

# Profiling differential gene expression of corals along a transect of waters adjacent to the Bermuda municipal dump

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## Abstract

A coral cDNA array containing 32 genes was used to examine the gene expression profiles of coral populations located at four sites that varied with distance from a semi-submerged municipal dump in Castle Harbour, Bermuda (previously identified as a point source of anthropogenic stressors). Genes on the array represent transcripts induced under controlled laboratory conditions to a variety of stressors both natural (temperature, sediment, salinity, darkness) and xenobiotic (heavy metals, pesticides, PAH) in origin. The gene expression profiles produced revealed information about the types of stressors. Consistent with other studies undertaken in Castle Harbour, the coral cDNA array detected responses to heavy metals, sedimentation, as well as oxidative stress.

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## 1. Introduction

Stress is the reduction of an organism's ability to maintain homeostasis as a result of cumulative modifications in multiple metabolic pathways (Gasch et al., 2000; Svensäter et al., 2000). The same stressor can elicit different responses depending on the organism's physiological status prior to exposure to the stressor(s). Factors such as the type of stressor, the period of exposure (both temporally and spatially), as well as previous physiological conditions can all influence the stress response. The health of an organism may be best characterized along a continuum from healthy to dead. An organism's position on such a continuum can be influenced by several variables including developmental stage, competition, predation, disease, or environmental influences. One means of determining whether corals are stressed is to compare the same response in different populations at the same time. Unfortunately, population/community level studies suggest that most coral populations

are experiencing at least some degree of stress, which has resulted in decreases in coral coverage and/or species diversity world wide (Jackson et al., 2001; Pandolfi et al., 2003). Working within the paradigm of "no corals are unstressed", valuable information can still be obtained by comparing different populations to see if each population is expressing the same suite of stress responses during the same time period. Biomarkers expressed in one population that are up/down regulated relative to other populations can be valuable indicators of the relative position of each population along the continuum of healthy/stressed responses as the organisms continually attempt to maintain homeostasis.

### *1.1. Anthropogenic point sources: an application of ecotoxicogenomics*

Identifying geographic locations as probable point sources of anthropogenic stressors offers ecotoxicologists the opportunity to investigate whether anthropogenic stressors are capable of being detected along an exposure gradient from the point source. The study sites identified during the First International Ecotoxicology and Coral

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Health Workshop held in Bermuda in September 2003 (Owen et al., 2005) provide a setting to investigate the effectiveness and sensitivity of a stress gene array in detecting a potential point source of xenobiotics, the island's semi-submerged municipal dump. In the developing field of ecotoxicogenomics, there are two main objectives: (1) to analyze the expression of several genes simultaneously in order to identify patterns of stress responses at the level of gene transcription and (2) to isolate and characterize the functionality of unknown genes which are differentially expressed in response to stressors. Studying how expression patterns of stress-induced genes are altered in different environments allows diagnosis of where the position of coral populations are along a healthy/stress continuum. With the development of stress gene arrays, possibilities now exist for comparing the expression of many genes simultaneously. An array composed of relatively few stress genes can provide valuable information regarding the ability of coral to respond under different environmental conditions, even if the actual stressor(s) remains unknown. The objective of this study was to use a 32 gene cDNA array to test corals from different sites in Bermuda in order to evaluate patterns of gene expression at each location.

## 2. Materials and methods

### 2.1. Study sites

Four study sites were chosen, three were located along a transect line originating in proximity to a semi-submerged municipal dump within Castle Harbour (CH), a patch reef lagoon ecosystem located in north east of Bermuda (for details, see Flood et al., 2005; Quinn et al., 2005). The site nearest the municipal dump was termed the Dump (site 1) and was located approximately 50 m from this potential point source, the Annex (site 2) was located 960 m from the dump, and Tuckers Town (site 3) was 2.73 km from the dump. North Rock, a site located 14.39 km from the dump on the edge of the outer fringing reef was chosen as a control site (site 4) since it is located in a region where open oceanic water exchange is high and where there is no direct circulation of water from Castle Harbour.

### 2.2. Coral sampling

Due to the limited coral diversity along the transect in Castle Harbour, *Diploria strigosa* was chosen as the coral to examine since it was one of the few species present at all sites. All samples were collected on 18 November 2003. Colonies sampled were taken from depths ranging from 2 to 4 m within CH, while samples from North Rock were taken from depths ranging from 3 to 7 m. Three centimeter square (3 cm<sup>2</sup>) cores from three different colonies at each site were collected. Coral tissues were brought onto the boat where they were immediately placed in liquid nitrogen in order to prevent degradation of nucleic acids. Frozen tissues were then shipped using a dry liquid nitro-

gen shipper to laboratories in Georgia for subsequent molecular analyses.

### 2.3. Extraction and manipulation of RNA

Excess calcium carbonate was removed with a chisel before total RNA was extracted from tissue by application of 4 ml of TRIzol<sup>®</sup> (Invitrogen, Carlsbad, CA, USA) to approximately 4–6 g of coral skeleton plus tissue. Homogenization was accomplished by pulverizing samples in a mortar and pestle. After the homogenization in TRIzol<sup>®</sup>, the samples were divided into 1 ml aliquots of the respective treatment groups and the RNA extraction protocol was completed for each replicate/sample. Integrity and concentration of total RNA from holobiont (i.e. coral and symbiotic zooxanthellae) was confirmed by electrophoresis of an aliquot of each sample on a 1% formaldehyde agarose gel (Sambrook et al., 1989) and was compared to a standard (MS2, Roche Diagnostics, Indianapolis, IN, USA). Total RNA was further purified by DNase I digestion followed by phenol/CHCl<sub>3</sub> extraction (Message Clean<sup>®</sup>, GenHunter, Nashville, TN, USA).

### 2.4. Probe development

After total RNA purification, reverse transcriptions of mRNAs were performed using 1 µg of total RNA, DIG-labeled UTPs (Roche Diagnostics, Indianapolis, IN, USA), oligo-dT primers, and reverse transcriptase (Superscript II, Invitrogen, Carlsbad, CA, USA) in 40 µl reactions. Concentrations of DIG-labeled cDNA produced were quantified by fluorimetry (DynaQuant, Amersham).

### 2.5. Detection of differential gene expression

DIG-labeled cDNA probes were used in Reverse Northern dot blot hybridizations to visualize the presence of target transcripts present in the total RNA samples from each site. Probe solutions (~30 ng/ml) from each site were hybridized to replicate membranes consisting of 32 genes that were previously isolated (Morgan et al., 2001; Morgan and Snell, 2002; Snell et al., 2003; Edge et al., 2005). Additional information about these 32 genes can be found in Edge et al. (2005).

Each gene was spotted in triplicate for a total of 96 spots on the cDNA array. Immunodetection of probe hybridization was accomplished using anti-DIG antibodies conjugated to alkaline phosphatase (Roche Diagnostics, Indianapolis, IN, USA) to CSPD<sup>®</sup> substrate (Roche Diagnostics, Indianapolis, IN, USA). Chemiluminescent visualization was accomplished by exposing blots to autoradiographic film for 17 h.

### 2.6. Determining the expression profile for each population

Although biologists commonly make comparisons based on fold-changes in expression, that approach was

not used in this study since it assumes that each population is expressing the same suite of genes. In addition, our previous studies have demonstrated that the genes on our array exhibit various levels of stressor specificity, therefore to make fold-change comparisons infers that each population is being exposed to the same stressors at comparable concentrations. Densitometry of blots was performed using computer software Image J (National Institutes of Health, Bethesda, MD, USA). Measurements were recorded from replicate blots for every detectable gene on each membrane. Control genes were identified by homology to rRNA protein sequences (BLASTX 2.2.9, NCBI nr database).

Background signals were quantified by measuring an area around each spot that represented twice the diameter measured within an individual spot. The intensity of each signal was initially determined by subtracting its adjacent background values. Expression of an individual stress gene was determined by replicate blot signals for that gene on two different membranes.

In order to compare signal intensities of multiple spots on different membranes, all data were log transformed since this manipulation is considered a valid approach for analyzing data where the effects in the data are believed to be multiplicative (Kerr et al., 2000). Analysis of variance (ANOVA) was then performed since it is capable of systematically estimating the normalization parameters on all relevant data (Kerr et al., 2000). Two different post hoc tests were used depending on the homogeneity of sample variances as identified by Levene's Test of Error Variances. If population variances were not significantly different from each other, then Student–Neuman–Keuls post hoc test was used. If population variances were significantly different, then Tamhane's T2 post hoc test was used.

## 2.7. Verification of expression

The purpose of this study was to apply the coral cDNA array as an initial screening tool to compare the expression profiles for selected populations. Other techniques with higher levels of sensitivity must subsequently be utilized to confirm expression of genes initially detected on the array. Northern dot blots were performed in manners previously described (Morgan et al., 2001; Morgan and Snell, 2002) to detect expression for genes particularly relevant to Castle Harbour.

## 3. Results

Fourteen of the 32 genes represented on the coral array were expressed at one or more locations within this study (Fig. 1). All four locations showed similar patterns of expression for all ribosomal genes that were used as controls (Fig. 2). Detectable log transformed expression signals ranged from 0.75 to 1.77 arbitrary units (au) above the background. Statistical analysis of log transformed control gene expression data revealed similar levels of variance in the control genes expressed at each location ( $P > 0.05$ , Laveane's Test Equality of Error Variances). There were however significant differences (One-way ANOVA,  $F_{3,92} = 6.58$ ,  $P < 0.001$ ) in the mean signal intensities of the different control genes (Table 1). Collectively, expression levels for the control genes were significantly different depending on location. The Annex was significantly higher in expression of control genes compared to all other sites (Student–Newman–Keuls,  $P < 0.05$ ). Tuckers Town was also significantly higher than the Dump (Student–Newman–Keuls,  $P < 0.05$ ) but not significantly different from North Rock ( $P > 0.05$ ).

Site	Genes										
	Ribosomal	Thioredox	TRAP	uPAR	Carboxy	Dibrom-long	Dibrom-short	Copper	Mercury	Poly-U	Methionine amino-peptidase
Dump											
Annex											
Tuckers Town											
North Rock											

Fig. 1. Representative dot blots for all genes expressed at each Bermudan study site. The ribosomal dot blot for each location is actually representative of four separate ribosomal genes.

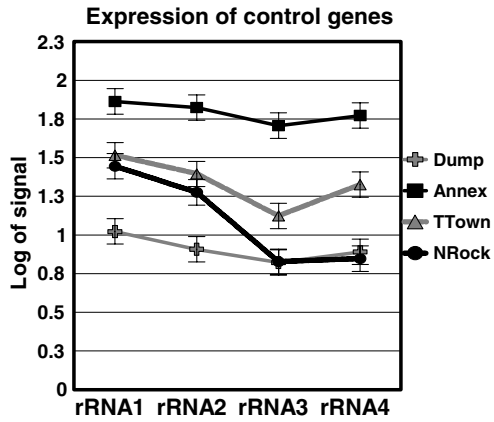


Fig. 2. Expression of control genes at all four Bermudan study sites. Control genes expressed at Annex are significantly different in their levels of expression compared to the other sites ( $F_{3,92} = 11.7, P < 0.01$ ). Expression levels of all four control genes within a location do not differ significantly from each other ( $F_{3,92} = 1.3, P > 0.5$ ).

Excluding control genes, coral from the Annex expressed the largest number of stress genes (10) followed by coral from Tuckers Town with six, the Dump with three, and North Rock with two (Fig. 1). There were three stress genes that were expressed at all sites in Castle Harbour (Thioredoxin, TRAPD, and uPAR). Two stress genes (Thioredoxin and uPAR) were expressed at all four sites (Fig. 1).

To confirm preliminary results, Northern dot blots were performed on a subset of genes originally expressed on the coral cDNA array. This subset represents transcripts that previously have demonstrated responsiveness to some of the same classes of stressors (e.g. metals) that have been previously reported within Castle Harbour (Burns et al., 1990). Results from Northern dot blots indicate that two genes (copper and uPAR) originally detected on the cDNA array were also detected on the Northern dot blots, however their expression profiles were not identical (Fig. 3). The Northern dot blot for corals from Tuckers Town had the highest average expression signals for the uPAR transcript. Corals from all locations within Castle Harbour were significantly different in their expression signals of uPAR compared to corals from North Rock (Student–Newman–Keuls,  $P < 0.05$ ). The copper transcript was detected by Northern dot blots at every location in this study. Descriptive statistics of log transformed copper data indicated unequal variances

(Levene’s Test of Equality of Error Variances,  $F_{3,8} = 4.44; P < 0.05$ ). Since univariate ANOVA is generally insensitive to heteroscedasticity, Tamhane’s T2 post hoc test was applied (Tamhane, 1979). Results of Tamhane’s test revealed that corals from all locations in this study exhibited similar expression levels of the copper transcript. The mercury and dibrom probes represented other signals detected on the array that were of particular interest. Northern dot blots were unable to detect target transcripts from the mercury and dibrom probes.

4. Discussion







4.1. Gene expression profiles

Results from the array suggest that each site is not expressing the same suite of genes (Fig. 1) and the overall level of transcriptional activity varies between sites (Fig. 2). Corals at the Annex appear to be most actively responding to stressor(s) by up-regulating 10 genes. Tuckers Town, located 1.5 km from the dump, showed elevated expression of six stress genes on the array, while corals from the Dump site up-regulated expression of four stress genes. Our results suggests that in general, the coral populations in Castle Harbour have the greatest level of stress gene expression compared to corals collected at North Rock. At the North Rock control site, the array detected expression of the ribosomal genes and two stress-responsive genes (Thioredoxin and uPAR). Variability between sites could be due to differences in concentrations of stressors. Previous studies using subsets of these gene probes have demonstrated that different concentrations of the same stressor can produce different levels of expression for individual transcript (Morgan et al., 2001; Morgan and Snell, 2002; Snell et al., 2003; Edge et al., 2005). The dump location exhibited a smaller number of transcripts which may be explained by differences in concentrations of the same stressor(s), or exposure to a different suite of stressors. Although every site in this study showed similar expression profiles of control genes, there were significant differences in the levels of transcriptional activity between sites. The reduced transcriptional activity observed at the dump would be consistent with those corals experiencing the greatest stress. Results from Northern dot blots demonstrated that expression of uPAR was significantly elevated at all three sites within Castle Harbour

Table 1  
Spot intensities ( $\bar{x} \pm SE$ ) of control genes on eight membranes

	Dump	Annex	Tuckers Town	North Rock	Gene $\bar{x}$ across sites ( $P > 0.05$ )
Ribosomal gene 1	1.02 ± .16	1.86 ± .13	1.51 ± .11	1.44 ± .14	1.46 ± .07
Ribosomal gene 2	0.91 ± .15	1.82 ± .15	1.39 ± .16	1.27 ± .17	1.35 ± .07
Ribosomal gene 3	0.82 ± .13	1.71 ± .16	1.12 ± .11	0.83 ± .23	1.12 ± .07
Ribosomal gene 4	0.89 ± .17	1.77 ± .22	1.32 ± .19	0.85 ± .22	1.21 ± .09
Gene $\bar{x}$ within a site ( $P < 0.01$ )	0.91 ± .08	1.79 ± .08	1.34 ± .08	1.01 ± .08	

Six replicates in each category were pooled to yield mean levels of expression. There were no significant differences of expression for any gene ( $F_{3,92} = 1.3, P > 0.05$ ) within site, but there were significant differences for expression levels between sites ( $F_{3,92} = 11.7, P < 0.01$ ). There was also a significant difference in the mean signal intensities of the different control genes (One-way ANOVA,  $F_{3,92} = 6.58, P < 0.001$ ).

Copper probe				
Location	Array blot	Array blot signal intensity	N. dot blot	N. dot blot signal intensity
Dump		ND		$\bar{x} = 0.75 \pm .16 \text{ SE}$
Annex		$\bar{x} = 1.36 \pm .09 \text{ SE}$		$\bar{x} = .098 \pm .16 \text{ SE}$
Tuckers Town		$\bar{x} = 0.83 \pm .11 \text{ SE}$		$\bar{x} = 1.31 \pm .16 \text{ SE}$
North Rock		ND		$\bar{x} = 1.11 \pm .16 \text{ SE}$









uPAR probe				
Location	Array blot	Array blot signal intensity	N. dot blot	N. dot blot signal intensity
Dump		$\bar{x} = 0.99 \pm .01 \text{ SE}$		$\bar{x} = 1.56 \pm .06 \text{ SE}$
Annex		$\bar{x} = 1.71 \pm .1 \text{ SE}$		$\bar{x} = 1.59 \pm .06 \text{ SE}$
Tuckers Town		$\bar{x} = 1.43 \pm .1 \text{ SE}$		$\bar{x} = 1.81 \pm .06 \text{ SE}$
North Rock		$\bar{x} = 0.82 \pm .15 \text{ SE}$		$\bar{x} = 1.51 \pm .06 \text{ SE}$

Fig. 3. Signal comparisons from the cDNA array and Northern dot blots for the copper and uPAR genes. ND indicates a signal was not detected.

(CH) when compared to the control site at North Rock. Northern dot blots for uPAR exhibited patterns of expression consistent with blots detected on the cDNA array. Responses to heavy metals and pesticides proved more variable. The copper transcript was initially detected on the array only for corals collected from the Annex and Tuckers Town sites, but Northern dot blots revealed its expression at comparable levels within all study sites. Northern dot blot analyses could not confirm the expression of the mercury or organophosphate transcripts which were initially detected on the array.

Even though preliminary results from cDNA array were more variable than Northern dot blots, collectively both assays help to identify subtle changes in physiology which suggest that corals at all sites are exposed to stressors capable of inducing expression of recognized stress genes. Thioredoxin (TRX) is known to be induced by a variety of oxidative stress conditions (Das and Das, 2000; Schallreuter and Wood, 2001; Das and White, 2002) and its expression at every location in this study suggests that all corals examined are experiencing this type of stress. This finding

is consistent with oxidative stress responses detected in other marine invertebrates at these sites during the same time period (Quinn et al., 2005). Expression of the copper transcript by corals at all study locations suggest exposure to this heavy metal is ubiquitous around Bermuda. This conclusion is also supported by bioassays conducted on other marine invertebrates (Quinn et al., 2005) as well as previously conducted chemical analyses (Burns et al., 1990). Elevated expression levels of the uPAR transcript in Castle Harbour are consistent with genes expressed by corals under laboratory induced sedimentation stress (data unpublished) as well as other studies that have quantified elevated levels of sedimentation within Castle Harbour (Flood et al., 2005). Our results suggest that the coral populations in this study are exhibiting suites of stress responses consistent with these known types of stressors.

#### 4.2. Probable sources of stressors

One plausible explanation for the pattern of gene expression observed in corals at the four sites would be

the proximity to the dump in Castle Harbour. It is hypothesized that the dump, which is semi-submerged, is leaching out a complex mixture of chemicals into the water which is circulated throughout Castle Harbour. A previous investigation of water and sediments in Castle Harbour has identified significantly elevated levels of organic and trace metal contamination at a site adjacent to the dump compared to a control site within CH (Burns et al., 1990). However, a number of other plausible sources of stressors also exist. Bermuda's international airport is located near the municipal dump and thus represents a potential source of chemical pollutants. A non-point source of toxicants may also be coming from accumulated sediment within the harbour that can be periodically resuspended by tides, currents, and hurricanes. Both recent (Linzey et al., 2003) and older studies (Burns et al., 1990) have demonstrated the presence of numerous organics (including pesticides) and heavy metals in the soils and/or sediments of Bermuda. Although no significant differences in the sedimentation rates at various sites within Castle Harbour have been detected over the study period (Flood et al., 2005), that study reported overall higher sedimentation rates in Castle Harbour compared to control sites (outside CH). Flood et al. (2005) also reported flow rates in the area of Tuckers Town that are significantly higher than other sites in CH such as the Annex (Flood et al., 2005). The responses we have detected are consistent with corals responding to resuspended and/or contaminated sediments.

#### 4.3. Gene expression profiles reveal information about types of stressors

Our results suggest that corals in Castle Harbour have been exposed to various levels of a complex mixture of stressors capable of inducing the gene expression profiles observed. Two probable anthropogenic stressors are heavy metals and organics. Morgan and Snell (2002) demonstrated that corals can exhibit different gene profiles even when exposed to a small range of copper and mercury concentrations. Anti-fouling agents such as Irgarol 1051 are known to leach copper (Boxall et al., 2000) and studies have shown that Bermuda's coastal waters are contaminated with this biocide (Connelly et al., 2001). Copper has also been detected in sediments of CH (Burns et al., 1990). Another plausible explanation for expression of genes known to be induced by heavy metals could be a compound that has not previously been examined using our stress gene probes. The anti-fouling agent Tributyltin (TBT) and its breakdown product (DBT) have been detected in the waters of Bermuda (Connelly et al., 2001), in spite of governmental regulations to restrict its use of TBT (Bermuda Government, 1989). Burns et al. (1990) detected elevated levels of zinc and lead in sediments in CH. Quinn et al. (2005) detected a suite of heavy metals in the soft tissues of bivalves (scallops) placed at each of these study sites over the period October–December 2003. They also reported elevated levels of metallothionein-like

proteins in the gills of scallops deployed at the Dump and Tuckers Town sites. In our study, expression of the metallothionein gene by corals was not detected on the cDNA array at any of the study sites. It should be noted that the copper, mercury, and metallothionein stress gene probes used in this study were developed by exposure of corals to a small number of stressors in a narrow range of concentrations (Morgan et al., 2001; Morgan and Snell, 2002). Future studies examining the expression of these genes should examine exposures to a wider variety of heavy metals including TBT, DBT, lead, cadmium, zinc, and nickel in order to clarify the specificity of their response. Analyzing metal concentrations in coral tissues would be useful for confirming exposure and providing estimates of dose.

Expression of two dibrom probes in this study suggests exposure of the corals to some type of organic. PAHs have been detected in sediments from CH (Burns et al., 1990). The long-dibrom probe has previously been shown to be a more general response in corals that were exposed to organophosphates and/or elevated levels of PAHs, whereas the short-dibrom probe has been shown to exhibit a greater specificity to the concentration of an organophosphate stressor (Morgan and Snell, 2002). A previous study (Owen et al., 2002a) has reported seasonal inhibition hemolymph acetylcholinesterase activity in bivalves deployed in the coastal waters of Bermuda, which may reflect exposure to organophosphates. That study, however, did not include Castle Harbour. Other types of organic residues have also been detected from soil samples in Bermuda, including DDE, DDT, Dicofol, Dieldrin, and PCBs (Linzey et al., 2003). The gene expression profiles for corals at the Annex and Tuckers Town sites are consistent with previous studies (Morgan and Snell, 2002; Owen et al., 2002a) that have demonstrated detection of responses to exposures of organophosphates in tropical marine invertebrates. Terrigenous sources of pesticides (Linzey et al., 2003) coupled with Castle Harbour sedimentation data (Flood et al., 2005) suggests that other sources and/or locations in CH besides the dump could be impacting the coral populations in this study. Since Northern dot blots could not confirm expression of these organophosphate probes, future studies should seek to further characterize the specificity of these probes in corals exposed diverse classes of organics.

The three stress genes expressed at all locations within CH have previously demonstrated photo-inducible expression in other organisms. Thioredoxin (TRX) is capable of sequestering reactive oxygen species (Das and Das, 2000; Das and White, 2002) and has also been identified as a component of an environmental stress response in model organisms (Gasch et al., 2000). Translocon-associated protein delta (TRAP- $\gamma$ ) is a subunit of a transmembrane protein complex located at the site where nascent secretory proteins enter the endoplasmic reticulum (Hartmann et al., 1993; Hothuis et al., 1995). Expression of TRAP- $\gamma$  has been linked to UVB exposure (Wang and VandeBerg, 2004) and has recently been identified as part of a ubiquitinated

protein complex that forms cytotoxic aggregates (Miyazaki et al., 2004). Urokinase-type plasminogen activator receptor (uPAR) has been induced by exposure to UVB (Marshall et al., 1999) and is also known to be actively involved in tissue remodeling such as wound healing by proteolytic degradation of extracellular matrices (Ploug, 2003). It would be tempting to attribute the expression of these genes by corals in CH to photon exposures. However, these genes are known to be induced under diverse conditions. In fact, we originally isolated TRX and TRAP- $\gamma$  from laboratory exposures of coral to heavy sediment load. Although the uPAR gene was originally isolated from corals kept in the dark for 2 days, we have also detected its expression under laboratory induced sedimentation stress (data unpublished). From the responses we have detected, expression of all three genes can be linked to exposure to elevated sedimentation. Coral species such as *D. strigosa* are known for their ability to thrive in environments of high sedimentation (Bak and Elgershuizen, 1976) and there is evidence that coral community structure in Castle Harbour has shifted towards this sediment-tolerant species (Flood et al., 2005). Expression of uPAR in this study is consistent with a coral tissue remodeling response in an environment of elevated sedimentation. Previous studies on correlating depth profiles and oxidative stress have concluded that coral populations at greater depths exhibit increased oxidative stress responses during periods of thermal stress (Downs et al., 2002). We cannot exclude the possibility that these genes (uPAR, Thioredoxin, TRAP- $\gamma$ ) could be induced by different stressors at different locations. However, it seems unlikely their expression is correlated to differences in depth at these high latitude study sites in November.

#### 4.4. Effects of multiple stressors

Chemical analyses and toxicity tests performed on soils, sediments, and waters in Bermuda indicate that multiple anthropogenic stressors are ubiquitous (Burns et al., 1990; Connelly et al., 2001; Owen et al., 2002b; Linzey et al., 2003). Previous studies have demonstrated that different stressors may elicit the same response. For example, elevated temperature (Jokiel and Coles, 1990; Gates et al., 1992; Fitt et al., 1993; Winter et al., 1998), exposures to heavy metals (Harland and Brown, 1989; Jones, 1997), UV irradiance (Gleason and Wellington, 1993; Lesser et al., 1990; Lesser, 1997) or cyanide (Jones and Steven, 1997) have all been shown to induce coral bleaching. Collectively, these studies illustrate how coral responses to diverse stressors converge into common biochemical pathways. Some of the genes used in this study may best be characterized as general stress responses since they are known to be induced by multiple stressors. The stress genes detected in this study have one common characteristic, they were all originally detected/isolated from corals exhibiting up-regulated responses to stress. Molecular responses represent individual components of a more comprehensive

physiological response and an attempt by corals to maintain and/or return to a stable homeostatic condition. The expression profiles for corals from all sites within CH indicate that corals are making site-specific physiological adjustments that are clearly different from each other as well as being different from the corals at North Rock.

#### 4.5. Temporal variability in stressor exposure

Without constant monitoring of Castle Harbour, intermittent exposures of anthropogenic stressors may go undetected. The gene expression profiles generated in this study represent a suite of responses measured at one point in time. Repeated sampling for extended periods of time may lead to different trends or may reinforce observed responses. Studies have shown that elevated levels of some anthropogenic stressors in Bermuda occur seasonally with, for example, increased boating activities (Owen et al., 2002b). Variability in exposure can also be due to sediment resuspension events that can occur regularly with tidal changes, seasonal rainfall, or as the result of intermittent natural events such as hurricanes (Bermuda experienced a category 3 hurricane in early September 2003).

#### 4.6. Tolerance

Development of tolerance associated with chronic stressor exposures may also explain the observed gene expression profiles. Corals are known to exhibit some metal tolerance (Harland and Brown, 1989) which could influence the abundance of specific transcripts. If the genes detected in this study are associated with a tolerance response, then dot blot signals could potentially be influenced by previous exposures. Another possibility is that the heavy metal concentration at the dump site exceeds the level necessary to induce expression of the genes. Metal concentration has previously been shown to be a key element in the expression of a coral metallothionein gene (Snell et al., 2003). The genes used in this study were originally isolated from corals that were exposed to heavy metals for only 4 h (Morgan et al., 2001; Morgan and Snell, 2002; Snell et al., 2003). Without knowing the dose-response characteristics of each of the genes used in this study, it is plausible that exposure length and/or concentration may influence expression of the target transcripts.

#### 4.7. Potential sources of variability

##### 4.7.1. Membrane effect

Statistical analysis of control gene expression suggest that observed differences between locations reflect true population differences and were not artifacts of membrane preparation. Each location expressed all control genes in similar patterns with comparable variances. Likewise, there was no significant difference among the blotted replicates for an individual control gene ( $F_{3,92} = 1.3$ ,  $P > 0.05$ ). Analysis of background signals indicated that all membranes

had similar levels of background ( $P > 0.05$ , data unpublished). These statistical analyses affirm that individual arrays were produced in a consistent manner. However, another element of membrane variability could exist in the form of independent PCR amplifications of replicate blots of rare stress genes. If each independent PCR reaction did not amplify the same quantity of product, then individual replicate blots may not contain the exact same amount of amplified target. Detectable thresholds for rare genes will be critically important. Most of the genes detected on the array gave signals in all three replicate blots. However, two genes (copper and uPAR) had less than three signals from the replicates and yet the results from Northern dot blots revealed that these genes were expressed (Fig. 3). The quantity of cDNA on replicate blots on the array was originally quantified by comparisons to DNA mass ladders using gel electrophoresis (Edge et al., 2005). Future versions of the coral array should quantify the amounts of blotted cDNAs by more sensitive techniques such as fluorimetry.

#### 4.7.2. Efficiency of probe labeling and/or annealing

The same amount of total RNA (1  $\mu\text{g}$ ) was used in the reverse transcription (RT) reaction for each population. The RT reactions for all four populations were performed at the same time to ensure uniformity of reagents and conditions. Each DIG-labeled probe produced was then quantified by fluorimetry in order to ensure the probe concentration (29 ng/ml) would be the same for the hybridization solution of each population. If we assume that the efficiency of the DIG-labeling reactions are not altered by the relative abundance of individual mRNAs, then signals expressed on the array should accurately represent relative differences in populations. In Fig. 1, we see that corals at each location are exhibiting similar patterns of transcriptional activity for the control genes even though the levels of activity are elevated for those at the Annex and Tuckers Town sites. Fig. 1 by itself would suggest that the efficiency of probe labeling may be different at the Annex compared to the other sites. When we compare the three stress genes commonly expressed within CH, we see there are no significant differences between either the genes or the locations (Two-way ANOVA,  $P > 0.05$ ). One would expect that elevated expression signals for the control genes observed at the Annex would also be exhibited in all other signals expressed at that location as well if probe labeling efficiency were an issue (see Fig. 2). By comparison, Northern dot blots detected expression signals of selected genes within all populations. Expression profiles of uPAR on both the cDNA array and Northern dot blots, coupled with statistical analyses, suggest observed differences in expression patterns may represent real population differences between sites within Castle Harbour and the control site of North Rock. Techniques with greater resolution (i.e. Northern dot blots) are capable of detecting expression patterns for selected genes that may indeed differ from the initial results obtained with the cDNA array. It should be noted that it is

not only more labor intensive but also more expensive to prepare 32 individual DIG-labeled probe reactions compared to one DIG-labeled reverse-transcription reaction. As a preliminary screening tool of populations, the cDNA array can generate new hypotheses to be investigated and should be complemented by other techniques. Discrepancies between the cDNA array and Northern dot blots suggest there were differences in the sensitivity of responses detected by each technique even though hybridization conditions were identical for all membranes (both cDNA arrays and Northern dot blots). Genes not detected on the array at some sites, were subsequently detected at those same sites using Northern dot blots. These differences are best characterized as false-negative results. Studies have shown that false-negative results are more common (compared to false-positives) in microarrays (Peplies et al., 2003). Investigations looking at strategies for optimizing microarray hybridizations have determined that secondary structures of target molecules can reduce the accessibility of probe binding sites (Peplies et al., 2003). To reduce the potential influence of interfering secondary structure, future investigations/applications using the coral cDNA array should optimize hybridization conditions. Studies have demonstrated that reducing the formamide concentration in the hybridization buffer and elevating the hybridization temperature to 46° will decrease the occurrence of false-negative results (Peplies et al., 2003). To consistently detect rarer transcripts and thus help reduce the variability observed in this study, future applications of the coral cDNA array should include mRNA enrichment in the probe labeling RT reactions. Since microgram quantities of mRNA are difficult to obtain, techniques capable of amplifying small amounts of mRNA have been developed that do not significantly distort the proportions of transcripts expressed (Baugh et al., 2001).

#### 4.8. Probe specificity in different species

All genes in these analyses were originally detected and isolated from laboratory induced exposures to various stressors on species other than *D. strigosa* used in this study (Morgan et al., 2001; Morgan and Snell, 2002; Snell et al., 2003; Edge et al., 2005). While some coral genes appear to be highly conserved (van Oppen et al., 1999), expression studies from other organisms suggest that a species may contain a gene which lacks a homolog in closely related species (Rubin et al., 2000). In this study, it appears from detectable expression signals that 14 of the genes examined do have significant homology between species (see Fig. 1). Other Cnidarian genomic studies have demonstrated that mitochondrial genomes of numerous species exhibit low genetic diversities (Shearer et al., 2002). Detection of target transcripts in other coral species broadens the applicability of our microarray in gene expression profiling. Our results demonstrate that molecular probes isolated from *Acorpora cervicornis* and *Montastrea faeolata* can be applied to other coral species such as *D. strigosa*. The data in this

study represents baseline information on inter-species specificity. The possibility exists that expression signals detected in *D. strigosa* are not representative of the target transcripts in *A. cervicornis* or *M. faeolata* even though hybridization washes were performed under high stringency conditions. Detectable results from “cross-hybridizations” between different species provides information about similar sequences, however sequence similarities do not automatically correlate with similar function (Milkos and Maleszka, 2001).

Even though 14 genes did give a signal, there were an additional 18 genes on the array that did not give a detectable signal. A number of plausible explanations exists for the lack of expression signals. First of all, studies from model genomic organisms have demonstrated that only subsets of known stress genes are expressed at any one time (Adams et al., 2000; Gasch et al., 2000; Hill et al., 2000). It therefore seems highly improbable that the coral cDNA array is entirely composed of stress genes that will all be expressed under the environmental conditions represented in this study. Secondly, *D. strigosa* genes may be highly homologous to our *Acropora* and *Montastrea* probes but these genes simply were not expressed in this study. Thirdly, *D. strigosa* may have expressed these genes but sequence similarities were insufficient to withstand the high stringency washes during hybridizations. Lastly, these genes were expressed but at levels below the level of detection for the techniques used in this study. Differences in detectable levels of expression can also account for the discrepancies between the expression signals initially detected on the array and the subsequent expression signals detected by Northern dot blots. Coupling real-time PCR with additional gene sequence information from *D. strigosa* should allow future studies to discern which explanation is most appropriate.

It is also possible that genes originally up-regulated in *Acropora* or *Montastrea* are constitutively expressed in other coral species such as *D. strigosa*. This option, however seems highly unlikely since constitutive expression should increase the probability of detecting signals for the majority of genes (if not all genes) on the array. Future genomic studies will be needed to determine whether the *D. strigosa* signals detected on the array represent expression of orthologous genes or well-conserved paralogs (related but perform different function) of *Acropora* and *Montastrea* genes.

## 5. Conclusions

Gene expression profiling represents an excellent tool for comparing and detecting subtle differences in the health/physiology of coral populations. Our gene array detected responses to heavy metals, sedimentation, and oxidative stress at the locations sampled in this study. These findings are consistent with the pollutants/stressors reported in other studies as well as our previous laboratory induced stress exposures. Expression of the thioredoxin,

copper, and uPAR genes at the control location (North Rock), some distance from mainland Bermuda, also reaffirmed previous conclusions that all coral populations are experiencing some level of stress. Although previous studies have reported high concentrations of various anthropogenic stressors within and surrounding Castle Harbour, there still are corals surviving at these locations. This suggests that the gene probes on our array detect sublethal responses to stress which reaffirms the concept that gene expression profiling provides insight into subtle alterations in physiology employed by organisms to maintain homeostasis.

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