study are 1) to show whether white grunt populations from Alina's Reef are exhibiting a significantly different cellular biomarker profile from those taken from the two reference reefs (White Banks and Molasses Reef) with similar community-structure attributes (response documentation), 2) if fish from Alina's Reef do exhibit differences from those at the reference sites, determine whether these differences constitute a stressed condition for that population, 3) formulate a

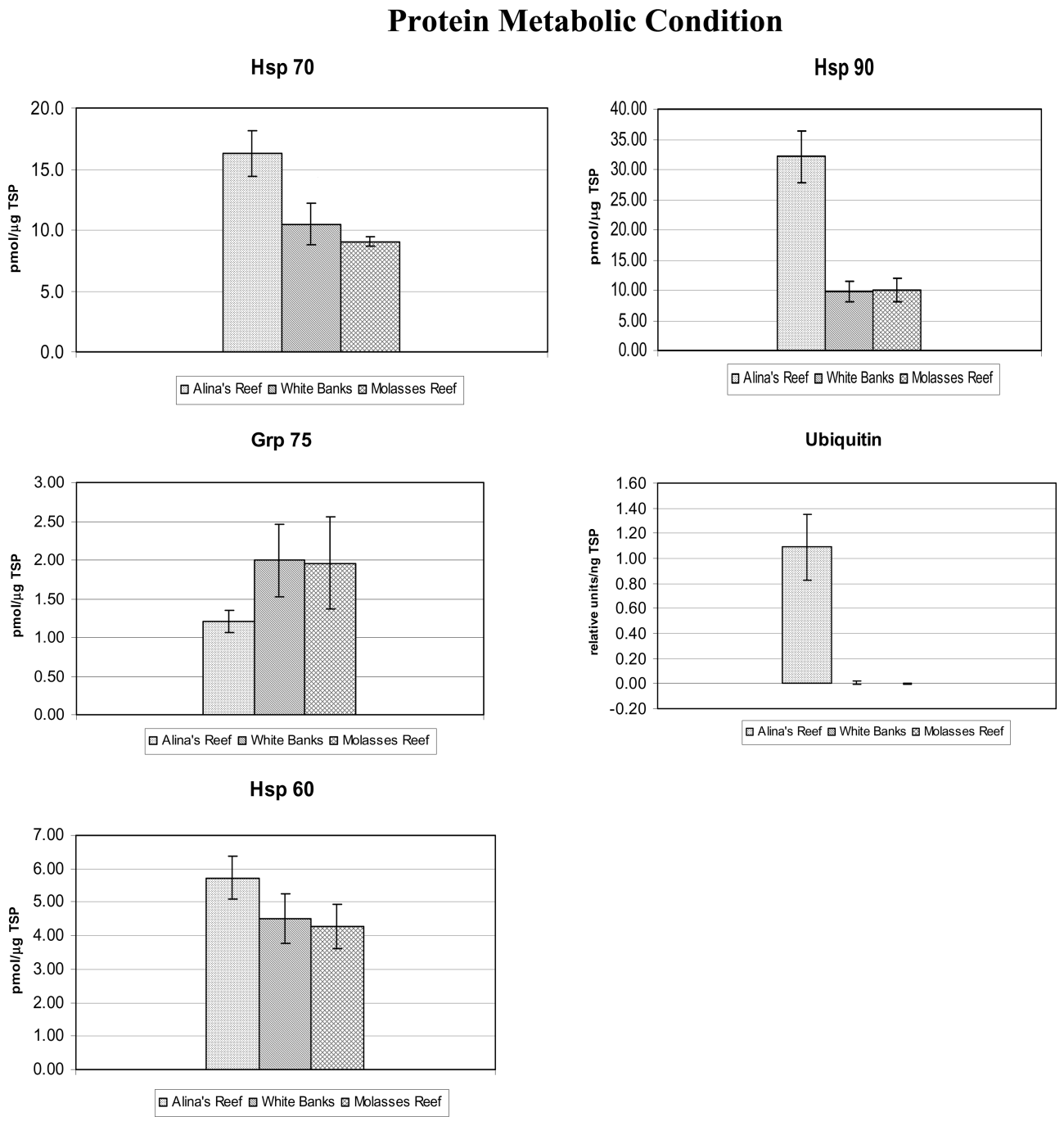

Figure 5. ELISA results for parameters of protein metabolic condition.

mechanistic model that may explain the underlying pathological condition, 4) suggest possible causative agents that could produce the mechanism of pathology, and 5) assay for the presence of these causative agents in the fish livers.

Protein Metabolic Condition

Hsp90 was significantly elevated in fish from Alina's Reef compared to fish from either White Banks or Molasses Reef (Figure 5). Hsp90 is an ATPase enzyme that is an abundant, highly conserved cellular chaperonin (Picard, 2002). Hsp90 is unique in that it engages in a diversity of functions as multi-chaperone complexes, though it is selective in its over 100 known substrates or client proteins (Buchner, 1999; Caplin et al., 2003, Sangster et al., 2004), thus conferring considerable specificity, unlike the general protein folding functions of Hsp60 and Hsp70. A wide range of physiological processes are influenced by Hsp90 including cell cycles, growth, development, evolution, apoptosis, cancer, and stress (Caplin et al., 2003;

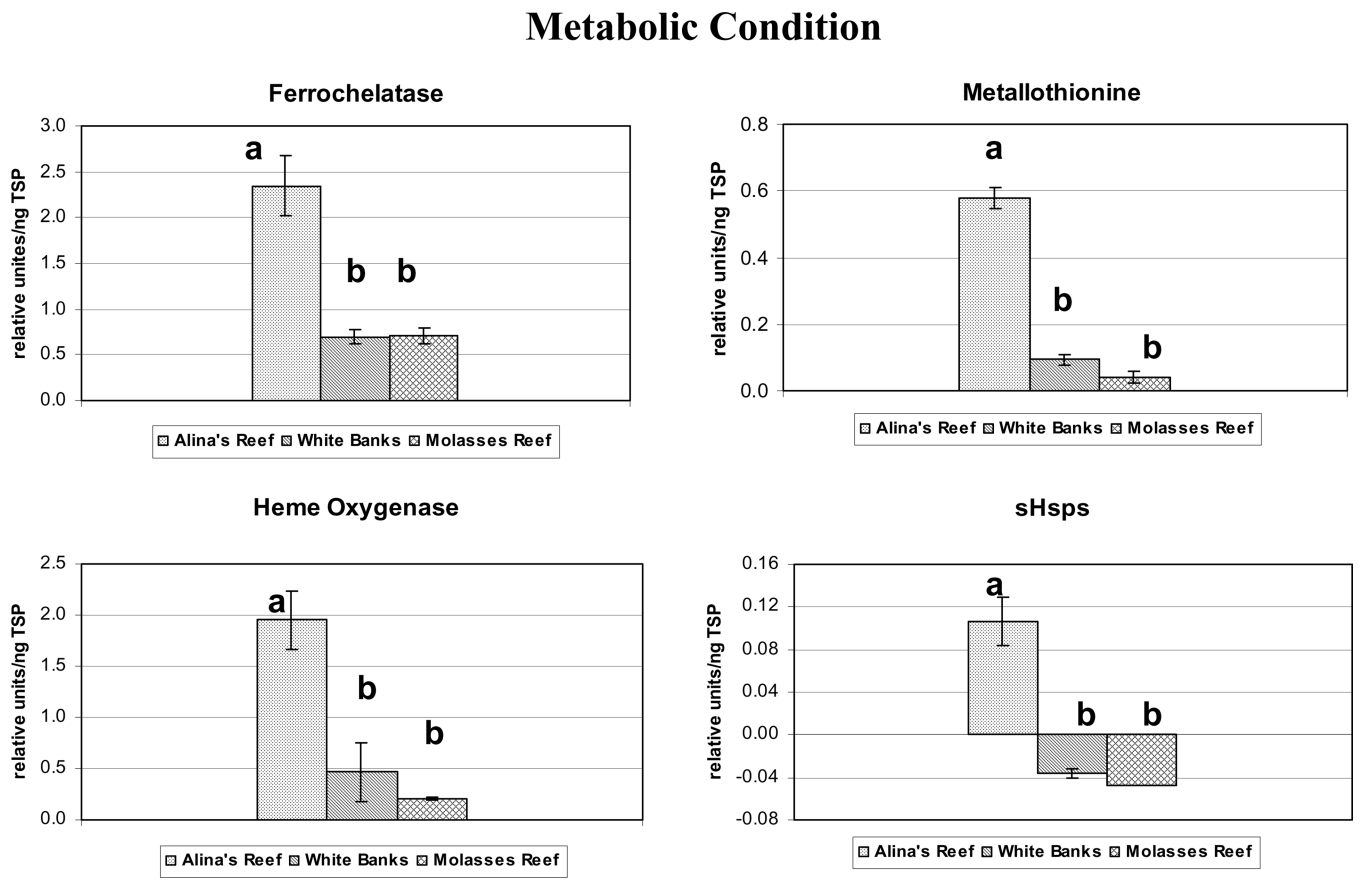

Figure 6. ELISA results for parameters of metabolic condition.

Holt et al., 1999; Lange et al., 2000; Rutherford and Lindquist, 1998). Hsp90 was first discovered as an essential chaperone in the maturation of the estrogen receptor and is known to be up-regulated by increased estrogen exposure (Bouhouche-Chatelier et al., 2001; Stein et al., 2001). Recently, evidence has shown the involvement of Hsp90 in the regulation of cytochrome P450 2E1 (CYP2E1) is essential for its degradation by unfolding the protein for degradation via ubiquitin/proteosome protein degradation pathway (Goasduff and Cederbaum, 2000). In relation to the known functions of Hsp90, an increase in Hsp90 activity viewed independent of other cellular parameters could indicate either a need for increased protein maturation or a response to increased protein degradation.

In the case of Alina's Reef, induction of Hsp90 seems to be a result of a shift in protein metabolic condition towards increased protein degradation, because ubiquitin levels in fish from Alina's reef accumulated more than ten orders of magnitude in comparison to ubiquitin levels at the other two sites (Figure 5). Ubiquitin is a 76-residue polypeptide that is conjugated to protein slated for degradation via the 26S proteosome (Ciechanover, 1998; Muratani and Tansey, 2003). Significant elevation of both of these biomarkers is suggestive that specific enzyme complexes are damaged and are targeted for rapid degradation. According to Goasduff and Cederbaum (2000), one such enzyme complex that is being specifically damaged and slated for rapid protein turnover could be the cytochrome P450 monooxygenase complexes, especially CYP P450 2-class.

Cytosolic Hsp70 is a chaperonin that is inducible by the cellular stress response and functions primarily to renature denatured proteins into the active state or to assist in degradation of proteins too damaged to be repaired and are slated for proteolysis (Ellis, 1996; Hartl, 1996). Hsp70 was significantly elevated in fish from Alina's Reef compared to the other two sites (Figure 5), supporting the interpretation that protein complexes were being damaged in the cytosol, and that the cell was compensating for the increase in protein turnover. Canonical correlation analysis corroborates the interpretation that there is a significant shift in protein metabolic condition between fish at Alina's Reef and fish at the two other sites (Figure 9).

Grp75 is a mitochondrial homologue of Hsp70 and functions in the maturation of newly imported proteins into the mitochondria, along with Hsp60, another mitochondrial protein maturation enzyme complex (Voos and Rottgers, 2002). Neither of these proteins significantly differed among the three fish populations, indicating that the mitochondrial protein metabolic condition was not overtly being affected and that the shift in protein metabolic condition is predominantly occurring in the cytosol (Figure 5).

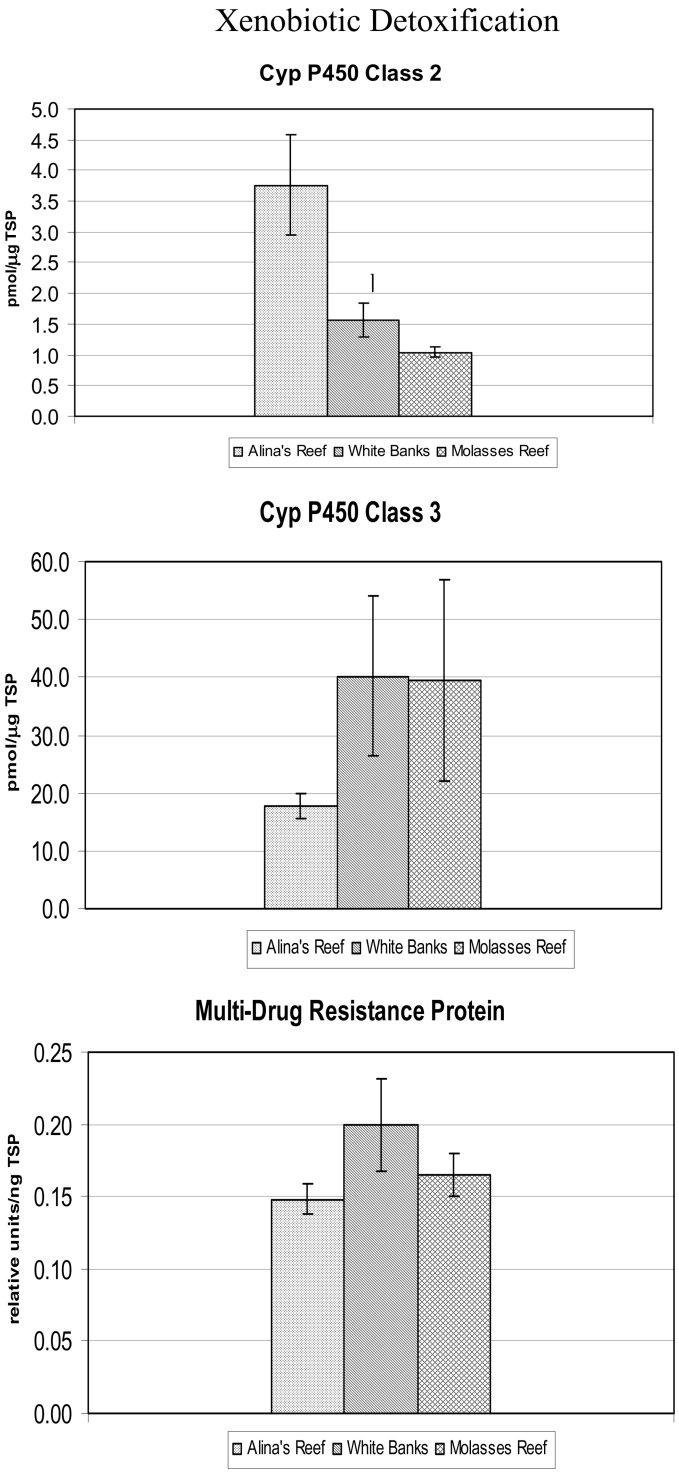

Figure 7. ELISA results for parameters of xenobiotic response.

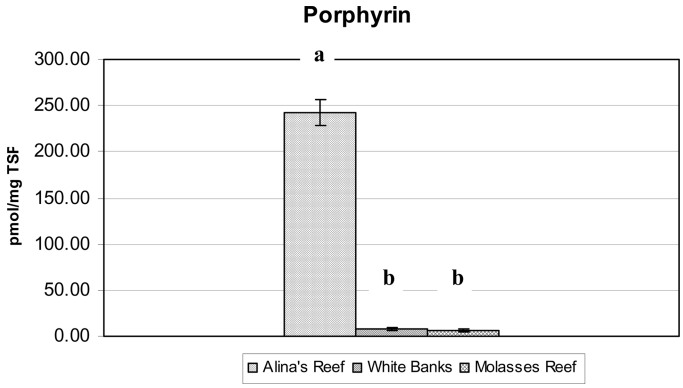

Figure 8. Concentration of porphyria species in white grunt liver.

Metabolic Condition

Metallothionein is often used as a biomarker of heavy metal exposure, though it is argued that this may be a misuse of this biomarker (e.g., Aspholm and Hylland, 1998; Galloway et al., 2004; Soazig and Mark, 2003). In the context of a cellular diagnosis in this study, we interpret changes in metallothionein levels as an indicator of broad changes in mitochondrial functional equilibrium. We base this assumption on recent work demonstrating that metallothionein type 1 localizes to the intermembrane space of the mitochondria and can regulate oxidative phosphorylation (Simpkins et al., 1994; Ye et al., 2001). Studies showing the interaction of zinc, metallothionein, and mitochondrial function further support the role of metallothionein as a regulator of cellular energy production and redox state (Coyle et al., 2002; Maret, 2000, 2003). Our position is additionally justified by toxicology studies with cadmium and metallothionein that demonstrate the interaction between cadmium and zinc metallothionein and mitochondrial dysfunction (e.g., Klassen et al., 1999; Simpkins et al., 1998; Tang and Shaikh, 2001).

Metallothionein was significantly elevated in the fish population at Alina's Reef compared to the other two sites (Figure 6). Induction of metallothionein is known to modulate mitochondrial oxidative phosphorylation activity, especially by uncoupling membrane potential, electron transport, and ATP production (Simpkins et al., 1994, 1998a, 1998b). A second-tier diagnostic investigation should include assays that examine mitochondrial function, as well as mitochondrial oxidative stress parameters, to determine if there is a mitochondrial function effect, and the nature of that effect. Though the cause of this induction for metallothionein is unknown, it should be noted that metallothionein gene expression in fish can be induced by exposure to chlorinated aromatic hydrocarbons (Gerpe et al., 1998), which is consistent with the high chlorinated aromatic hydrocarbon load in the liver of these fish (Table 1).

Ferrochelatase levels were almost fives times higher in fish from Alina's Reef compared to fish from the other two sites (Figure 6). Ferrochelatase is the enzyme that catalyzes the final step in the production of hemin (Dailey et al., 2000). Induction of this protein in fish from Alina's Reef indicates that there is a high cellular demand for hemin. This induction also begs the question as to the nature of the cellular demand for hemin. Is the demand for hemin a necessity to replace damaged hemins, or is it a requirement to fulfill demands resulting from increased production of enzyme complexes that require hemin as a prosthetic group?

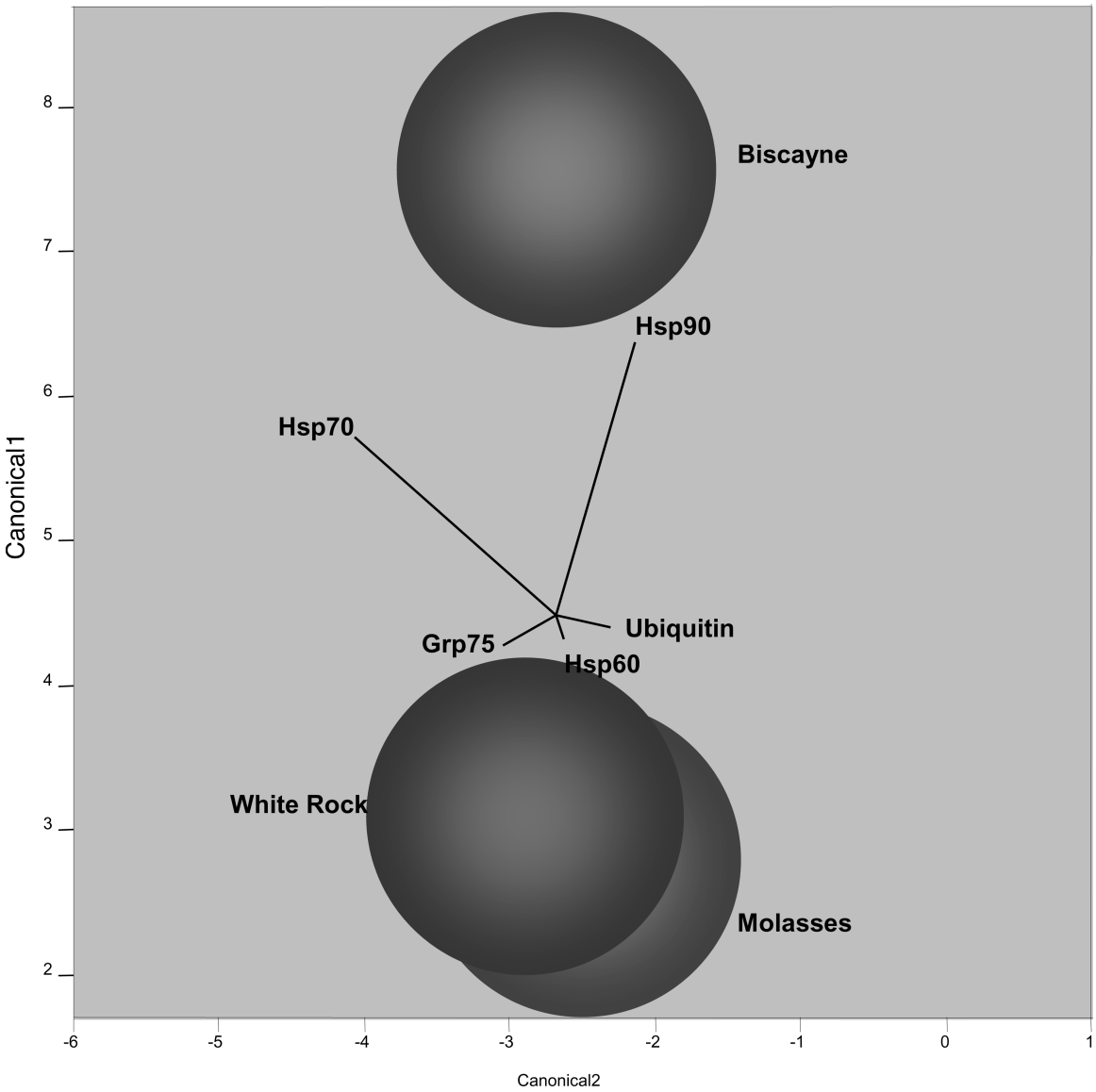

Figure 9. Canonical centroid plot of protein metabolic condition biomarkers. Original variates were biomarker levels expressed as a percentage of the control value in each treatment. Circles show the 95% confidence intervals around the distribution centroid of each stressor. Biplot rays radiating from the grand mean show directions of original biomarker responses in canonical space. Overlapping centroids indicate that those populations are not significantly different from one another, while non-overlapping centroids indicate a difference.

The four-fold higher accumulation of heme oxygenase in fish from Alina's Reef compared to fish from the other two sites provides some measure of an answer to the preceding question. Heme oxygenase is an enzyme that catalyzes the decomposition of heme to biliverdin, carbon monoxide, and ferrous iron (Schwartzburd, 2001). Biliverdin is further catalyzed to bilirubin, which is a powerful lipophilic antioxidant (Stocker and Ames, 1987). Increase of heme oxygenase at Alina's Reef could be occurring for two reasons: 1) a need for membrane-associated antioxidants and/or 2) an increased demand for the breakdown of hemin as a result of cytochrome P450 "suicide reactions" and the production of N-alkyl porphyrins (Dailey, 1990; Guengerich, 2004). Induction of alkyl porphyrins at Alina's Reef supports the second mechanism—degradation of hemin that has been damaged.

Induction of the small heat-shock protein class is generally indicative of the cell experiencing a severe stress. This protein is usually not present during a normal physiological state but is induced to high levels during a stressed condition that is associated with dire cellular insult. Unfortunately, the exact function of this protein is unknown in fish, but shows induction patterns that are consistent with homologues found in mammals and invertebrates. Accumulation of this protein in fish from Alina's Reef corroborates the interpretation that the livers of these fish are experiencing a severe cellular stress.

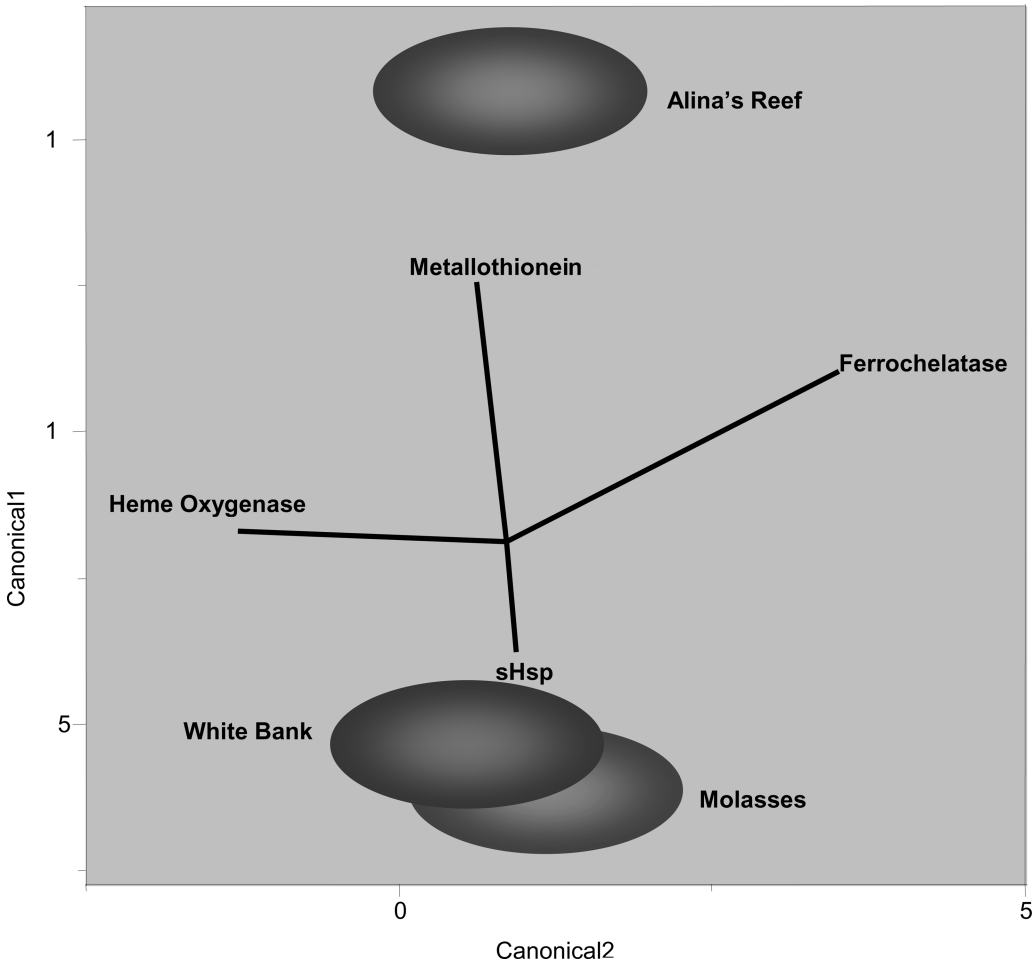

Figure 10. Canonical centroid plot of metabolic condition biomarkers. Original variates were biomarker levels expressed as a percentage of the control value in each treatment. Circles show the 95% confidence intervals around the distribution centroid of each stressor. Biplot rays radiating from the grand mean show directions of original biomarker responses in canonical space. Overlapping centroids indicate that those populations are not significantly different from one another, while non-overlapping centroids indicate a difference.

Canonical correlation analysis corroborates the interpretation that all four biomarkers of metabolic condition indicate a significant shift in some basic metabolic pathways (Figure 10).

Xenobiotic and Detoxification Response

Regulation and detoxification of potentially harmful xenobiotics occurs via a three-phase process. In Phase I, polar groups such as a hydroxyl (—OH), carboxyl (—COOH), thiol (—SH), or an amino (—NH_2) group are enzymatically adducted to the xenobiotic (for review, see Jokanović, 2001) by enzymes found in the superfamily of cytochrome P450s, the flavin-containing monooxygenases, and various esterases. These newly modified polar xenobiotics can lead to toxic compounds from an otherwise non-toxic xenobiotic, while these modifications in other xenobiotics cause increased toxicity compared to the parent compound. These new polar metabolites are conjugated with endogenous substrates such as sulfates, acetates, glutathione, and glucuronides in Phase II by enzymes that include the family of glutathione-s-stransferases, sulfotransferases, and UDP-glucuronosyltransferases (Negishi et al., 2001). These now water-soluble products can be managed by the cell for transport to lysosomes for further metabolism, sequestered into lysosome-like structures for containment, or excreted from the cell through active diffusion transporters, such as the ATP-binding cassette transporters (e.g., multi-drug resistance protein 1, MDR1; Borst and Elferink, 2002).

In this study we examine levels of two major components of this system, CYP P450 2-class, CYP P450 3-class, and MDR 1. Cytochrome P450 (CYP P450) enzymes are a superfamily of membrane-bound hemoproteins that are generally localized to microsomes, endoplasmic reticulum, and mitochondria and catalyze the oxidation of a wide array of substrates (Guengerich, 2004). Animals contain at least 50 isoforms of CYP P450, which are crucial components in pathways such as steroid metabolism and lipid biofactors, in addition to their role in Phase I toxification and detoxification (Omura, 1999).

Liver levels of CYP P450 3-class and MDR1 were not significantly different among all three populations (Figures 7 and 11). However, CYP P450 2-class enzyme levels were more than

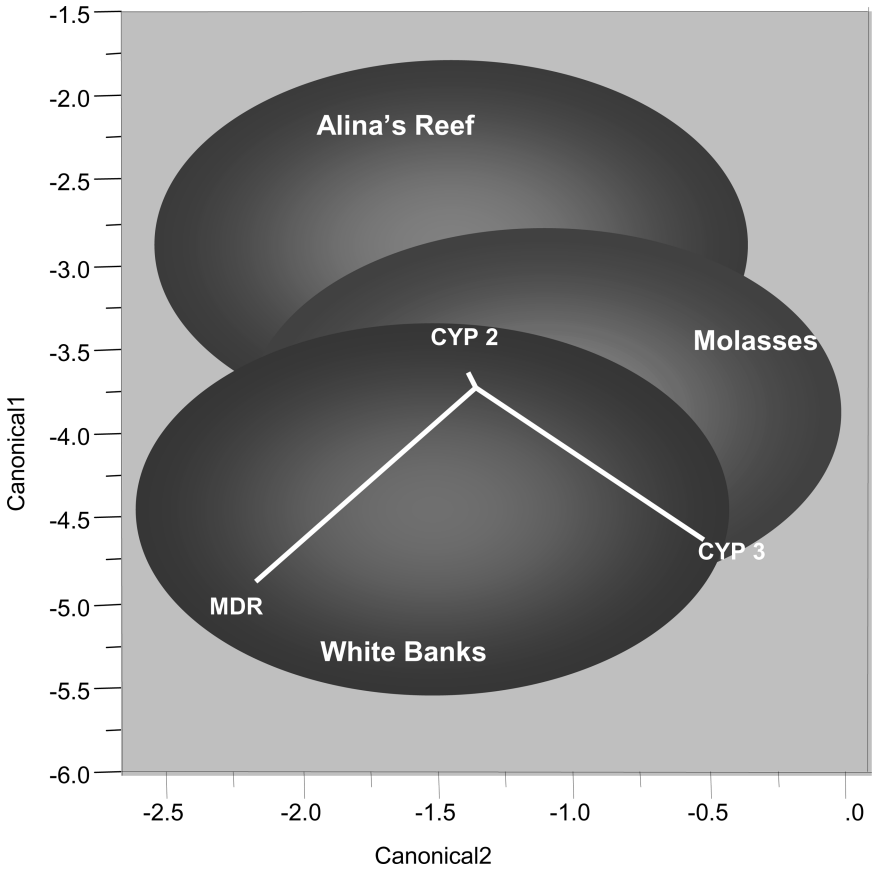

Figure 11. Canonical centroid plot of xenobiotic response biomarkers. Original variates were biomarker levels expressed as a percentage of the control value in each treatment. Circles show the 95% confidence intervals around the distribution centroid of each stressor. Biplot rays radiating from the grand mean show directions of original biomarker responses in canonical space. Overlapping centroids indicate that those populations are not significantly different from one another, while non-overlapping centroids indicate a difference.

doubled in fish from Alina's Reef compared to those from White Banks or Molasses Reef. CYP P450 2-class enzymes are best known for their role in xenobiotic transformation (Snyder, 2000). Accumulation of CYP P450 2-class protein levels could reflect two possible molecular mechanisms. The first mechanism is the controlled up-regulation of CYP P450 2-class and the concomitant increase enzymatic activity for this protein complex. The alternative explanation for increased protein levels is that the perceived accumulation of this protein is the accumulation of both functional and damaged CYP P450 2-class protein. Certain xenobiotics that are metabolized by CYP P450s will result in a damaging effect to the CYP P450 enzyme complex, usually by alkylating the porphyrin with the xenobiotic. Alkylated porphyrin results in a non-functional enzyme complex, and as a result, the cell must produce additional CYP P450 enzyme complexes to maintain a homeostatic state. This phenomenon would be perceived as increased CYP P450 protein levels, though there would be a decrease in overall CYP P450 activity levels. The only way to directly resolve which mechanism is occurring is to measure activity levels of a number of different 2-class CYP P450 species. But indirect evidence gleaned from the protein metabolic and metabolic condition data supports the CYP P450 damage mechanism. This is further corroborated by the significantly higher levels of alkylprotoporphyrin accumulation in fish from Alina's Reef compared to the other two sites.

Multi-drug resistance protein 1 (MDR 1) is an ATP-binding cassette transporter that exports glutathione-conjugated compounds out of the cell (Borst and Elferink, 2002). The lack of significant difference in MDR1 between all three populations suggests four possible interpretations: 1) the populations were not being exposed to a xenobiotic, 2) the level of MDR1 was sufficient to meet the demands of substrate export, 3) xenobiotics or toxins were being degraded to an extent that the remnants of the compound did not require excretion from cell, or 4) the xenobiotic compound was of a class that was not recognized by the MDR1 transporter (Bard, 2000; Tampal et al., 2003). The alkylated porphyrin hypothesis is consistent with the fourth interpretation, since the CYP P450 metabolized form of the xenobiotic would readily alkylate with the hemin group of CYP P450, and thus not recognized for transport.

Concentration of N-Alkylprotoporphyrins and Porphyrin Mixture

The higher levels of N-alkylprotoporphyrins in fish from Alina's Reef compared to the other two populations demonstrate not

only that the suicide reaction of cytochrome P450s is occurring, but is consistent with the diagnosis derived from the other biomarker data indicating that porphyrins are being damaged. It is indiscernible from the present data whether these N-alkylprotoporphyrins are acting as inhibitors of ferrochelatase, but a second-tier diagnostic investigation of assaying for ferrochelatase activity should conclusively resolve this issue.

Porphyria species were significantly higher by about 20-fold in the Alina's Reef samples in comparison to the other two populations. The method used in this study cannot discriminate which porphyria species is accumulating, only that non-metal porphyrins are accumulating. Discrimination of the type of porphyrin/porphyrinogen that is accumulating can be accomplished by HPLC analysis, thereby determining exactly which enzyme in the pathway of heme metabolism is being inhibited. These porphyria species are indicative of an organismal-level pathology (Thunell, 2000).

Model of Cellular Pathology

Fish from Alina's Reef were experiencing an altered cellular physiological condition that is consistent with a porphyria pathology. The crux of the model is the action of CYP P450 2-class and its interaction with the unknown xenobiotic. CYP P450 2-class exhibited a higher level of protein, of which is most likely non-functional enzyme complexes. This inhibition is a result of a suicide reaction of this CYP P450 and an unknown xenobiotic resulting in an N-alkylated protoporphyrin. Hsp90 is recruited to aid in the degradation of the "broken" CYP P450 by the ubiquitin-proteosome pathway (Correia et al., 2005). This CYP P450 is most likely associated with the microsomal fraction and/or the outer mitochondrial membrane, requiring cytosolic Hsp70 to aid in protein chaperoning of newly made CYP P450 proteins to replace the broken CYP P450s. N-alkylprotoporphyrins can inhibit ferrochelatase and other heme synthesis enzymes, resulting in an increase in porphyria species. A number of xenobiotics can directly interfere with enzymes in the heme synthesis reaction, as well as inhibiting CYP P450 activity, inducing the accumulation of porphyria species (Marks, 1985). Inhibition of the CYP P450s, either by alkylation or by competitive inhibition is known to cause oxidative stress. Oxidative stress can adversely affect mitochondrial function, specifically oxidative phosphorylation, which, in turn, can alter metallothionein and small Hsp behavior (Downs et al., 1999; Theocharis et al., 2003). Based on this mechanism of cellular pathology, the most likely toxic xenobiotics that could produce such a cellular profile are tin-based organics, diazinon, hexachlorobenzene, lindane, heptachlor, the dioxin-like PCBs, 2,4-dichlorophenoxyacetic acid (2,4-D), 2,3,7,8-tetrachlorodibenzo-p-dioxin, and acetylenes such as allylisopropylacetamide and sibling compounds. Other potential suspects are arsenics, gold, sodium thiomalate, cadmium, and thiol-based toxicants such as hydrogen sulfide and methylmercaptan (Downs, 2005; Marks, 1985, and references therein).

Contaminant Analysis of Liver for Pesticides and PCBs

The presence of a wide range of persistent and bioaccumulative agricultural biocides in the livers of the fish from all three populations was unexpected. The highest contaminant in all three populations was DDT, which was higher than the threshold concentrations for safe consumption for wildlife consumers (e.g., fish that subsist on grunts) and humans (Environment Canada, 1997; National Academy of Sciences, 1973; USEPA, 1997). DDT and its metabolites (e.g., DDD, DDE) persist in the environment with a half-life of over 15 years and are toxic to a wide range of aquatic species, as well as humans (USEPA, 1997; WHO, 1989). DDT is a phenobarbital-like inducer of the cytochrome P450 2-class family. The level of DDT in livers from Alina's Reef could have produced the induction of the CYP P450 2-class, but if this were so, this same class of proteins would be induced in both the Molasses Reef and White Banks Reef samples. Hence, there is a low probability that DDT and its metabolites are the inducers of the biomarker pattern we see in these three populations. It should be noted that the lack of DDE present in Alina's Reef samples along with the high levels of DDT in these samples strongly suggests that the exposure to DDT by this population is fairly recent. DDD and DDE are metabolites of DDT, hence only in a fairly recent exposure would it be expected to see no or a low level of DDD and DDE metabolites. Following a similar argument, the DDT/DDD ratio in tissue is also used as a proxy of the time of initial exposure; a ratio of more than one indicates a recent exposure, while less then 1 indicates an exposure to DDT that has allowed enough time for the metabolites to accumulate in tissue to a significant extent (Sapozhnikova et al., 2002). Fish from both Molasses Reef and White Banks, as well as Alina's Reef, had DDT/DDD ratios greater than 1, supporting the argument for a recent exposure.

Pesticides and their metabolites, such as malaoxon, chlorpyrifos, chlordane, lindane, heptachlor, endosulfan, aldrin, and oxadiazon were all present in the three populations, but showed no correlation between biomarker profiles and concentration. Four pesticides did show a trend between concentration in tissue and biomarker profiles: hexachlorobenzene, endrin, PCB105, and mirex.

Hexachlorobenzene is renowned for its ability to induce porphyria, which includes inducing the accumulation of ferrochelatase, cytochrome P450s, and heme oxygenase (Marks, 1985; Rietjens et al., 1997; Stonard et al., 1998). Hexachlorobenzene will also induce the accumulation of porphyrin species as a result of inhibition of uroporphyrinogen decarboxylase and ferrochelatase (Marks, 1985). An industry-funded risk assessment study of hexachlorobenzene in marine fish concluded that the worst-case predicted environmental concentration (PEC) for hexachlorobenzene in marine fish is 3 ng/g wet weight (Euro Chlor, 2002). The 1 ng/g hexachlorobenzene found in Alina's Reef samples is within the range for this worst-case PEC. The data from Figures 2 through 4, Figure 8, and Table 1 strongly support the argument that hexachlorobenzene is a primary suspect for the altered cellular condition seen in

the fish of Alina's Reef. Evidence that may be either irrelevant or contrary to this argument is that there is nothing in the literature, as far as we know, indicating the induction of N-alkylprotoporphyrins by hexachlorobenzene. The accumulation of N-alkylprotoporphyrins in fish from Alina's Reef may be the result of an unknown mechanism of toxicity for hexachlorobenzene in fish, or it may be the result of a toxic mechanism affected by different contaminants or the result of a synergistic effect of hexachlorobenzene and another contaminant (Marks, 1985).

Endrin is a polyhalogenated cyclodiene. Mechanisms of toxicity in both mammalian and invertebrates are poorly described, but recent studies indicate that endrin blocks GABA-chloride channels as well as induces a severe oxidative stress that seems to originate with endrin's interaction with the mitochondria (Bagchi and Stohs, 1992; Narahashi et al., 1992). Secondary effects of this mechanism include lipid peroxidation, adverse changes in lipid viscosity, and DNA damage (Bagchi et al., 1992). Characterization of cellular responses to endrin is very limited, though it has been demonstrated that endrin induces the accumulation of both the alpha and beta isoforms of Hsp90 (Bagchi et al., 1996). Nothing in the literature indicates that endrin has been tested for its ability to induce porphyria, affect heme metabolism, or even induce suicide reactions of cytochrome P450 enzymes. Further intensive studies on the mechanisms of cellular toxicity need to be conducted before any conclusions can be drawn of a link between endrin exposure in fish and cellular pathology.

2,3,3′,4,4′-Pentachlorobiphenyl (PCB 105) is a dioxin-like acting compound that is a known carcinogen and a suspected endocrine disruptor. This compound is known to interfere in a dose-dependent manner with heme metabolism, producing a reduction in available heme pools and increasing fluorescent protoporphyrin and porphyrinogen species (Chu et al., 1998). PCBs, especially those that have dioxin-like activity, stimulate EROD activity, up-regulate a number of cytochrome P450 species, and induce oxidative stress (Cheung et al., 2002; Chu et al., 1998, and references therein). If PCB 105 or similar PCB species were responsible for this alternation in the cytochrome P450 2-class and heme metabolism pathway, it would also be expected that MDR levels would not be affected by PCB exposure—which is exactly the case (Tampal et al., 2003). Exposure to toxic levels of PCB 105 could very well induce the biomarker pattern seen in Alina's Reef, except that the concentration of this compound in the liver of these fish is significantly below the expected threshold concentration (500 ng/g) to induce such a cellular altered state, though this threshold level has been extrapolated from acute exposure studies in mammals (Chu et al., 1998). With the available data, PCB 105 cannot be ruled out as a primary or contributing culprit. A dose-response experiment needs to be conducted to better ascertain the relationship between this compound and its ability to alter cellular function.

Mirex is a chlorinated organic used as a pesticide, a fire-retardant additive, and a supplement to fireworks. Mirex is a known carcinogen, and produces a wide variety of systemic effects with exposure (Buelke-Sam et al., 1983; Faroon et al., 1995). Mirex has broad effects on endocrine function, not only impacting reproductive communication and development, but also on angiogenic processes (Faroon et al., 1995). It should also be noted that mirex can induce embryotoxicity (El-Bayomy et al., 2002). Unfortunately, little is known about the cellular mechanisms of toxicity of mirex, other than its ability to induce cytochrome P450 species and the degradation of mirex via an iron-prophyrin model (Faroon et al., 1995; Holmstead, 1976).

Conclusions

Previous studies have indicated a chronic degradation of reef condition in the Florida Keys. A study examining white grunt populations at these sites over the time span of this study demonstrated a significant decrease in biomass and density at Alina's Reef compared to the similar habitat of White Banks (Fisher et al., 2005). We have presented here a snapshot of the health condition of three reefs within the upper Florida Keys using white grunt as a biological indicator. We used cellular diagnostics to investigate whether white grunts at these locations were 1) sustaining physiological injury based on cellular pathological signs and 2) defined a signature for possible causative agents using the observed biomarker response profiles. The cellular biomarker response profile using gene-encoded markers suggested a pathology involving perturbations in porphyrin metabolism in the livers of fish from Alina's Reef compared to fish from the reference reefs. The elevated concentrations of porphyrins and alkylated porphyrins in fish from Alina's Reef supports this diagnosis. The biomarker-response profile, coupled with the supporting evidence of elevated porphyrin-related compounds, leads to a proposed list of xenobiotic compounds, which with sufficient exposure could elicit the observed cellular pathology. We screened a panel of possible contaminants (pesticides, PCBs, and PAHs) to determine their concentration in the livers of these fish. Our task was then to determine whether the extent of the available evidence was sufficient to provide the weight of evidence necessary to establish a causative link between any of the agents present and the biomarker response profiles. Examination of the contaminant loads in the context of their amounts, biomarker response profiles, and known mechanisms of toxicity allowed us to exclude a number of possible causative agents. This resulted in two remaining agents that were present in the fish and have known biological effects that are consistent with the biological evidence. Although we were able to exclude a number of possibilities, the available data could not unequivocally establish a direct link between either of these two agents and the detected pathology. This is due, in part, to a deficiency in thorough toxicological characterization of these compounds in controlled laboratory experiments. Such experiments serve to establish the mechanism of toxicity for a given compound and establish biological response signatures. These data often are not available for fish, or frequently for any organism; thus limiting the available points in the compound's biological response signature for matching. In part, a firm causal link was not achieved, because reagents to identify specific metabolites

(i.e., antibodies specific to xenobiotic-conjugated porphyrins) have not been developed or data from functional activity tests were not available.

This study serves to illustrate the challenges and complexities of protecting and managing our coral reef ecosystems against injury and damage associated with anthropogenic activities. Detection of an alleged natural resource damage event puts into motion a process whose criteria must be met in order to assign responsibility and affect a change. Under current U.S. regulations, the process requires not only detection of an injury, but quantifying it and establishing causation (USEPA, 2000). To show causation requires demonstrating the mechanism of toxicity for a given contaminant, documenting exposure of the resource to the contaminant(s), and establishing an associated injury sustained by the resource.

We have a building body of evidence that points to land-based sources of pollution as a significant factor in the global degradation of coral reefs; however, our knowledge of coral reef toxicology lags far behind that of other systems (i.e., freshwater systems, inter-tidal zones, salt marshes) and our ability to establish these causal links is tenuous. To effectively assist in the proper management of land-based activities in proximity to our coral reef ecosystems, we must begin focusing on developing the knowledge base necessary to establish mechanisms of toxicity of those contaminants used in and around our reefs. Along with increasing our understanding of reef toxicology, we must also employ the most appropriate and sensitive methodologies for detecting injury and causative agents, and continue developing technologies that allow precise "fingerprinting" of causative agents with the ability to allocate the degree of injury in a mixed stressor environment. Only when these elements are in place can we expect to be able to identify the "smoking gun," determine the extent of injury, and provide reasonable guidance for restoration.

Acknowledgements

We thank Elizabeth Fisher of the University of South Florida for her help in sample collection and Samantha Ryan, Sonomi Hikima, and Tye Pettay of the NOS CCEHBR laboratory for their invaluable help in sample dissections and processing. We also would like to thank NOAA's Undersea Research Center (NURC) in Key Largo, Florida, for providing housing. Finally, we thank Dick Rutkowski of Hyperbarics International, Inc., for his encouragement of this work in the Florida Keys.

This project was funded by the National Sea Grant Marine Biotechnology Program, Award No. NA86RG0052, Am.7; partial support was also provided by NOS CCEHBR and EnVirtue Biotechnologies, Inc. JEF's contributions were in part supported by the National Science Foundation grant DEB 9727039. Samples were collected under permit from the Florida Keys National Marine Sanctuary, FKNMS-2001-008; Florida Fish and Wildlife Conservation Commission, 01S-517; and Biscayne National Park BISC-2002-SCI-0022.

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