

Development of a coral cDNA array to examine gene expression profiles in *Montastraea faveolata* exposed to environmental stress

Sara E. Edge^{a,*}, Michael B. Morgan^b, Daniel F. Gleason^c, Terry W. Snell^a

^a School of Biology, Georgia Institute of Technology, 310 Ferst Drive, Atlanta, GA 30332-0230, USA

^b Berry College, School of Mathematical and Natural Sciences, Mount Berry, GA 30149-0430, USA

^c Department of Biology, Georgia Southern University, Statesboro, GA 30460-8042, USA

Abstract

The development of a cDNA array of coral genes and its application to investigate changes in coral gene expression associated with stressful conditions is described. The array includes both well-characterized and previously unidentified coral genes from *Acropora cervicornis* and *Montastraea faveolata*. Corals were exposed to either natural or anthropogenic stressors to elicit the expression of stress genes for isolation and incorporation onto the array. A total of 32 genes involved in protein synthesis, apoptosis, cell signaling, metabolism, cellular defense and inflammation were included on the array. Labeled cDNA from coral (*Montastraea faveolata*) exposed to elevated seawater temperature, salinity and ultraviolet light was tested against the microarray to determine patterns of gene expression associated with each stressor. Carbonic anhydrase, thioredoxin, a urokinase plasminogen activator receptor (uPAR) and three ribosomal genes demonstrated differential expression across all replicates on the array and between replicate colonies. Specific gene expression patterns produced in response to different stressors demonstrate the potential for gene expression profiling in characterizing the coral stress response.

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1. Introduction

A variety of natural and anthropogenic stressors impact coral reef ecosystems in many regions of the World. Increases in mean annual seawater temperatures (Hoegh-Guldberg, 1999; Buddemeier and Ware, 2003), elevated levels of ultraviolet radiation (Gleason, 2001) and increased salinity fluxes have been recorded on several reefs (Halley et al., 1994; Porter et al., 1999). Individual organisms respond to changing environments

by regulating metabolic pathways to prevent or abate physiological damage. These cellular events precede population-level changes and could be useful biomarkers if linked to specific physiological or ecological events.

Physiological responses such as shifts in respiration (Ferrier-Pages et al., 1999; Anthony and Hoegh-Guldberg, 2003), photosynthetic efficiency (Warner et al., 1996; Jones et al., 1999), changes in growth rate (Ferrier-Pages et al., 2000) and bleaching (Lesser et al., 1990; Gleason and Wellington, 1993; Fitt and Warner, 1995) have been commonly employed as measures of coral health. In many instances, observations of such responses indicate coral in serious physiological decline. However, most physiological measurements do not

* Corresponding author. Tel.: +1 404 385 4434; fax: +1 404 894 0519.

E-mail address: gte405r@mail.gatech.edu (S.E. Edge).

identify the stressor or the underlying molecular mechanisms controlling a response. Changes in gene expression and protein production are key elements of the stress response and usually occur before physiological damage is evident (Tsuji et al., 2000; Hohmann, 2002). For example, in response to the production of damaging oxygen radicals, genes such as superoxide dismutase, cytochrome c peroxidase, ferritin and thioredoxin are transcribed and translated to bind and neutralize these reactive oxygen species (Cairo et al., 1995; Gasch et al., 2000). Existing genomic techniques, developed in biomedical research, are capable of diagnosing and quantifying the impact of stressors on corals. Molecular tools, from the fields of proteomics and transcriptomics, are commonly used to investigate how an individual's genome regulates biological functions. The application of this technology in assessing coral response to environmental change has the potential to advance the field of coral biology and provide valuable management tools for the rapid assessment of coral reef health.

1.1. Transcriptomics

All living organisms have thousands to tens of thousands of unique genes encoded in their genome, of which only a small fraction are expressed at a given time. Therefore, it is the temporal and spatial regulation in gene expression that determines metabolic activity (DeRisi et al., 1997). The subset of genes transcribed in a given organism is called the transcriptome. It is the dynamic link between the genome, the proteome and the phenotype. Transcriptomics allows the monitoring of differential gene expression which can identify gene function, elucidate the mechanisms behind a biological response and produce a partial snapshot of cellular machinery in action (Snape et al., 2004). To facilitate the discovery of differentially expressed genes, a variety of methods have been developed including differential display PCR (ddPCR) (Liang and Pardee, 1992), subtractive suppression hybridization PCR (SSH PCR) (Diatchenko et al., 1996), representational difference analysis (RDA) (Pastorian et al., 2000; Hubank and Schatz, 1999), serial analysis of gene expression (SAGE) (Velculescu et al., 1995), real-time quantitative PCR (Gibson et al., 1996), in situ hybridization (Angerer et al., 1987) and hybridization to gene arrays (Larkin et al., 2002; Held et al., 2004; Voelckel et al., 2004). Although each method has distinct advantages and limitations, the general methodology to detect differentially expressed genes has advanced to automatic high throughput methods such as hybridization-based gene arrays. Thus, to study non-model organisms, such as coral, it is necessary to identify a variety of informative genes and design a cDNA array to functionally characterize the genes and their expression profiles.

1.2. Molecular advances in Cnidarian research

Applications of well-established molecular methods in Cnidarian research are evident and promise to continually expand in the field. For example, genomic information on Cnidaria is growing at an ever increasing rate in databases such as the National Center for Biotechnology Information (NCBI) and the European Bioinformatics Institute (EBI). In addition, the Cnidarian Evolutionary Genomics Database, or *Cnidbase* (<http://cnidbase.bu.edu>), is a publicly accessible database that stores gene expression data for Cnidarians such as *Hydra*, the sea anemone *Nematostella*, the stony coral *Acropora*, and the jellyfish *Podocoryne* (Ryan and Finnerty, 2003). Although *Cnidbase* focuses primarily on evolutionary changes and structural genomics, it facilitates the functional characterization of newly discovered Cnidarian genes by providing a central resource where comparisons of new experimental data can be made with existing data.

Several projects have been initiated to sequence a Cnidarian genome. In 2003, a group of coral researchers, using a forum available through NOAA's Coral Health and Monitoring Program (CHAMP), discussed the importance of sequencing a coral genome and selecting a representative species. Based on this discussion, a proposal was made to the National Human Genome Research Institute (NHGRI) of the National Institutes of Health (NIH). In addition, The Coral Genomics Group, part of the Comparative Genomics Center at James Cook University in Queensland Australia, has developed a genetic library of expressed sequence tags (ESTs) from *Acropora millepora* in a study of metazoan divergence and evolution (Kortschak et al., 2003). Finally, in 2004, the US Department of Energy Joint Genome Institute (<http://www.jgi.doe.gov/>) was reportedly nearing completion of a project to sequence the genome of the Cnidarian, *Nematostella vectensis* (Monica Medina, *personal communication*).

1.3. Molecular biomarkers

As of early 2005, the NCBI database held over 1,50,000 nucleotide sequences and nearly 3000 protein sequences for Cnidaria. Scleractinian corals represented just over 2% of the nucleotide sequences and 29% of the protein sequences. However, it should be noted that more than half of the sequences are ribosomal or mitochondrial and a significant portion of the remaining sequences are repetitive. Nevertheless, for the development of molecular biomarkers, the database provides a valuable resource for coral researchers. Molecular biomarkers are potentially useful for detecting early signs of change in an organism's physiological state, such as stress or injury due to environmental factors, which may be chemical, physical or biological. Identifying

suites of proteins or genes that are expressed in response to specific conditions is the first step in developing a set of biomarkers capable of diagnosing key stressors in an environment.

A few well-characterized molecular biomarkers currently identified in Cnidaria, include a metallothionein coding gene (Snell et al., 2003), heat shock proteins (Black et al., 1995; Sharp et al., 1997) and heat shock genes (Gellner et al., 1992; Ryan and Finnerty, 2003), carbonic anhydrase (Weis and Reynolds, 1999), a ceruloplasmin gene (Morgan and Snell, in press), a symbiosis gene (Reynolds et al., 2000; Mitchelmore et al., 2002), glutathione peroxidase (Schroth et al., 2005) and an oxidative stress protein mRNA (Schroth et al., 2005). Other potential biomarkers involved in developmental regulation (Lohmann et al., 1999), evolution (Romano and Palumbi, 1997; Romano and Cairns, 2000) and speciation (van Oppen et al., 1999) have also been identified in various orders of Cnidaria. It is now technically feasible to incorporate hundreds of coral stress gene biomarkers onto an array and monitor their expression in a single experiment.

1.4. cDNA array analysis in ecotoxicogenomics

Once genes of interest are identified and isolated from an organism, they are spotted onto glass slides or nylon membranes for expression profile analyses. A major advantage of gene expression analysis using array technology is the ability to characterize a likely stressor and identify underlying mechanisms of the physiological response. For example, in yeast with a genome of approximately 6200 genes, hundreds of genes are differentially expressed in response to a variety of stresses (Gasch et al., 2000). This response has been called the environmental stress response (ESR) and regulates the metabolism of many biochemical pathways for the purpose of preventing damage to membrane structure and maintaining normal cellular functions. Larkin et al. (2002) isolated cDNA clones from sheepshead minnow induced by 17- β -estradiol exposure using ddPCR. Several estrogen responsive genes including vitellogenin, vitelline envelope protein (ZP2) and the iron transport protein transferrin, along with 17 constitutive genes were spotted onto a nylon membrane. Of the 54 cDNAs spotted on the array, 15 were up-regulated by estradiol exposure, 7 were down-regulated, and 32 were unaffected. In a similar study, Williams et al. (2003a,b) used degenerate primers and suppressive subtractive hybridization (SSH) to construct a 160-gene microarray for European flounder. The array compared differential expression between fish from polluted and relatively unpolluted estuaries. Eleven transcripts were found to be differentially expressed in male flounder, 7 were up-regulated and 4 were down-regulated in fish from the polluted site. Expression profiling using gene arrays has become a

powerful tool for comparing stress responses between experimentally exposed organisms and a control population. The ability to identify which stressors are responsible for an observed pattern of expression is a major advantage of this technology.

1.5. Toxicogenomics and ecotoxicology

Molecular tools have been employed by a few researchers to detect stress responses in corals (Black et al., 1995; Fang et al., 1997; Tom et al., 1999; Ammar et al., 2000; Downs et al., 2000; Morgan et al., 2001; Morgan and Snell, 2002). At the present time, most of these responses represent phylogenetically conserved cellular expressions of well-characterized proteins, such as molecular chaperones. The developing field of toxicogenomics examines stress responses at the molecular level with the objective of identifying new patterns of gene expression and/or previously unknown genes in organisms important to ecotoxicology (Snell et al., 2003). The application of toxicogenomics in coral research provides opportunities to discover new molecular responses related to specific stressor exposures. Responses at the level of gene expression (transcription) will always precede protein production (translation) of the same transcript. Therefore toxicogenomics offers researchers a tool that is capable of discovering new molecular biomarkers which represent responses that are sublethal and/or precede major physiological events like bleaching. Toxicogenomic investigations also provide coral researchers a direct avenue to access extensive and growing genomic databases supported by well-funded biomedical research. Access to such data offers the possibility of identifying genes based on sequence similarity and suggests potential functions where annotations are available. In addition, such genomic information may help generate new hypotheses to investigate.

In this paper, we describe the development and application of a cDNA array consisting of 32 coral genes that are differentially expressed in response to various stressors. The array is used to investigate changes in transcript abundance in coral (*Montastraea faveolata*) responding to controlled exposures of elevated temperature, salinity and ultraviolet light. To create the array, complementary DNA (cDNA), representing the 32 coral genes, is fixed to a membrane (probes). The arrayed probes are subsequently hybridized to reverse transcribed and labeled total RNA (targets) isolated from the coral population being investigated. Chemiluminescent detection reveals where annealing occurs between a probe and its target (Zhang et al., 1996; Dilks et al., 2003). In this study, gene expression patterns varied across different treatments of stressors, providing preliminary evidence for unique gene expression profiles associated with specific stressors.

2. Materials and methods

2.1. Expression profiling of laboratory exposed corals: exposures and target development

2.1.1. Coral collections

Fragments of *M. faveolata*, approximately 40 cm², were collected at a depth of 4 m from East Turtle Shoal (24°40'N, 80°55'W) in the middle Florida Keys, USA, in April 2001. Conditions on the reef were 25 °C surface temperature and 38 ppt salinity. Coral fragments were transported in closed recirculating containers of natural seawater to the Florida Keys Marine Laboratory (FKML) on Long Key.

2.1.2. Controls and exposures

Prior to acute stressor exposures, coral fragments were maintained in an outdoor water table with flow through seawater pumped directly from the bay behind the Florida Keys Marine Laboratory. The water on the table was roughly 15 cm deep and ambient light intensity was reduced by 40% with shade cloth. During exposure to temperature, salinity, and light, corals were placed in closed tanks containing 10 l natural seawater that was constantly recirculated with a submersible pump. Manipulations of temperature, salinity and light exposures lasted 4 h. Sediment and dark experiments were carried out on the water table with 48 h exposures.

2.1.2.1. Temperature. Two coral fragments, from different colonies, were placed in each treatment tank and a control tank. Temperature in the control tank was maintained at 25 °C and treatment tanks were elevated to 28 and 31 °C, respectively, using an aquarium heater. Salinity in each tank was 40 ppt, which was the salinity of the recirculating natural seawater in the FKML system.

2.1.2.2. Salinity. Two coral fragments, from different colonies, were placed in each treatment tank and a control tank. Instant Ocean® was mixed with natural seawater collected from the reef to elevate salinity from 38 to 43 ppt and 46 ppt. The control tank was natural seawater at 38 ppt. Temperature in each tank was maintained at 25 °C.

2.1.2.3. Light. Four coral fragments from two different colonies were divided evenly between two tanks. The fragments were exposed to either visible or ultraviolet light using fluorescent tubes in the laboratory. Treatments consisted of either two 20 W daylight lamps (OSRAM Sylvania, Westfield, Indiana) or a combination of one 20 W UVA ($\lambda_{\text{max}} = 368$ nm, light emission range = 310–420 nm) and one 20 W UVB ($\lambda_{\text{max}} = 313$ nm, light emission range = 270–390 nm) fluorescent lamp (Ultraviolet Resources International, Cleveland, OH). All lamps were 60 cm in length and treatments

were separated with thick black vinyl to prevent leakage of light to adjacent corals. In both treatments, coral fragments were placed 28 cm from the light source in 38 l aquaria containing 10 l of sea water. Instantaneous measures of irradiance being received by corals in each treatment were determined across a wavelength range of 300–700 nm in 2 nm increments using an underwater scanning spectroradiometer fitted with a cosine collector (LI-1800UW, LiCor Inc., Lincoln, NE). The cosine collector was submerged 2 cm below the water surface and visible and ultraviolet light were recorded in units of Wm⁻². These light values are representative of the dose rate being received by the corals in each treatment during the 4 h exposure. Temperature in the tanks during the exposure period was 26 °C and salinity was 40 ppt.

2.1.2.4. Sediment. Six coral fragments, two from three different colonies were maintained in the outdoor water table with flow through seawater. Water temperature fluctuated from 24 to 28 °C throughout the day and salinity was 40 ppt. Three of the fragments received 20 mg/cm² of sediment twice a day for two days. This rate of application and sediment load allowed corals enough time to clear most of the sediment before the next application and avoided mortality due to excessive sedimentation. The sediment used was commercial Matt Stone® brand leveling sand (ASTM C-33 standard gradation) and grain size was refined by filtering through a 150–250 µm screen.

2.1.2.5. Dark. Two coral fragments from different colonies were placed in a 20 l opaque Rubbermaid® plastic container and placed on the outdoor water table with flow-through seawater. Holes were drilled in each end of the container to allow water flow, which was maintained with a submersible pump. Coral fragments were exposed to dark conditions for two days to simulate shading due to suspended sediments.

2.1.3. Extraction of RNA

After exposure, colonies were removed from control and treatment tanks and immediately processed for RNA extraction. Most of the coral skeleton was removed with a hammer and chisel, leaving only the top few mm of living tissue. The tissue was ground in 60 ml of a phenol based solution (TRIzol®, Invitrogen™) with a mortar and pestle. Homogenization in TRIzol® stops cellular activity for long-term RNA storage and preservation. Total handling and processing time did not exceed 8 min. A single 2 ml aliquot from each homogenized coral fragment was isolated and total RNA was extracted following the manufacturer's protocol (based on Chomezynski and Sacchi, 1987). RNA concentrations were estimated by ultraviolet absorbance at 260 nm (Stratalinker 1800, Stratagene) and integrity

was confirmed by electrophoresis on a 1% formaldehyde agarose gel.

2.1.4. Target development

After purification, replicate aliquots of up to 2 μg of total RNA from each treatment were reverse transcribed using SuperScript™ II reverse transcriptase (Invitrogen™) and an oligo (dT) primer (Operon Biotechnologies, Inc.). During reverse transcription, DIG labeled dUTPs (digoxigenin-11-2'-deoxy-uridine-5'-triphosphate, alkali-labile; Roche Diagnostics) were incorporated into the transcribed cDNA for subsequent detection using chemiluminescent visualization. To enhance the amplification of longer transcripts in the cDNA pool, the manufacturer's protocol (Invitrogen™) was modified by adding a ramped temperature incubation period. The transcription conditions were 37 °C for 1 h, followed by 42 °C for 1 min with a one-degree temperature increase every minute until 50 °C for 1 min (Pastorian et al., 2000). The reaction was then incubated at 70 °C for 15 min to stop the reaction. The resulting cDNAs were quantified with a fluorometer following the manufacturer's protocol (DyNA Quant™ 200, Amersham Biosciences). For each treatment, an aliquot of cDNA was added to a high sodium-dodecyl-sulfate (SDS) buffer (Roche, Indianapolis, IN, USA) resulting in cDNA concentrations of 33 ng ml⁻¹ (salinity), 47 ng ml⁻¹ (temperature) and 50 ng ml⁻¹ (light). These DIG-labeled cDNA solutions were subsequently used as targets to assess changes in gene expression within treatments using the array.

2.2. Expression profiling of laboratory exposed corals: array development

An experimentally designed coral cDNA array was used to evaluate differential gene expression in temperature, salinity and light targets. ESTs on the array represent 32 different cDNAs isolated from corals *Acropora cervicornis* and *M. faveolata* exposed to various natural and anthropogenic treatments. These gene fragments were isolated using subtractive hybridization, differential display PCR, or reverse transcription PCR with designed primers (Snell et al., 2003), details of which are summarized below.

2.2.1. Probes from previous studies

Pesticide, PAH and heavy metal probes on the array were isolated by Differential Display PCR (ddPCR) in laboratory experiments using *A. cervicornis* (Morgan et al., 2001; Morgan and Snell, 2002). Corals were exposed for 4 h to treatments of copper (25 and 50 $\mu\text{g l}^{-1}$), mercury (5.0 and 50 $\mu\text{g l}^{-1}$), permethrin (1 and 10 $\mu\text{g l}^{-1}$), dibrom (0.5 and 5.0 $\mu\text{g l}^{-1}$), or naphthalene (50 and 300 $\mu\text{g l}^{-1}$). In addition, a metallothionein probe was also developed using the 50 $\mu\text{g l}^{-1}$ copper treatment

and reverse-transcription PCR (Snell et al., 2003). These probes range in size from 150 to ~500 bp. Each probe demonstrates various degrees of response specificity to a small suite of stressors. Most probes show no significant homologies to sequences in GenBank, with the exception of metallothionein isolated from 50 $\mu\text{g l}^{-1}$ copper (Snell et al., 2003), and ceruloplasmin, isolated from 50 $\mu\text{g l}^{-1}$ naphthalene (Morgan and Snell, in press) (Table 1).

2.2.2. Recently isolated probes

Probes on the array derived from exposure to temperature, salinity, darkness, or sediment were isolated from *M. faveolata* using suppressive subtractive hybridization PCR (SSH PCR) or reverse transcription PCR and designed primers (Snell et al., 2003), Table 1.

2.2.2.1. Suppressive subtractive hybridization PCR. Clontech's PCR-Select™ cDNA Subtraction Kit (Cat# K1804-1) was used to perform two different subtractive hybridizations on coral fragments from the sediment or dark treatment and the control. Total RNA was extracted with TRIzol® (Invitrogen) and mRNA was purified with Oligotex™ (Cat# 72022, Qiagen). Messenger RNA concentrations were estimated by ultraviolet absorbance and integrity was confirmed by gel electrophoresis. After the final round of PCR to enrich for differentially expressed sequences (subtracted product), cDNA libraries were constructed of sequences up-regulated in response to sedimentation and darkness exposures. The subtracted PCR products were shotgun cloned into a vector (pCR® II-TOPO, Invitrogen, Carlsbad, CA, USA), and recombinant plasmids were inserted into competent *E. coli* cells and stored at -80 °C in 15% glycerol solution.

Differential screening was performed following the manufacturer's protocol to eliminate false positives (PCR-Select™ Differential Screening Kit, Cat# K1808-1, Clontech). Random clones were picked from the subtracted cDNA libraries, a colony PCR was performed and amplified cDNA inserts were spotted onto nylon membranes following the Hybond-N+ protocol for dot blotting nucleic acids (Amersham Pharmacia Biotech, Inc.). DIG labeled dUTPs (digoxigenin-11-2'-deoxy-uridine-5'-triphosphate, alkali-labile; Roche Applied Science) were incorporated into subtracted and unsubtracted cDNAs during a PCR using primers provided in Clontech's SSH PCR kit. Conditions consisted of 20 cycles at 94 °C for 10 s, 68 °C for 30 s and 72 °C for 1.5 min, ending with a 5 min extension step at 75 °C. Nylon arrays consisting of the subtracted ESTs were hybridized to DIG-labeled subtracted and unsubtracted cDNA targets following the protocol described in Morgan et al. (2001), Morgan and Snell (2002). Membranes were wrapped in clear plastic and exposed to CL-Xposure* Film (Pierce Biotechnology, Inc.) for 16 h.

Table 1

A list of all 32 ESTs represented on the coral cDNA array including the environmental conditions associated with each gene and putative identification as determined by BLAST results

Clone	Primer sequence	E-value	Exposure conditions	Putative ID	Accession # of homolog
1 S(B) H5	No primer	BlastN: 1e-171	Sediment	Ribosomal RNA	AY026375.1
2 S(C) A6	No primer	BlastN: 1e-134	Sediment	Ribosomal RNA	AY026365.1
3 S(C) C9	No primer	BlastX: 2e-19	Sediment	Thioredoxin	CAA76654.1
4 S(C) E7	No primer	TblastX: 5e-59	Sediment	Ribosomal RNA	AY026375.1
5 S(C) E9	No primer	BlastX: 7e-21	Sediment	Trap-D	S59869
6 S(D) B11	No primer	TBlastX: 1e-65	Sediment	Ribosomal	AY026375.1
7 Dk(C) F1	No primer	BlastN: 7e-34	Darkness	Ferritin	CAC84555.1
8 Dk(C) G10	No primer	NS	Darkness	Uncharacterized	NS
9 Dk(C) G11	No primer	RPS-Blast: 7.6	Darkness	uPAR	CDD 14821
10 T1	(F) catggatgtgtcgcagttc (R) ggagcaatgaatcctccagt	BlastX: 1e-22	Thermal: 28 °C	Met-aminopeptidase	AAM61284.1
11 T2	(F) gctgccagaaattacaaggat (R) ggtcaaatgggtttccctct	NS	Thermal: 28 °C	Uncharacterized	NS
12 T3	(F) gatgctgtcgtgttacaatg (R) tcatgcctccacagtttc	NS	Thermal: 28 °C	Uncharacterized	NS
13 T4	(F) atgaaagaggtagccgaagc (R) acgagaaccacgtcatgga	NS	Thermal: 28 °C	Uncharacterized	NS
14 T5	(F) agaggaataataatcaagctgt (R) acctttccactttcttg	BlastX: 5e-09	Thermal: 28 °C	NALP	AAO18165.1
15 T6	(F) aagatgaacaggtcacagt (R) tctggatatggaactcct	NS	Thermal: 28 °C	Uncharacterized	NS
16 T7	(F) aagatgaacaggtcacagt (R) tctggatatggaactcct	NS	Thermal: 28 °C	Uncharacterized	NS
17 C30	No primer	NS	50 µg l ⁻¹ Copper	Uncharacterized	BI534458 [†]
18 P22	No primer	NS	1 µg l ⁻¹ Permethrin	Uncharacterized	NS
19 Dbs	No primer	NS	0.5 µg l ⁻¹ Dibrom	Uncharacterized	BI534457 [†]
20 Dbl	No primer	NS	0.5 µg l ⁻¹ Dibrom	Uncharacterized	BI534456 [†]
21 H30	No primer	NS	5 µg l ⁻¹ Mercury	Uncharacterized	BI534459 [†]
22 N40	No primer	TblastX 4e-29	50 µg l ⁻¹ PAH	Ceruloplasmin	DN167139
23 Mt 1	(F) agcccttgaattgcattga (R) cgaacaactggagtcacattta	BlastX: 0.87	50 µg l ⁻¹ Copper	Metallothionein	DR681654
24 CAN con 60	(F) gatggctgcaccaaattggg (R) aaagaagccctgacgttgctg	NS	Thermal: 25 °C	Uncharacterized	NS
25 CX sal 19A	(F) acatgcatggcaatgaag (R) caactttcacaagatgattgt	BlastX: 1e-4	Salinity: 43 ppt	Ankyrin	gi 28373837 pdb 1N0R A
26 PU sal 17B	(F) aagacactcaccggcaag (R) accttcttatcttggatctt	TBlastX: .073	Salinity: 43 ppt	Poly Ubiquitin	TVU27577
27 DNAG T28 19A	(F) agtgtctttacttagcagga (R) tcaccaaatgaatgtgt	NS	Thermal: 28 °C	Uncharacterized	NS
28 HX sal 17C	(F) acctggacgaaaggattca (R) ttgatgctctcacgatccac	NS	Salinity: 43 ppt	Uncharacterized	NS
29 GST con 10A	(F) atctccagatattggcc (R) cgtagtaaaaagcgttgaaa	BlastX: 1e-07	Thermal: 25 °C	Bombesin-like peptides	NM_015548.2
30 CAN T28	(F) gatggctgcaccaaattggg (R) aaagaagccctgacgttgctg	BlastX: 8e-04	Thermal: 28 °C	Carbonic anhydrase	AAD32675.1
31 CX T28 19A	(F) acatgcatggcaatgaag (R) caactttcacaagatgattgt	BlastX: 2e-29	Thermal: 28 °C	Phosvitin	AAA98791.1
32 PU cope 17A	(F) aagacactcaccggcaag (R) accttcttatcttggatctt	BlastX: 3e-13	Thermal: 25 °C	Poly Ubiquitin	BAA09860.1

Accession numbers represent sequences within GenBank that showed significant similarities to the ESTs on the array. ESTs on the array with their own accession numbers are identified by the symbol †. NS indicates those ESTs with no significant homology to sequences in GenBank. For ESTs generated using RT-PCR and designed primers, primer sequences are shown in the 5' to 3' direction. F = forward primer, R = reverse primers.

Dark spots on the film, corresponding to specific transcripts in the target cDNA pool, provided visual identification of expressed probes. A difference in the level of expression of a transcript between subtracted and unsubtracted samples was quantified using the program

ImageJ (National Institute of Health). The background around each spot was measured repeatedly and the average measurement calculated from the spot intensity. An EST was considered differentially expressed if it was at least two-fold darker in the subtracted cDNA pool.

2.2.2.2. Reverse transcription PCR. A bioinformatics approach was used to isolate several other genes incorporated on the final array. Consensus sequences of well-characterized stress-induced genes from several animals were identified in GenBank (National Center for Biotechnology Information) and aligned using the program ClustalW (European Bioinformatics Institute). Primers of 20–25 base pairs were designed from regions of high similarity with the program Jellyfish v3.0 (Lab-Velocity). Total RNA was isolated from *M. faveolata* fragments exposed to previously described treatments and quality was checked on a 1% formaldehyde gel. A RT-PCR kit (Titan One Tube RT-PCR Kit, Roche Applied Science) and a pair of designed primers were used to reverse transcribe RNA and PCR amplify the resulting cDNA in a single step. The conditions consisted of an incubation period at 45 °C for 1 h followed by 2 min at 92 °C. Cycling parameters included 35 cycles of 30 s at 92 °C, 45 s at the appropriate annealing temperature for the primer pair and 68 °C for 1 min. Amplified cDNA was separated and visualized on a 2% agarose gel.

Bands corresponding to the correct sequence size were purified using a QIAquick™ gel extraction kit (Qiagen). The extracted product was re-amplified to check for the possibility of comigrating bands. PCR parameters consisted of incubation at 94 °C for 2 min, followed by 25 cycles of 20 s at 94 °C, 45 s at the appropriate annealing temperature and 68 °C for 1 min. A 7 min period at 68 °C finalized the reaction and completed the sequence extension. Agarose gel electrophoresis confirmed that a single band was amplified and the PCR product was cloned into a vector (pCR® II-TOPO, Invitrogen). The recombinant plasmids were inserted into competent *E. coli* cells and stored in a glycerol solution.

Probes isolated with SSH PCR and RT-PCR were sequenced at the University of Georgia Molecular Genetics Facility (Athens, Georgia, USA) and characterized based on sequence similarity to known genes. Searches were carried out using a basis local alignment tool (BLAST), available on-line at NCBI (www.ncbi.nlm.nih.gov). Table 1 provides information on the isolated ESTs and indicates putative IDs for those with significant sequence similarity to a known gene.

2.2.3. Membrane and probe preparation

Plasmids containing the 32 gene specific probe inserts were isolated from *E. coli* stocks using a commercial kit (QIAprep Spin Miniprep Kit, Cat# 27104, Qiagen). Inserts were PCR amplified using plasmid specific primers, M13 forward and M13 reverse. The conditions consisted of a denaturing step at 94 °C for 45 s followed by 25 cycles of 95 °C for 15 s and 50 °C for 3 min. The amplified inserts were visualized on a 2% agarose gel and quantified by visual comparison with a DNA size/mass ladder.

The probes were prepared in 0.2M NaOH and incubated at 37 °C for 15 min. Denatured cDNA probes were blotted in triplicate onto BioBond™ Nylon membranes (Sigma-Aldrich, USA) in 2 µl volumes at concentrations ranging from 0.25 to 0.5 µg µl⁻¹. Membranes were neutralized in 0.5 M Tris-HCl and cDNA was fixed to the membrane by UV cross-linking.

Three separate PCRs were performed for each replicate probe on a membrane, and all membranes were blotted on the same day. Thus, replicate spots across membranes came from the same PCR pool and were identical, while replicate spots within a membrane came from different PCR pools and could vary in concentration. The average of replicate spots on a membrane, representing a single probe, was compared between membranes, but replicate spots were not compared within a membrane. This method may create variability in the degree of expression between replicate spots on a membrane, but it controls for false results produced by anomalous polymerase chain reactions.

2.3. Expression profiling of laboratory exposed corals: cDNA hybridization

Resulting arrays were hybridized with DIG-labeled targets to visualize which probes were expressed in the total RNA from each treatment. Target cDNA was isolated from coral exposed to temperature, salinity or ultraviolet light (see Section 2.1). The hybridization protocol is described in Morgan et al., 2001 and Morgan and Snell, 2002. After overnight exposure of the membranes to X-ray film, dark spots appeared that corresponded to expressed transcripts in the target cDNA pool. A difference in the level of expression of a transcript between control and exposed samples was compared. Hybridizations were performed at least two times using labeled cDNA from different colony fragments in each exposure. Membranes were used only once to ensure a consistent correlation between spot intensity and transcript concentration.

2.3.1. Array analysis

Densitometry of blots on the array was performed using ImageJ software (NIH). 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 gene was determined by average signal intensities of the three replicate spots of each gene on each membrane.

In order to compare signal intensities of multiple spots on different membranes, all data were log transformed. This manipulation is considered a valid approach for data analysis 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). Lavene's test for Equality of Variance was applied to determine whether compared populations exhibited similar variances. For multiple comparisons where variances were similar, the Student–Neuman–Keuls posthoc test was performed to determine which populations were significantly different. Since ANOVA is generally insensitive to heteroscedasticity, Tamhane's T_2 posthoc test was applied (Tamhane, 1979) to multiple comparisons where equal variances were not assumed. In experiments with only one stressed condition (UV and PAR), a Student T -test was performed to compare expression levels between control and exposed populations.

2.4. Expression profiling of laboratory exposed corals: Northern blot confirmation

To confirm gene expression results detected on the array, Northern dot blots were performed for the uPAR gene. Total RNA was isolated from coral fragments exposed to temperature and salinity treatments. RNA concentrations were estimated by ultraviolet absorbance and integrity was visually confirmed by agarose gel electrophoresis. One microgram of total RNA from each treatment (see Section 2.1) was blotted onto positively charged nylon membranes (Hybond-N+, Amersham Pharmacia Biotech Inc.) and cross-linked by exposure to UV light. Plasmids containing the uPAR inserts were isolated from *E. coli* stocks (Qiagen QIAprep kit) and amplified using M13 primers. PCR conditions were 94 °C for 1 min followed by 20 cycles of 94 °C for 15 s, 52 °C for 15 s and 72 °C for 30 s ending with a 72 °C extension step for 5 min. Amplified cDNA was visualized on a 2% agarose gel. The band corresponding to uPAR was excised and purified from the gel (Qiagen QIAquick™ Gel Extraction kit) and the purified cDNA was DIG labeled using conditions described above for amplification of the plasmid insert (Roche Diagnostics). The amplified uPAR gene was quantified by ultraviolet absorbance and the incorporation of DIG labeled bases was confirmed by gel electrophoresis. Hybridization of the uPAR probe to the RNA dot blot array was performed and the results visualized on X-ray film. Differential gene expression was determined by densitometry measurements of the dot blot signals. Statistical tests (previously identified) were performed to compare differences between uPAR expression in treatment and control RNA.

3. Results

3.1. cDNA array composition

The coral cDNA array, as used in this experiment, includes thirty-two ESTs representing ribosomal genes and genes expressed in response to xenobiotic and natural stressor exposures. The ESTs range from approximately 150 to 600 bases in length and were isolated from *Acropora cervicornis* or *M. faveolata*. Location on the membrane of each EST probe was determined randomly. However, to prevent spatial biases, adjacent spotting of replicate probes was avoided (Machl et al., 2002).

3.2. Putative identification of ESTs

Various BLAST programs were used to search genomic and proteomic databases for significant sequence similarities. Forty-four percent of the coral ESTs showed significant similarities (E -values $<10^{-4}$) to database sequences and putative identifications were assigned (Table 1). Another thirteen percent of the ESTs had E -values $>10^{-4}$ and putative IDs were assigned only after it had been determined that conserved amino acids were identified in positions characteristic of a particular protein. The remaining 33% of ESTs showed no significant similarities to database sequences searched in June 2004. All of the genes represented on the array were isolated from corals that had been exposed to some type of stressor; therefore it is not surprising that coral genes with significant E -values from BLAST would be similar to stress-induced genes in other organisms.

3.3. Gene expression profiles in controlled conditions

Using the array, gene expression changes were compared for *M. faveolata* exposed to different levels of temperature, salinity, or UV stress. To estimate within treatment variance, all probes were spotted in triplicate on a membrane and at least two membranes were used for each exposure. Genes with significantly different average intensities ($P < 0.05$) between treatments were considered differentially expressed. Of the 32 genes screened, six exhibited significant differential expression. Of these, three ribosomal genes were consistently expressed but showed slight variation in response to treatments. The remaining three genes (carbonic anhydrase, thioredoxin and a urokinase plasminogen activator receptor, uPAR) varied to a much greater extent in response to treatments. Descriptions of these genes can be found in Table 1.

3.4. Baseline expression of control genes

The expression level of control genes, three 28S large subunit ribosomal fragments, was averaged (per treatment) and compared across seven different membrane

hybridizations. Baseline conditions of 24 °C water temperature, 38 ppt salinity, and PAR (20 W daylight bulb) were used to make comparisons to laboratory induced stressors. Statistical analysis (Univariate ANOVA, SPSS Inc.) of log transformed control gene expression data for these baseline conditions revealed unequal variances for the control genes expressed on each membrane ($P < 0.05$, Lavenne's test equality of error variances). Since equal variances were not assumed, Tamhane's T_2 posthoc test was applied and revealed there were no significant differences ($P > 0.05$) in individual membranes or in the expression of individual control genes for all baseline conditions.

3.5. Expression of control genes under stressed conditions

Expression levels of control genes differed significantly under various stress conditions. Corals exposed to 46 ppt salinity showed elevated expression of control genes (Figs. 2 and 4, $P < 0.05$, Tamhane's T_2 test) compared to 38 and 43 ppt salinity exposures. Corals exposed to UV stress also demonstrated significant up-regulation of these genes (Figs. 3 and 4, $P < 0.001$, Student's T -test). By comparison, these same control genes exhibited a different trend for corals experiencing thermal stress where their expression decreased significantly with increasing temperature (Figs. 1 and 4, $P < 0.05$, Tamhane's T_2 test).

3.6. Expression of stress genes under stressed conditions

The array detected different expression profiles for corals exposed to elevated salinities, temperature shock

treatments, or UVB. Three genes (thioredoxin, carbonic anhydrase, and uPAR) each demonstrated distinctively different patterns of expression.

3.6.1. Temperature shock

Carbonic anhydrase showed significant decreases in expression at each temperature ($P < 0.05$, Tamhane's T_2 test). Expression of uPAR showed significant up-regulation at 28 °C as well as significant down-regulation at 31 °C ($P < 0.05$, Student–Neuman–Keuls). Thioredoxin was not up-regulated under these laboratory controlled conditions (Figs. 1 and 4).

3.6.2. Elevated salinities

Thioredoxin (TRX) and carbonic anhydrase (CAN) were both significantly elevated at salinities 43 and 46 ppt ($P < 0.05$, Student–Neuman–Keuls). Expression of uPAR was significantly elevated only at 46 ppt ($P < 0.05$, Student–Neuman–Keuls) (Figs. 2 and 4).

3.6.3. UV exposure

Carbonic anhydrase showed significant up-regulation in expression after exposure to UV ($P < 0.001$, Student T -test). In a similar pattern, uPAR also showed significant up-regulation ($P < 0.05$, Student T -test). Thioredoxin was not up-regulated under these laboratory controlled conditions (Figs. 3 and 4).

3.7. Northern dot blots

A Northern dot blot was performed to verify results obtained using the cDNA array. The uPAR gene was up-regulated in the 28 and 31 °C temperature treatments

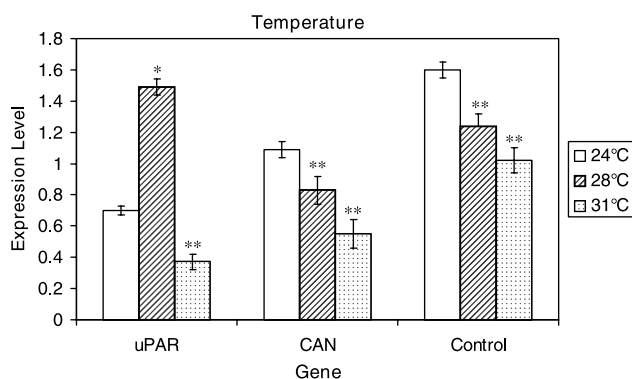


Fig. 1. Mean expression of genes exhibiting differential responses to temperatures of 24 °C (ambient), 28 °C or 31 °C. Genes are uPAR—urokinase plasminogen activator receptor, CAN—carbonic anhydrase, Control—average of three ribosomal genes. Expression level is the mean signal intensity of log transformed data for each gene in a treatment. The expression level of a gene was compared between treatments but not between genes. Bars are equivalent to standard error. A single asterisk indicates significant up-regulation ($P < 0.05$) and double asterisks indicate significant down-regulation ($P < 0.05$) compared to ambient conditions.

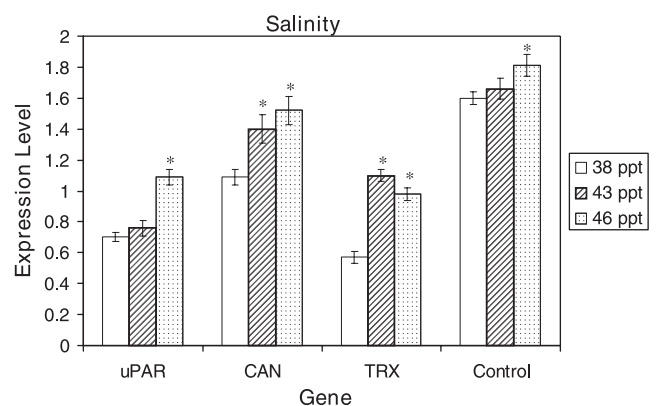


Fig. 2. Mean expression of genes exhibiting differential responses to salinities of 38 ppt (ambient), 43 ppt or 46 ppt. Genes are uPAR—urokinase plasminogen activator receptor, CAN—carbonic anhydrase, TRX—thioredoxin, Control—average of three ribosomal genes. Expression level is the mean signal intensity of log transformed data for each gene in a treatment. The expression level of a gene was compared between treatments but not between genes. Bars are equivalent to standard error. An asterisk indicates significant up-regulation ($P < 0.05$) compared to ambient conditions.

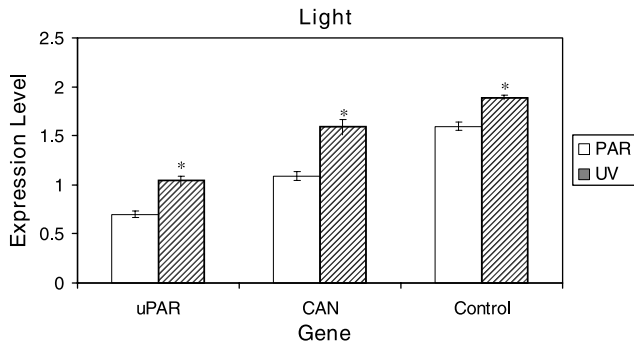


Fig. 3. Mean expression of genes exhibiting differential responses to modified light intensities, PAR (ambient) or UVR. Genes are uPAR—urokinase plasminogen activator receptor, CAN—carbonic anhydrase, Control—average of three ribosomal genes. Expression level is the mean signal intensity of log transformed data for each gene in a treatment. The expression level of a gene was compared between treatments but not between genes. Bars are equivalent to standard error. An asterisk indicates significant up-regulation ($P < 0.05$) compared to ambient conditions.

compared to the 24 °C control treatment ($P < 0.05$, Student–Neuman–Keuls). At the elevated salinities of 43 and 46 ppt, uPAR expression levels were significantly up-regulated compared to the 38 ppt control treatment ($P < 0.05$, Student–Neuman–Keuls), Fig. 5.

4. Discussion

Organisms react to environmental change by altering the expression of suites of genes within a repertoire of thousands of genes in the genome. Changes in gene expression can be general, as in the induction of chaperones in response to a variety of stressors (Hofmann et al., 2002), or specific such as a gene that is only expressed upon exposure to an organophosphate insecticide (Morgan and Snell, 2002). The expression pattern derived from a combination of general and specific genes can be interpreted to indicate the effect of stress on cellular functions (Gasch et al., 2000; Williams et al., 2003a,b).

4.1. Differential expression

Each treatment (temperature, salinity, UV) evaluated in this study resulted in a unique gene expression pattern due to the up- or down-regulation of a subset of genes on the coral array. Thioredoxin (TRX), which was isolated from coral exposure to sediment loading (Section 2.1 above; Table 1) was expressed during salinity stress, but not temperature or UV stress, indicating some degree of specificity in the response. Studies using yeast show that TRX is superinduced during hyperosmotic shock (Gasch et al., 2000; Posas et al., 2000). In addition, TRX acts as a singlet oxygen quencher and hydro-

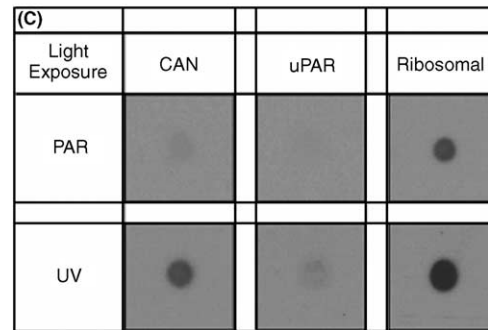
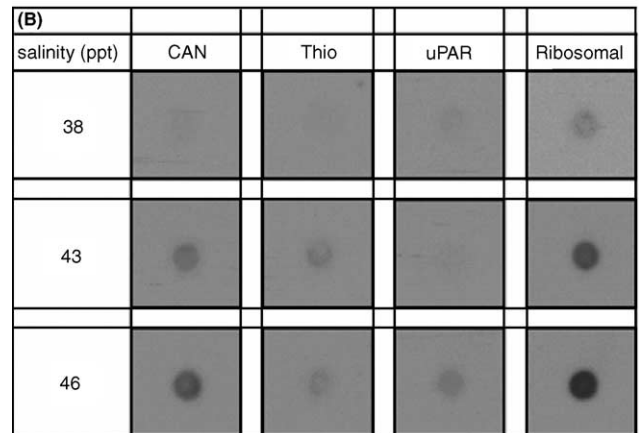
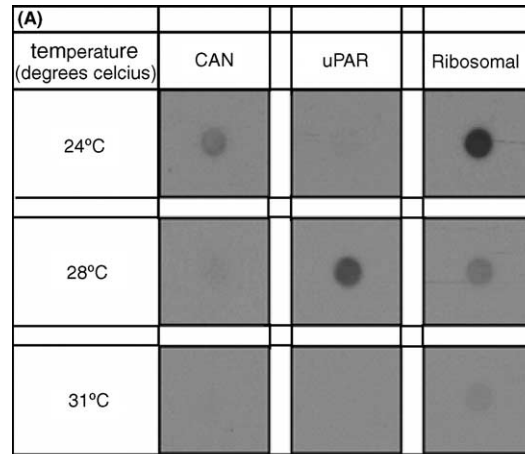


Fig. 4. Varying gene expression in response to different treatments displayed as blot intensity and visualized using chemiluminescent detection. A representative ribosomal gene is shown to represent the average expression of all three expressed ribosomal genes. (A) Temperature. CAN is down-regulated at 28 and 31 °C, uPAR is up-regulated at 28 °C and down-regulated at 31 °C, ribosomal genes exhibit down-regulation with elevated temperatures. (B) Salinity. CAN, thioredoxin and the ribosomal genes are up-regulated in response to elevated salinity; uPAR is up regulated at the highest salinity of 46 ppt. (C) Light. CAN and uPAR are up-regulated in response to UV; ribosomal genes are also up regulated but to a lesser degree.

xyl radical scavenger maintaining redox balance within eukaryotic cells (Das and Das, 2000). The absence of expression by TRX in other treatments in this study could be due to insufficient exposure time or dose.

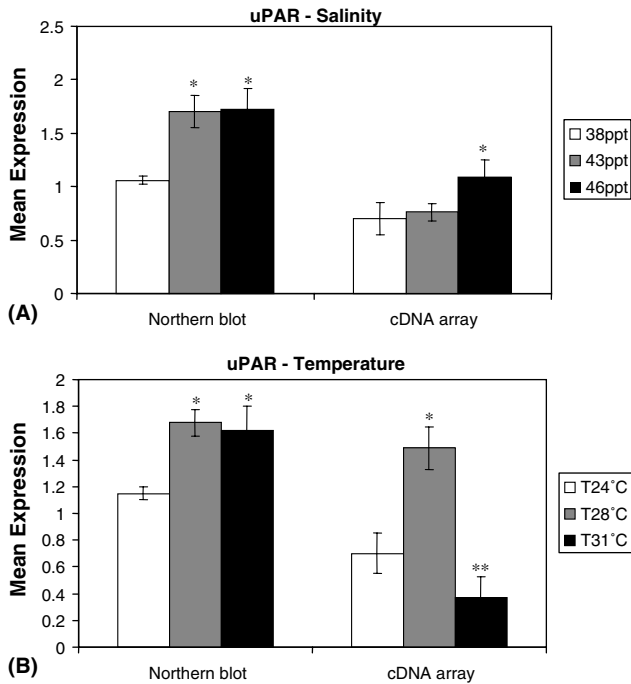


Fig. 5. Results from Northern dot blot compared to cDNA array analysis. (A) Salinity results for uPAR at 38, 43, and 46 ppt. (B) Temperature results for uPAR at 24, 28, and 31 °C. Bars indicate standard error. Mean expression is the average signal intensity of log transformed data for uPAR. The expression level was compared between treatments but not between analyses. A single asterisk indicates significant up-regulation ($P < 0.05$) compared to ambient conditions (38 ppt or $T = 24$ °C), double asterisks indicate significant down-regulation ($P < 0.05$) compared to ambient conditions.

A general stress response was demonstrated by elevated expression of the uPAR gene in all treatments. The highest temperature exposure was an exception where all genes, including ribosomal controls, were significantly down-regulated. Pepper et al. (1993) demonstrated that uPAR, which is involved in proteolysis, wound healing and inflammation, was significantly up-regulated in mechanically wounded endothelial cells.

Carbonic anhydrase (CAN) demonstrated variable expression; this increased upon exposure to elevated salinity and UV, but decreased when exposed to thermal stress. CAN plays an important role in symbiotic Cnidarian respiration (Weis and Reynolds, 1999) and photosynthesis (Weis, 1993; Furla et al., 2000; Estes et al., 2003), as well as acid–base balance, ion regulation, and osmoregulation in marine organisms (Wheatly and Henry, 1992; Whiteley et al., 2001; Wilson et al., 2002). Gilbert and Guzman (2001) demonstrated that activity levels of carbonic anhydrase decreased in anemones exposed to metal contamination. In contrast, carbonic anhydrase activity correlates positively with densities of zooxanthellae and light intensity (Weis, 1991; Weis and Reynolds, 1999).

4.2. Temperature

The effect of temperature on corals is widely studied and has been shown to impact photosynthetic efficiency (Warner et al., 1996; Jones et al., 1998), reduce respiration (Nystrom et al., 2001), induce oxidative stress (Lesser, 1997), impair CO₂ fixation in symbionts (Jones et al., 1998) and alter the configuration of membrane lipids (Tchernov et al., 2004). Additionally, temperature has long been ascribed as one of the major causes of coral bleaching (Gates et al., 1992; Brown, 1997; Hoegh-Guldberg, 1999). In this study, acute exposure of *M. faveolata* to elevated temperatures caused a significant decrease in carbonic anhydrase mRNA, while the uPAR-like gene increased expression at mildly elevated temperatures but returned to background levels at the highest temperature. Down-regulation of gene expression may be related to the observed decreased in ribosomal function at the highest temperature. The repression of ribosomal protein genes and genes involved in a variety of other cellular functions has been observed during the stress response in yeast (Gasch et al., 2000). Well-characterized thermal genes were not observed during this experiment. This could be due to a maximum temperature exposure of only 31 °C. Most studies using protein biomarkers have not reported the expression of heat shock elements at temperatures lower than 33 °C (Black et al., 1995; Sharp et al., 1997).

4.3. Salinity

Elevated salinity affects the ionic regulation (Dietz et al., 1997), acid–base balance (Whiteley et al., 2001) and natural osmoregulation of an organism (Whiteley et al., 2001; Wilson et al., 2002). In yeast, hyperosmotic shock causes cell wall and cytoskeleton reorganization (Slaninová et al., 2000). At the molecular level, yeast cells increase expression of genes in the high-osmolarity glycerol (HOG) pathway (Hohmann, 2002), and super-induce other environmental stress response genes, including oxidoreductases, cytosolic catalase, and Cu, Zn-superoxide dismutase (Gasch et al., 2000; Garay-Arroyo et al., 2003). In addition, the induction of molecular chaperones during osmotic stress has been demonstrated in several organisms (Smith et al., 1999; Spees et al., 2002). Coral exposed to varied salinity concentrations reveal alterations in respiration, photosynthesis, and total protein content (Moberg et al., 1997; Ferrier-Pages et al., 1999). Ferrier-Pages et al. (1999) demonstrated that colonies of *S. pistillata* maintained at an elevated salinity of 40 practical salinity units (psu) died during a 3-week exposure. To date, no studies have used specific biomarkers to investigate the effects of changes in salinity on Cnidaria. All genes observed in this study, including CAN, uPAR, TRX and ribosomal

controls, exhibited up-regulation in response to acute hypersaline conditions.

4.4. Ultraviolet radiation

Exposure to ultraviolet radiation (UVR) leads to protein damage, tissue inflammation, DNA damage and cell death either directly, or by generating reactive oxygen species (Miralles et al., 1998; Lesser et al., 2001). The formation of oxygen radicals disrupts protein synthesis and damages cell membranes, resulting in decreased photosynthetic rates (Lesser, 1996; Shick et al., 1996). Organisms respond by up-regulating suites of genes that code for transcription factors, growth factors and proteases, which have been characterized in mammals as the UV response (Devary et al., 1992; Miralles et al., 1998). The up-regulation of genes that initiate DNA repair or apoptosis has been reported in fish exposed to UVR (Lesser et al., 2001), while human epithelial cells induce the expression of uPAR mRNA (Marschall et al., 1999). Marine organisms defend against solar radiation by producing natural UV-absorbing sunscreens, identified as mycosporine-like amino acids (MAAs) (Shick and Dunlap, 2002). In addition, the accumulation of antioxidants, such as carotenoids, which quench oxygen radicals, offers protection from the harmful effects of ultraviolet radiation (Mobley and Gleason, 2003). In our study, acute exposure of *M. faveolata* to UVR, significantly increased transcription of CAN and uPAR genes.

4.5. Verification

Results from the Northern blot verification were consistent with the cDNA array data in that a general trend was apparent. In both analyses, uPAR was up-regulated exclusively in treatment conditions as compared to ambient conditions (Fig. 5). This was consistent for both salinity and temperature exposures. Discrepancies between the results can be attributed to differences in sensitivity of the techniques. It has been documented that Northern blot analysis is more sensitive than cDNA array analysis (Taniguchi et al., 2001; Chuaqui et al., 2002; Dieck et al., 2003). However, both techniques produce a degree of experimental variability. The cDNA array results were not completely identical with that of the Northern blot results, but there were clear parallels between the two analyses. This demonstrates that cDNA arrays provide quantitative data, but underscores the importance of validating results with a more sensitive method.

4.6. Putative identification of ESTs

Database search results of the sixteen cDNAs amplified by RT-PCR using designed primers (2.2. above)

produced no significant sequence similarities for eight of the cDNAs (unknown), five sequences showed similarity to non-target genes, and three sequences showed similarity to the genes of interest (Table 1). Several factors play a role in the amplification of non-target sequences. Primers must be very specific for the desired template to be amplified. Cross reactivity with non-target DNA sequences results in non-specific amplification of DNA. In addition, excess DNA polymerase and low annealing temperature can result in mispriming. The PCR primers designed for this study were specific for the targeted genes based on similarities between sequences from well-characterized organisms in GenBank (NCBI). The relatively low specificity of some of the primers may be due to the limited diversity of Cnidarian sequences in the databases at the time the primers were designed. However, the amplification of non-target sequences is not necessarily a negative result, since novel and interesting genes were identified (Harris et al., 2004).

Each EST on the coral array may have multiple roles. Based on annotations from homologs, classifications emerge that group some of the coral genes into multiple functional categories. One subset of genes on the array could be classified as representative of oxidative stress (TRX, ceruloplasmin (Cp), ferritin, Mt). The translocon associated protein- γ (TRAP-D), although not considered an antioxidant, has recently been shown to form cytotoxic aggregates with mutant forms of superoxide dismutase (Miyazaki et al., 2004), which is a powerful antioxidant. Another functional subset of genes may be associated with inflammation (Cp, NALP, uPAR). Mullen et al. (2004) points out that inflammation is not well-studied in stony corals, but amoebocytes are believed to be involved. However, an inflammatory response has been qualitatively and quantitatively characterized in at least one gorgonian (Mezzaros and Bigger, 1999). Additionally, histological examinations of coral tissue show evidence of tissue remodeling under stressed conditions (Mullen et al., 2004), which may be related to inflammation. A third subset of genes are induced upon exposure to UV light (TRAP-D, uPAR, TRX, Mt, Poly-U). While not grouped with other genes, the Bombesin-like peptide receptor is worth noting since its function appears to be involved with modulating stress in order to maintain homeostasis (Moody and Merali, 2004).

4.7. Strengths and limitations of this technology

Like any technology, cDNA array analysis has certain limitations. Manipulation during hybridization and image processing can add variability to the results. Such sources of fluctuation include the efficacy of reverse transcription, efficiency of target labeling, non-uniform PCR amplification of probes, accuracy of spotting, spot morphology and production of background on the

array (Schuchhardt et al., 2000; Cook and Sayler, 2003). Biological variability can also affect results. Genetic heterogeneity, previous exposure to stress, and differences in symbiont communities may mask changes associated with the stressor in question. Replication and validation increase the reproducibility of an experiment. Taking replicate samples from the same colony and multiple coral colonies estimates biological variability, while incorporating multiple spots of the same gene on duplicate arrays permits the statistical removal of experimental variability. Most variability is eliminated by proper experimental design and robust statistical analyses, such as log transformation and ANOVA (Kerr et al., 2000; Nadon and Shoemaker, 2002).

The validity of a hybridization experiment depends on the sensitivity, specificity and reproducibility of results. Sensitivity refers to the ability to detect a single probe out of a population of target cDNAs and to reliably determine a difference in expression between samples. It is directly dependent on the amount of target used in the hybridization mix (Bertucci et al., 1999), and is positively correlated with the length of a probe (Zhou and Thompson, 2002). Several studies have demonstrated that cDNA array technology provides good specificity and reproducibility (Sчена et al., 1995; De-Risi et al., 1997; Brutsche et al., 2001) even between different array systems (Bertucci et al., 1999). For example, Larkin et al. (2003) showed that gene arrays are sensitive enough to detect changes in gene expression of a contaminant at environmentally relevant concentrations. Sensitivity can be maximized by starting with quality RNA, optimizing RT-PCR, improving target labeling and optimizing probe length.

Specificity refers to the proportion of probes that correctly hybridize to target sequences and is dependent upon hybridization stringency, sequence identity, and probe length (Zhou and Thompson, 2002). High stringency conditions reduce cross-hybridization events significantly. Miller et al. (2002) examined cross-hybridization between five closely related genes. Hybridization stringency was high (0.6XSSC at 68 °C) and probes consisted of full length clones. Cross-hybridization between genes from different families was not observed unless sequence identity exceeded 94%. A similar study using four of the same gene families and less stringent conditions (0.1XSSC at 45 °C) observed cross-hybridization at 80% sequence identity (Evertsz et al., 2001). Thus, conditions can be adjusted to achieve a broad range of detections. The hybridization conditions in our study were very stringent (0.2XSSC at 68 °C), so cross-hybridization to non-target genes is probably inconsequential.

Probes on the coral stress gene array were isolated from different coral species (*M. faveolata* and *A. cervicornis*). It is important to know whether the hybridization system being employed has the ability to identify altered gene expression across species. Several studies

have shown cross-reactivity between species in microarray experiments. For example, human cDNA microarrays have been used to investigate cross-species hybridizations in pig (Moody et al., 2002), monkey (Chismar et al., 2002) and bovine (Adjaye et al., 2004) RNA with the majority of genes generating highly reproducible data. Cross-hybridization is expected between species with low genetic diversity. For example, there is approximately 5% sequence difference between the genomes of humans and rhesus monkeys (Chismar et al., 2002). Research has shown low genetic diversity between Anthozoans (van Oppen et al., 1999; Shearer et al., 2002) suggesting that cross-hybridization between coral species is feasible. In addition, a study by Morgan et al. (2005) has shown differential expression in the brain coral *Diploria strigosa* using the coral stress array. In our experiment, 6 of the 32 genes on the array exhibited reproducible expression. The lack of expression by other genes could be due to the absence of the probe in the target cDNA pool, low sequence identity, or high stringency conditions. Finally, even though we have focused on the response of the coral holobiont (i.e. the coral host and algal symbionts in total), detection of symbiont genes utilizing this array was most likely minimal. BLAST results of probe sequences did not reveal significant similarities to any known plant genes. Cross-reactivity between Cnidarian and zooxanthellate genes and between genes of different coral species should be investigated further.

In spite of the limitations outlined above, cDNA array technology is a powerful tool for investigating complex gene expression relationships. The biological function of a gene determines when and where it is expressed, and deviations from homeostasis cause changes in the level of transcription of many genes. Therefore, detailed information regarding the state of an organism can be gleaned from patterns produced by the expression of multiple genes. cDNA arrays provide a practical way of examining the expression of multiple genes in a single experiment. The application of this technology to coral research can facilitate the rapid screening of coral health in the field and identify molecular mechanisms responsible for an observed physiological response. The isolated and arrayed sequences in this study are potential genetic biomarkers of stress, which reveal specific gene expression patterns in response to different conditions. Utilizing even a small suite of biomarkers can direct attention appropriately and fuel future projects that investigate coral stress using integrated technologies.

4.8. Future studies

The cDNA array used in this study represents the first version of a coral stress microarray developed by our lab. Future versions of the array will be robotically

spotted on glass slides and include a minimum of ten replicates per gene. In addition, our lab has currently isolated and characterized approximately 15 new sequences for incorporation onto the array. More sensitive protocols will complement this technology, such as robust quantification of PCR probes and digital detection of gene expression. A series of positive and negative controls will be spotted on the array and spiked controls will be added to the labeled target cDNA. Finally, the standards set by the minimum information about a microarray experiment (MIAME) will be incorporated into all future experimental designs (Brazma et al., 2001).

Future studies investigating coral gene expression should address the relationship between exposure time and dose response. Gene transcription in response to a stressor may be transient or sustained depending on the type of stressor and level of exposure. Experiments will be carried out to compare gene expression patterns between acute and chronic exposures of the same stressor. In addition, field experiments will be performed to determine if responses in the laboratory correlate with those in the field. Tests comparing species specificity and cross-reactivity with symbiont genes will be conducted and incorporated into experiments addressing responses by the holobiont. Finally, using integrated approaches, questions of whether gene expression detected by the cDNA array translates into protein production can be addressed. This technology is based on changes in gene transcription. It does not consider the effect of stress on translation, post-translational modification, protein localization, or protein degradation. However, gene expression changes that are not associated with altered protein production are still interesting and relevant, especially if these changes represent reliable, sensitive, and selective markers of a response to specific conditions (Pennie et al., 2000).

The toxicogenomic approach we have described provides great potential for diagnosing sublethal coral stress in the field associated with exposure to stressors within multi-stressor environments. One issue to consider is how gene expression patterns for corals collected from these environments are interpreted. The accurate interpretation of gene expression may only be possible when experiments are conducted as part of an integrated approach to understand observed responses at the physiological or biochemical levels. There is a vast amount of information already available on the molecular mechanics of the stress response, particularly in yeast (Gasch et al., 2000). Experiments should be designed to make the most of these data. Directed research using array technology in conjunction with other proven methodologies can produce new fundamental knowledge about coral biology and response to environmental factors. This study represents an initial attempt at characterizing gene expression profiles in coral exposed to multiple lev-

els of different stressors using a cDNA array and demonstrates how analyzing gene expression could be useful in interpreting the coral stress response.

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