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Characterizing stress gene expression in reef-building corals exposed to the mosquitoside dibrom ☆

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Abstract

We characterize two genes expressed in *Acropora cervicornis* upon exposure to 0.5 μg/l of dibrom, a pesticide used for mosquito control in the Florida Keys. Fragments of these genes were isolated, sequenced, and developed into chemiluminescent probes for Northern slot blots. Expression of target transcripts was detected in corals exposed to a variety of stressors including organophosphates, organochlorines, heavy metals, naphthalene, and temperature. Within the context of stressors examined, the D25 probe demonstrates toxicant and concentration specificity for organophosphates, whereas the D50 probe had broader specificity, detecting transcripts in corals exposed to dibrom, naphthalene, and temperature stress. After characterizing specificity in the lab, these probes were used on field samples taken from the Florida Keys. Both probes detected their targets in samples taken from the upper Florida Keys in August 2000. Preliminary search of sequence databases suggest similarity exists between D25 and a thioesterase.
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Keywords: *Acropora cervicornis*; Florida keys; Dibrom; Biomarkers; Stressor specific response; Stress gene expression

1. Introduction

Corals are exposed to a variety of stressors of both natural and anthropogenic origins. Common natural stressors include temperature (Burns, 1985; Odgen et al., 1994; Leichter et al., 1996), hurricanes (Done and Potts, 1992; Goreau, 1992; Blair et al., 1994; Hughes, 1994; Hughes and Connell, 1999), salinity (Leichter et al., 1996; Porter et al., 1999), sediment (Jordán-Dahlgren, 1992), or ultra-violet (UV) exposure (Gleason and Wellingtion, 1993; Brown, 1997). The influence of natural stressors can be demonstrated on long time scales (millennia) with changes in sea level (Pandolfi, 1999) or on shorter time scales (seasonal or even daily basis) where stressors can exert relatively brief, episodic influences (Leichter et al., 1996). In addition to natural stressors, corals are also exposed to a variety of anthropogenic sources of stress resulting from human modifications of their environment. Anthropogenic stressors include eutrophication (Dubinsky and Stambler, 1996), terrestrial habitat destruction resulting in sediment runoff (Rawlins et al., 1998; Bastidas et al., 1999), altered water flow, pesticide use to control agricultural or household pests (Glynn et al., 1986; Glynn et al., 1989; Richmond, 1993; Rawlins et al., 1998), or even physical damage from boats, anchors, and divers (Causey et al., 2000). Acknowledging that many stressors may simultaneously be impacting a coral reef, the ability to prioritize the impacts of individual stressors is especially difficult. Tools are needed that allow identification and ranking of coral responses to multiple stressors to determine which stressors are having the greatest effects.

Studies are now emerging which demonstrate that the molecular analysis of gene expression can evaluate the relative impact of stressors, identifying responses specific to individual stressors (Morgan et al., 2001). The utility of these techniques are not only promising when they are applied to field populations (Ammar et al., 2000), but as nucleotide information in databases continues to grow, so will the probability that stressors can be identified by investigating patterns of stress gene expression.

☆ The sequences reported here are deposited in the Genbank database (accession numbers BI534456 for probe D50, BI534457 for probe D25, BI534458 for probe C30, and BI534459 for probe H30).

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1.1. Pesticides in the Florida Keys

With a moderate tropical climate and rich natural resources, it is not surprising that the number one industry in the Florida Keys is tourism which attracts over four million visitors annually. In addition, there are currently over 100,000 residents and this number is expected to rise 65% over the next 20 years (Causey et al., 2000). A critical element that has made the Florida Keys an attractive location is the effective control of mosquito populations. Presently, dibrom is one of two mosquito adulticides used in the mosquito abatement program. It is applied in early morning in ultra-low volumes from aircraft to reach mosquito infested areas (Pierce, 1998). While mosquito pesticides represent only a small fraction of myriad of potential stressors that could impact coral in the Florida Keys, they are unique as anthropogenic stressors since their applications are controlled and their effects have been previously documented to elicit specific responses in other organisms (Devonshire and Field, 1991; Chevillon et al., 1999).

1.2. Dibrom and its application

Previous studies have shown that aerial applications of dibrom in the Florida Keys can result in pesticide drift into non-targeted areas (Hennessey et al., 1992; Pierce, 1998). Dimethyl 1,2-dibromo-2,2-dichloroethyl phosphate (Dibrom), also known as Naled, is an organophosphate commonly used to control a variety of insects. It is known to be highly toxic to aquatic invertebrates with 96-h LC-50 values ranging from 0.4 μg/l in *Daphnia* to 18 μg/l in *Pontoporeia hoyi* and *Gammarus fasciatus* (Johnson and Finley, 1980). The effects of dibrom are similar to other organophosphates which include inhibition of cholinesterase (Gallo and Lawryk, 1991). It is the pesticide of choice because in the presence of water and sunlight it is rapidly broken down with a half-life of about 2 days (Tietze et al., 1996). The primary degradation product of dibrom is another organophosphate that is highly soluble, dichlorvos or DDVP (2,2-dichlorovinyl dimethyl phosphate). The LC-50 for DDVP indicates that it is much more toxic, especially in the presence of UV light and it has a longer half-life than dibrom (Gallo and Lawryk, 1991; Howard, 1991; Kidd and James, 1991).

This study examined the influence of dibrom on stress gene expression in the reef-building coral *Acropora cervicornis*. To control the application and concentration of exposure, corals were initially exposed to dibrom in the laboratory. Fragments of two genes induced from the pesticide exposure were isolated and developed into molecular gene probes. To characterize the specificity of response, Northern slot blots were performed using RNA extracted from corals exposed to a variety of other stressors. Different patterns of expression were exhibited by these two genes. Expression of one gene appears to be toxicant as well as concentration specific for dibrom, while the other gene can be induced by a variety of stressors including temperature. Stress gene expression induced by dibrom exposure was also analyzed using additional molecular probes developed from other stressors. The utility of these gene probes for detecting coral stress due to dibrom exposure was examined on field samples taken from the Florida Keys. Although these genes could be induced by dibrom exposure in the laboratory, corals were found to be expressing both gene transcripts at one field site. Nucleotide database searches suggest one of these dibrom-induced genes is significantly similar to an esterase.

2. Materials and methods

2.1. Coral collection

A. cervicornis was collected from the Middle Keys at West Turtle Shoal (24°700′N, 80°.967′W) in June 1997 and Tennessee Reef (24°771′N, 80°763′W) in April and August 1999. Colonies were also collected from a site in the Upper Keys known as Admiral Reef (25°02.690′N, 80°23.693′W) in August 2000.

2.2. Toxicants and controls

Coral fragments ~7 cm long were taken from Tennessee Reef in April 1999 and divided into pesticide treatments and controls. Coral fragments were exposed to dibrom at 0, 0.5, and 5.0 μg/l concentrations in the laboratory for four hours in aquaria filled with 10 l recirculating natural seawater at 26 °C and 36 ppt salinity. Three types of controls for *A. cervicornis* were used in this study. A field control was processed on the boat immediately following removal from the reef in order to estimate stress due to handling and transport. Laboratory control coral fragments were transported back to the lab and placed in an aquarium with 10 l of recirculating natural seawater. A solvent control consisted of coral fragments exposed to 100 μl acetone/l placed in 10 l of recirculating natural seawater for the duration of the pesticide exposures. Acetone was necessary as a solvent for dibrom to ensure adequate water solubility.

A second dibrom exposure of *A. cervicornis* was conducted in August 2000 at Admiral Reef where coral fragments from the same colony were divided into control and 0.5 μg/l exposed groups then placed in plastic bags and left on the reef for a four hour exposure. Ambient conditions were 31 °C and 36 ppt salinity. Some fragments were bagged while remaining attached to the colony and the substrate. Other fragments were broken off from the parent colony and placed in bags which sat on the substrate. Each bag was filled with 6 l

of seawater and sealed with drawties. For those coral fragments receiving pesticide, the necessary mass of dibrom was dissolved in 1 ml of acetone and injected into the designated bag with a 5 cc tuberculin syringe. At the end of the exposure, all attached corals were broken off from parent colonies while remaining sealed in the plastic bags. All coral fragments whether attached or unattached were brought aboard the boat and the coral tissue was immediately either washed in TRIzol® to begin the RNA extraction. Additional coral fragments from colonies not bagged were also collected and processed similarly in order to determine if there was a bag effect.

2.3. Extraction and manipulation of RNA

Total RNA was extracted from living tissue by direct application of 1 ml of TRIzol® (Invitrogen, Carlsbad, CA, USA) to $\approx$10 cm^2 of coral surface as a modification of the manufacturer's protocol which is based on Chomezynski and Sacchi (1987). All coral fragments from an individual control or treatment were placed in beakers and washed in appropriate volumes of TRIzol® for one hour on a gyratory shaker. Washing in TRIzol® was equivalent to the homogenization step in the manufacturer's protocol. After the TRIzol® wash, the tissues were divided into 1 ml working volume aliquots within respective treatment groups and the RNA extraction protocol was completed for an individual replicate/sample. RNA concentrations were estimated by excitable fluorescence at 485 nm (RiboGreen, Molecular Probes, Portland, OR, USA). Integrity of total RNA was confirmed by electrophoresis of an aliquot of each sample on a 1% formaldehyde agarose gel (Sambrook et al., 1989). Total RNA was further purified by DNase I digestion followed by phenol/$CHCl^3$ extraction (Message Clean®, GenHunter, Nashville, TN, USA). After purification, reverse transcriptions of mRNAs were performed using freshly diluted total RNA (0.1 μg/l) and MMLV reverse transcriptase (GenHunter, Nashville, TN, USA). Five samples were reverse transcribed. The samples were two replicate aliquots from the lowest dibrom dose (0.5 μg/l), and one aliquot from each of the three different control samples (field, lab, acetone). The cDNAs produced in this reverse transcription step were used in all subsequent differential display polymerase chain reactions (PCRs).

The cDNAs produced by reverse transcription were amplified by PCR using all combinations of the eight degenerate and three anchored primers provided in the RNA Image® Kit 1 (GenHunter, Nashville, TN, USA). The differential display PCR reactions were carried out using a Perkin Elmer™ Cetus thermocycler. Conditions used to amplify transcripts were 94 °C for 30 s followed by 40 °C for 2 min, 72 °C for 30 s, for 40 cycles, followed by 72 °C for 5 min. Separation of cDNA fragments was accomplished by electrophoresis of samples on a 6% denaturing polyacrylamide gel (Sambrook et al., 1989). After electrophoresis, the gel was transferred to blotting paper and dried under vacuum for one hour at 80 °C. Visualization of products was possible by incorporating ^{35}S dATP (deoxyadenosine 5′-[-α-^{35}S]-thiotriphosphate, Amersham Pharmacia Biotech, Piscataway, NJ, USA) during PCR and exposure of the radioactive cDNA fragments to autoradiographic film. The polyacrylamide gel permitted cDNA fragments (bands) in the size range of 100–600 bp to be analyzed for each primer pair. There were a total of 24 primer pair combinations used for differential display PCR in this study. Individual PCR reactions varied in the number of bands generated. To be considered a gene differentially up-regulated due to toxicant exposure, a band needed to be expressed in both replicates of the exposed group and absent in all control samples. The rationale for these criteria are: (1) to account for any potential genotypic variation amongst different colonies that were combined into a particular treatment and (2) to reduce the potential for choosing a false positive which is known to occur with ddPCR (Debouck, 1995; Zhang et al., 1996).

2.4. Development of molecular probes

The cDNA fragments exhibiting differential expression were extracted from the polyacrylamide gel and amplified twice by PCR using the corresponding primer pair and the same PCR conditions. The cDNAs were then reamplified again using the same PCR conditions to incorporate DIG labeled dUTPs (Digoxigenin-11-2′-deoxy-uridine-5′triphosphate, alkali-labile; Roche Diagnostics, Indianapolis, IN, USA) for subsequent use as gene expression probes. Electrophoresis of samples from each cDNA was performed in a 2% Tris Borate EDTA (TBE) agarose gel with a 50 bp ladder (Invitrogen, Carlsbad, CA, USA) before and after DIG incorporation to determine probe size and verify DIG incorporation. To reduce the time required for each PCR amplification, a RoboCycler Gradient 40 (Stratagene, La Jolla, CA, USA) was used for these PCR reactions. The manufacturer's suggested modification to amplification conditions were 94 °C for 41 s, 40 °C for 2 min and 26 s, 72 °C for 46 s, for 40 cycles, followed by 72 °C for 5 min, and concluding with 6 °C holding temperature until storage at −20 °C.

2.5. Verification of differential gene expression

DIG-labeled probes were used in Northern dot blot hybridizations to visualize the presence of target transcripts in total RNA samples. The blots consisted of replicates and/or samples of total RNA not previously

used in the ddPCR reactions. 1 μg of total RNA from one control, one low dose (0.5 μg/l) exposure, and one high dose (5.0 μg/l) exposure were blotted onto positively charged nylon membranes (Roche Diagnostics, Indianapolis, IN, USA) and cross-linked by UV light (Stratalinker 1800, Stratagene, La Jolla, CA, USA) or baked at 65 °C for 1 h. Hybridization of Northern dot blots consisted of prehybridizing a membrane in high SDS (sodium dodecyl sulfate) buffer (also known as Church buffer) for 1 h at 42 °C, then hybridizing the membrane at 42 °C for 18 h in high SDS buffer, followed by two 15 min room temperature 2X standard saline citrate (SSC) washes, and concluding with two 15 min 0.2X SSC high stringency washes at 68 °C (van Miltenburg et al., 1995). The hybridization solution consisted of 2 μl of probe solution (~25 ng cDNA/μl) per 1 ml of high SDS buffer.

Immunodetection of probe hybridization was accomplished by anti-DIG antibodies conjugated to alkaline phosphatase (Roche Diagnostics, Indianapolis, IN, USA) in the presence of CSPD® substrate (Roche Diagnostics, Indianapolis, IN, USA). This allowed for chemiluminescent visualization by exposing blots to autoradiographic film for 30–120 min. Changes in levels of expression were determined by comparing densitometric scans of blots from control and exposed samples using the computer program NIH Image. Each membrane was used only once to ensure linear correlation between optical density and RNA concentration of scanned images (Krueger and Williams, 1995). Hybridizations were conducted a minimum of three times to determine the variability of target transcript abundance among blots. Used in a variety of combinations were three different controls, three different replicates of the low dose exposure, and three different replicates of the high dose exposure. After hybridizations, an aliquot from each probe was amplified again by PCR, then purified (QIAquick PCR spin columns, Qiagen, Valencia, CA, USA), and sent to University of Georgia Molecular Genetics Laboratory to be sequenced. Sequence information was used to confirm that the probe represented a single transcript with identifiable primer sites. In addition, the sequence information was also used to gain insight into the function of the differentially expressed genes.

2.6. Obtaining additional sequence information from the probe D25

Each probe represents only a portion of an expressed gene and the nucleotide sequence therein can be used as a template for obtaining additional transcript information. A PCR was performed using the differential display primers AP-2 and H-T_{11} G (RNA Image kit, GenHunter, Nashville, TN, USA) to generate additional copies of the original probe sequence. The PCR conditions were as previously described. Amplified PCR products were electrophoresed on a 2% agarose gel along with a 50 bp ladder to confirm probe size and then removed from the gel and purified (QIAquick, Qiagen, Valencia, CA USA) in order to proceed with inserting the product into a vector. The probe was inserted into a vector (pCR® II-TOPO, Invitrogen, Carlsbad, CA, USA), and the recombinant plasmids were then inserted into competent *E. coli* cells and cultured for 16 h. After incubation, cloned plasmids were then extracted from the cultured cells (Wizard Mini-preps, Promega, Madison, WI, USA) and a 150 μl aliquot of prepared plasmid was sent for sequencing to the University of Georgia's Molecular Genetics Facility. The nucleotide information obtained from sequencing was then used to identify the regions most suitable for developing gene specific primers (GSP) which are necessary for the 5′ rapid amplification of cDNA snds (5′ RACE) protocol. Following the instructions outlined by the manufacturer (Invitrogen, Carlsbad, CA, USA), 5′ RACE was performed on the shorter dibrom probe D25. Amplified RACE products were cloned into the vector (pCR® II-TOPO, Invitrogen, Carlsbad, CA, USA), as previously described and then sequenced to confirm the additional nucleotide sequence obtained.

2.7. Determining the full length of a target transcript

The size of the full length transcript for the dibrom probe D25 was determined by probe hybridization to a Northern blot of total RNA. 5 μg of total RNA from a dibrom exposed sample were loaded onto a 1% formaldehyde agarose gel along with a 0.24–9.5 kb RNA ladder and then electrophoresed at 75 V for 2 h. Ethidium bromide was added to the ladder sample for visualization after the electrophoresis. The distance each ladder component traveled was recorded to the nearest 0.1 cm and used to generate a standard curve in order to correlate the distance traveled and the approximate size of the band produced from the Northern blot. After electrophoresis, the gel with its unstained sample were rinsed in deionized water for 30 min followed by a rinse in 10X SSC. The prepared gel was then placed in a vacuum blotter (BioRad, Hercules, CA USA) over a positively charged membrane and immersed in 10X SSC. A vacuum transfer was performed at 5 lbs/in^2 of pressure for 90 min. Afterwards, the membrane was removed from the blotter and the RNA was cross-linked by UV light (Stratalinker 1800, Stratagene, La Jolla, CA, USA). Probe hybridization and detection followed the protocol previously described. Transcript length was determined by measuring the distance the transcript traveled from the loading wells and correlating with the regression analysis of the electrophoresed RNA ladder.

2.8. Further characterizations of dibrom probes

2.8.1. Confirming coral genome contains genes expressed

When analyzing the *A. cervicornis* molecular responses to dibrom exposure, an important question is whether the observed responses are coming from host coral tissues or from the endosymbiotic zooxanthellae. One means of answering this question would be to expose each symbiont separately however, this is technically difficult since each organism would experience stress in the separation process. Even when there is separation of the animal and algal components, studies have shown that bleached corals still contain some zooxanthellae (Fitt et al., 1993; Fagoonee et al., 1999) and that coral endodermal cells are detached when zooxanthellae are expelled (Gates et al., 1992). The little existing information about zooxanthellae taxonomy suggests there is high species diversity and an uncertainty about the genetic relatedness of those species (Rowan, 1998). This fact suggests that to use zooxanthellae from sources other than the *A. cervicornis* collected for this study may give results that could be inaccurate (Rowan, personal communication). Currently, there are no known sources of cultured zooxanthellae from *A. cervicornis* taken from the locations examined in this study. Acroporids are known to hybridize with other species within the genus (Willis et al., 1997; van Oppen et al., 2000). This genetic relatedness is further supported by the fact that at least some acroporid genes are highly conserved (van Oppen et al., 1999). To determine whether the genes expressed are within the genome of the host coral, a southern blot of digested azooxanthellate genomic coral sperm DNA from *A. formosa* was probed with each dibrom probe following the protocol outlined in Morgan et al. (2001). Furthermore, we have confirmed that the vast majority (>98%) of zooxanthellae remain intact in our extraction protocol, making it even more unlikely that the stress genes that we have isolated are from zooxanthellae.

2.8.2. Inducing expression of target transcripts by different toxicants

A membrane was prepared and modeled after the concept of DNA gene-arrays (Lovett, 2000). This "mini array" was intended to demonstrate the specificity of each probe in detecting target transcripts within the selected RNA pools. 1 μg of total RNA was used for each blot. The membrane contained 15 different blots which represented three controls (field, lab, and acetone) and two concentrations of six different toxicants which included permethrin (1 and 10 μg/l), lindane (15 and 30 mg/l), copper (25 and 50 μg/l), mercury (5 and 50 μg/l), naphthalene (50 and 300 μg/l), and dibrom (0.5 and 5.0 μg/l).

2.8.3. Comparing the influences of temperature and dibrom

Coral fragments of *A. cervicornis* collected during early spring, early summer, and late summer were used to determine if changes in water temperature could also influence the expression of the target transcripts induced by dibrom. The water temperatures were 26 °C in April 1999, 29 °C in June 1997, and 31 °C in August of both 1999 and 2000. These sampling periods represent field conditions that are considered above and below the 30 °C water temperature which is known to cause coral stress in prolonged exposures (Jokiel and Coles, 1990). Some of the coral fragments collected in April 1999 were divided into three groups and each group was exposed to a different temperature (25, 30, or 35 °C) in laboratory aquaria for four hours. Each fragment was immediately placed in a designated temperature treatment without acclimation from the ambient temperature of 26 °C. All coral fragments used in this experiment were transported and maintained in the conditions previously described. Total RNA was extracted from the field samples as well as the temperature shock treated samples and blotted onto membranes in order to further characterize the specificity of each probe. Blots from eleven different sources were compared. Those sources were one 29 °C lab control, one 26 °C lab control, one 0.5 μg/l dibrom treatment at 26 °C, one 25 °C treatment, one 30 °C treatment, one 35 °C treatment, one 31 °C bagged field control, one 31 °C bagged field 0.5 μg/l dibrom treatment, one 31 °C unbagged field control from Admiral Reef, and one 31 °C unbagged field control from Tennessee Reef. By comparing these samples it is possible to determine if a target transcript is induced by exposure to dibrom under different conditions as well as sudden changes in temperature.

2.8.4. Searching sequence databases

One of the richest sources of biological information currently available are the DNA and protein sequences that can be accessed through public and private databases. What makes sequence information so valuable is the ability to make sequence comparisons and develop hypotheses about relationships and possible functions. Basis local alignment search tool (BLAST) (Altschul et al., 1990, 1997) is the most commonly used suite of programs used to search the databases for sequence similarities. The BLAST program TBLASTX was used to search for sequence similarities at National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). Beauty (Worley et al., 1995), an enhanced version of BLAST at that the Baylor College of Medicine (BCM) was also used to search for protein sequence similarities (http://searchlauncher.bcm.tmc.edu/seq-search/protein-search.html). Although the Beauty databases are significantly smaller than the databases at NCBI, the Beauty program has the capacity to uniquely

incorporate information about conserved patterns and motifs present in both the queried and database sequences which makes it capable of detecting sequence similarities of otherwise weak or distantly related transcripts. The statistical basis for determining if a probe sequence is significantly similar to a query sequence in a database is calculated by and *E* value (Altschul et al., 1990, 1997). Only database sequences with *E* values $<$ 0.01 were considered as having some degree of similarity with the gene probe sequences in this study.

3. Results

The laboratory exposures of dibrom on *A. cervicornis* show differential expression of two of the up-regulated cDNA fragments isolated during differential display PCR. The D25 probe detects a target transcript induced by 0.5 μg/l of dibrom, but when the concentration reaches 5.0 μg/l this transcript is no longer expressed (Fig. 1). The second differentially expressed transcript represented by the D50 probe detects its target at both 0.5 and 5.0 μg/l of dibrom. Sequences of both D50 and D25 probes are presented in Figs. 2 and 3, respectively.

Since the D25 probe was relatively short at 186 bases, additional nucleotide sequence would be desirable to increase the probability of detecting similarity to genes of known function. To obtain additional nucleotide sequence, three GSPs were developed from the available D25 probe sequence. Required for the RACE protocol, each GSP in combination with Invitrogen's abridged universal primer was used in a nested PCR reaction to produce subsequently shorter PCR fragments. Using GSP1, a base fragment $>$ 425 bases was amplified, followed by GSP2 which generated a ~400 base fragment, and concluding with GSP3 which generated roughly a 350 base fragment. The RACE experiments collectively yielded an additional 250 bases to the 5′ end of the probe sequence resulting in a 436 base sequence fragment of the entire mRNA transcript. A Northern blot determined that the full length mRNA transcript is ≈1.35 kilobases in length (Fig. 4). The RACE results combined with the Northern blot suggest that one third of the transcript sequence for this acroporid gene can now be identified. A Northern blot was not performed using the D50 probe since this probe already had significantly greater amounts of sequence information.

These differentially expressed cDNA fragments may be specific for the toxicant examined. Alternatively, the cDNA fragment may represent a transcript that is differentially expressed upon exposure to a number of stressors. The two dibrom probes show different degrees of specificity when hybridized to the 15 different total RNA samples blotted on the mini-array. The D25 probe hybridized only to the 0.5 μg/l concentration of dibrom (Fig. 5). By comparison, the D50 probe exhibited the greatest hybridization to both concentrations of dibrom as well as the high concentration of naphthalene (Fig. 6). It should be noted that these dibrom probes did not hybridize to coral tissues exposed to organochlorines (permethrin and lindane) or heavy metals (copper and mercury). To further illustrate the utility of the mini-array, a copper probe C30 and a mercury probe H30 were also investigated. These additional probes were generated from toxicant exposures of 50 μg/l of copper and 5 μg/l of mercury using previously published protocols (Morgan et al., 2001). The copper probe C30 only detected its target transcript in coral tissues exposed to 50 μg/l of copper (Fig. 7), while the mercury probe H30 detected its target in the high and low concentrations of both mercury and dibrom, as well as in 50 μg/l copper and 300 μg/l naphthalene (Fig. 8). These hybridization results demonstrate how each probe is capable of detecting its target transcript within the collective mRNA pool (see Figs. 5–8). The results of the H30 probe hybridization not only shows probe specificity, but also illustrates how very different stressors (i.e. heavy metals vs. organophosphates) can induce the same response (see Fig. 8).

Probe ID	Probe length (Number of bases)	Dibrom concentration (μg/L)			Mean fold increase in gene expression ± SE
		0	0.5	5.0	
D25	186				5.3 ± 1.5
D50	525				22.3 ± 7.7

Fig. 1. Differential gene expression of *A. cervicornis* in response to dibrom exposure. Each blot has 1 μg of total RNA from the different dibrom treatments. Means ± standard errors are calculated from three replicates for the dibrom concentration (0.5 or 5.0 μg/l) yielding the greatest increase in expression over controls (0 μg/l). Mean increases represent dot density relative to controls.

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D50   1 K  L  R  L  Y  G  C  F  S  K  A  K  Q  I  F
      1 AAGCTTCGACTGTATGGTTGCTTTAGCAAAGCAAAACAAATATTC

     16 I  L  H  M  P  I  R  K  R  K  I  Q  R  S  V
     46 ATATTGCATATGCCAATAAGAAAACGAAAAATACAGCGCAGTGTT

     31 I  I  K  Q  E  G  Q  T  S  S  L  I  A  A  Q
     91 ATTATAAAGCAAGAAGGCCAGACGTCGTCCCTCATAGCTGCGCAA

     46 I  S  I  S  H  F  S  K  T  R  D  L  R  S  G
    136 ATAAGCATTTCTCATTTTAGTAAAACGAGAGATTTGAGATCTGGT

     61 A  R  R  S  C  N  I  I  L  K  S  I  S  M  H
    181 GCAAGACGTTCCTGCAATATCATTTTGAAGTCGATTTCCATGCAT

     76 T  K  T  G  R  K  N  K  M  L  K  S  V  R  A
    226 ACAAAGACAGGAAGAAAAAATAAGATGCTCAAAAGTGTGCGTGCA

     91 S  T  K  R  H  N  L  C  R  C  Q  T  S  *  *
    271 TCCACAAAACGTCATAATTTATGCCGTTGTCAAACATCGTAATAA

    316 TTTTATTGACATAAATTAAATCTGAACCTAGAGAAGTTGAGACTT

    361 CTGCGTTTTTTCACAAGCACACTTAAAGATGGACGTTAATGTTAA

    406 GTACAGGGTTGGGGTAGGAGGTAACATCGCTGGTGTCGACTGGCG

    451 TATAATGAAATTACCTATCGGAAAGATTGGAAAAGTGATTACTCT

    496 TGTCAAAAGATGTCTAGTCATCCACTGAAAAAAAAAAA
                                   poly-A region
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Fig. 2. Nucleotide sequence for the D50 dibrom probe in the 5′–3′ direction. Probe length is the entire 533 nucleotides including the truncated poly-A region. The corresponding 103 translated amino acids in the +1 reading frame are also presented above the nucleotides.

In order to determine the utility of these dibrom probes in the field, small branches (~15 cm) of *A. cervicornis* were bagged and exposed to 0.5 μg/l of the pesticide while on the reef. These samples were then compared to other field samples and lab controls. Findings from the bag experiment suggest that all samples taken from Admiral Reef in August 2000 were expressing the target transcript for the D25 probe (see Fig. 9C). The August 1999 sample taken from Tennessee Reef was the only other field sample collected when the water temperature was 31 °C and this sample did not express the D25 target transcript. The D50 probe detected its target transcript in all August samples from both 1999 and 2000 (see Fig. 10C). The specificity of response was also examined using temperature as a natural stressor. Temperature shock treatments of 25, 30, and 35 °C were used to see if the target transcripts induced by dibrom could also be induced by temperature stress. The target transcript for the D25 probe is not significantly induced upon exposure to temperature shock treatments (Fig. 9B), whereas expression of D50

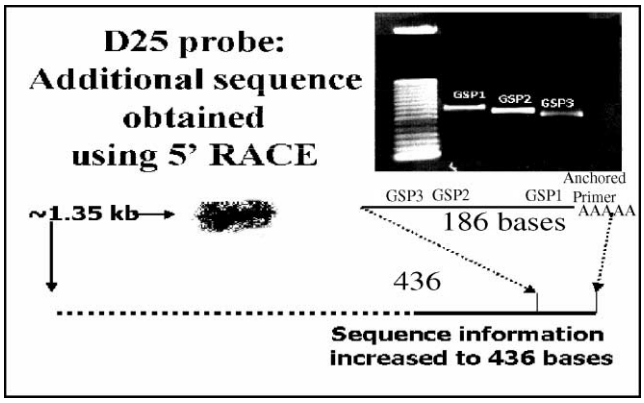

Fig. 4. The approximate size of each amplified RACE fragment is compared to a 50 bp DNA ladder on the ethidium bromide stained gel (see inset). The relative locations of the three GSPs are shown above the original probe length of 186 bases. The composition of the 436 base fragment includes the original probe length plus the additional bases obtained by 5′ RACE. The Northern blot determined the 1.35 kb length of the entire transcript.

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D25    1 D  T  F  M  L  I  E  T  L  L  N  C  L  M  S
       1 GATACCTTCATGTTGATAGAGACACTTCTAAACTGCCTTATGTCT

      16 S  H  H  L  L  D  T  F  A  F  N  K  M  N  D
      46 TCTCACCATTTATTGGACACCTTTGCTTTTAACAAAATGAATGAT

      31 Q  C  F  K  V  L  L  P  N  T  E  H  F  L  K
      91 CAATGTTTTAAAGTCCTCCTACCAAATACTGAACATTTCTTGAAA

      46 M  S  L  E  D  K  H  S  S  *
     136 ATGAGCCTTGAAGATAAGCACAGTAGCTAAAGGATTGAGAGATTT

     181 AATACTGGGCTAAGAGTCATAGGAAGCTTGCCTTGTATTCATTGC

     226 CAGGAATGCTTGGCGACTGTACCAACAGAGAATAATAGTTATTGT

     271 ATGTGGCTAGCAAGTAGATATGGCAAGTGGTGGTCCTACATGAGT

     316 TTGAAGAGTGCAACTCCTTGTTTAATGTCGTTCAGATGTAACTAG

     361 TGTCCATATACCAGCGGAGAACAGCAATGTCTATTAAAGAAATGT

     406 GTCACTGACATCCAAAAAAAAAAA
                                   poly-A region
```

Fig. 3. Nucleotide sequence in the 5′–3′ direction for the D25 dibrom probe plus additional sequence generated from 5′ RACE. The probe is 186 nucleotides including the truncated poly-A region and is identified by the underline. The corresponding 54 translated amino acids in the +1 reading frame are also presented above the nucleotides.

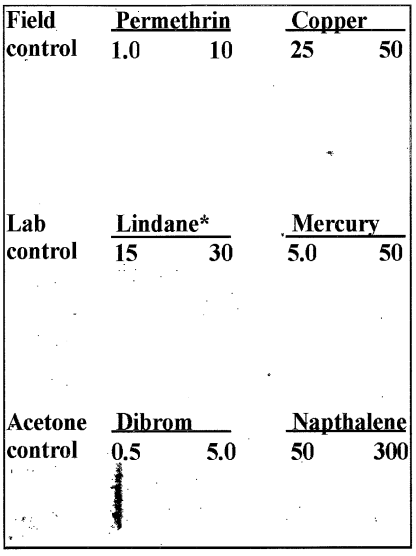

Fig. 5. Mini-array characterization of D25 probe specificity. The mini-array consists of 15 different total RNA samples of *A. cervicornis*. All toxicant concentrations are in μg/l except for Lindane* which is mg/l. The acetone concentration in the solvent control is 0.1 ml/l.

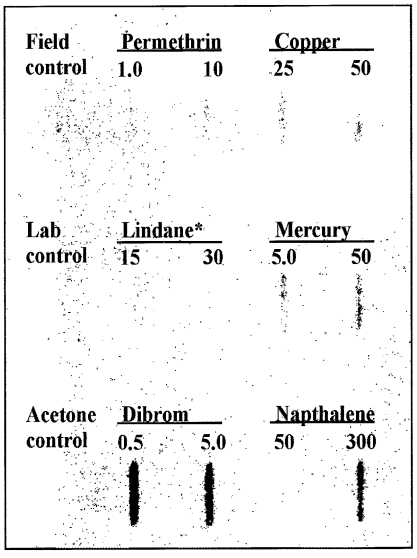

Fig. 6. Mini-array characterization of D50 probe specificity. The mini-array consists of 15 different total RNA samples of *A. cervicornis*. All toxicant concentrations are in μg/l except for Lindane* which is mg/l. The acetone concentration in the solvent control is 0.1 ml/l.

target transcript appears to be up-regulated by those treatments (Fig. 10B).

The gene probes developed in this study not only provide sensitive measures of detecting stress responses, they also provide gene sequence information which can be used to access available databases. Results of database searches provided no significant sequence similarities for probes D50, C30 or H30. The Beauty program did identify a significant similarity ($E_{value} = 0.002$) between the 54 translated amino acid sequence of D25 and a thioesterase (Fig. 11).

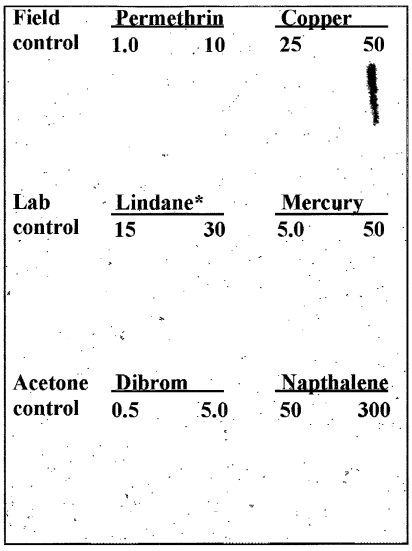

Fig. 7. Mini-array characterization of the copper probe C30 specificity. The mini-array consists of 15 different total RNA samples of *A. cervicornis*. All toxicant concentrations are in μg/l except for Lindane* which is mg/l. The acetone concentration in the solvent control is 0.1ml/l.

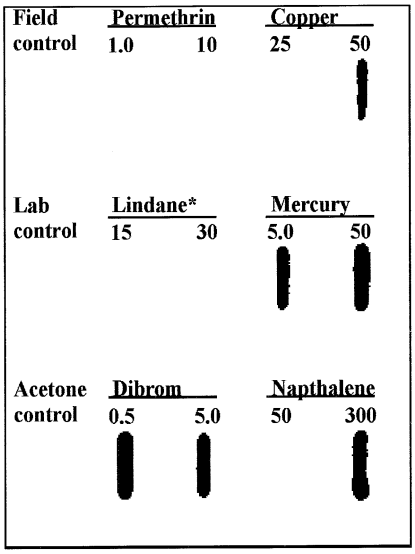

Fig. 8. Mini-array characterization of the mercury probe H30 specificity. The mini-array consists of 15 different total RNA samples of *A. cervicornis*. All toxicant concentrations are in μg/l except for Lindane* which is mg/l. The acetone concentration in the solvent control is 0.1 ml/l.

4. Discussion

4.1. Toxicant specificity

Results represented in Figs. 1 and 3 suggest the dibrom probe D25 is capable of detecting a target transcript that is toxicant and concentration specific within the context of the stressors utilized in this study. Further

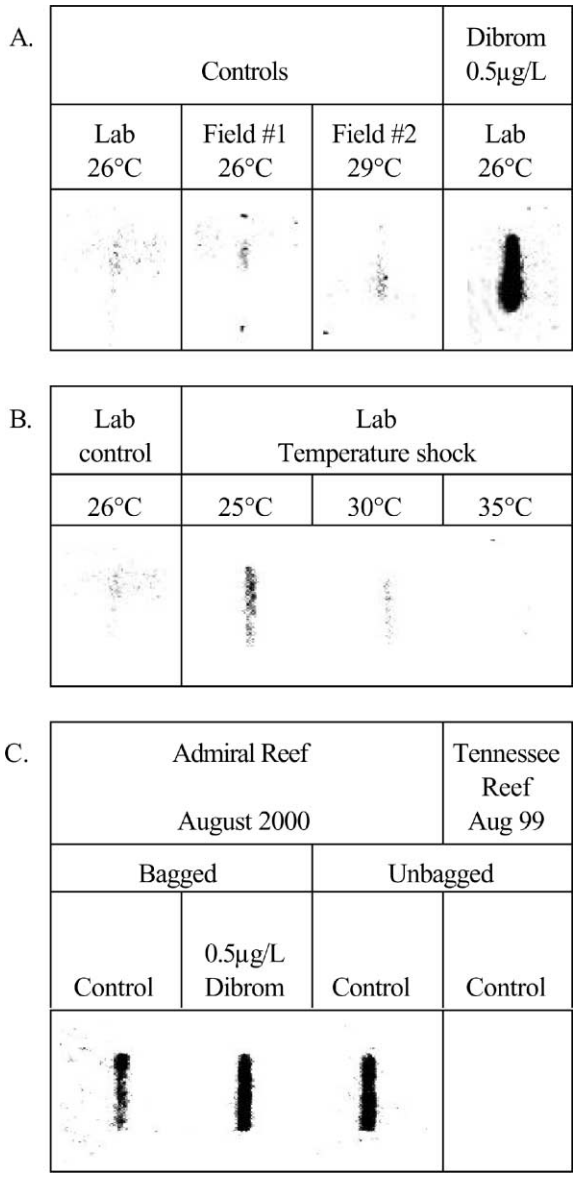

Fig. 9. Detecting D25 in field samples and temperature treatments. (A) Comparison of different controls to a laboratory exposed dibrom treatment. (B) Expression of dibrom induced transcript after 4 h exposure of three temperature shock treatments. (C) Detection of transcript from field samples taken from Admiral Reef, August 2000. Colonies were bagged for 4 h and the dibrom exposure was 0.5 µg/l of the pesticide while the ambient water temperature was 31 °C. Additional colonies not bagged were also taken to compare bag effects.

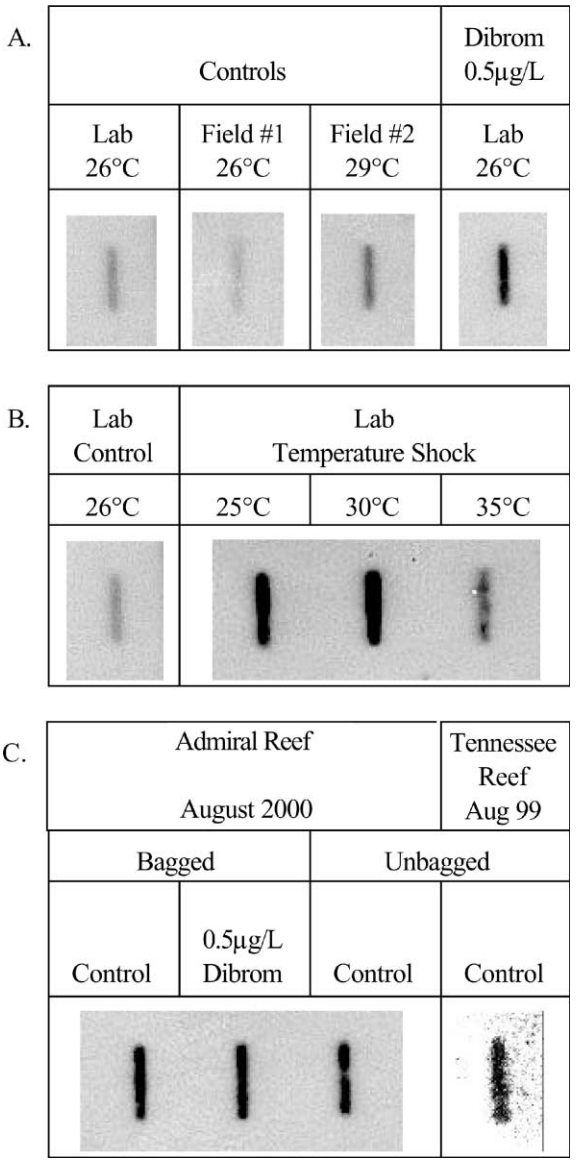

Fig. 10. Detecting D50 in field samples and temperature treatments. (A) Comparison of different controls to a laboratory exposed dibrom treatment. (B) Expression of transcript after four hour exposure of three temperature shock treatments. (C) Detection of transcript from field samples taken from Admiral Reef, August 2000. Colonies were bagged for four hours and the dibrom exposure was 0.5 µg/l of the pesticide while the ambient water temperature was 31 °C. Additional colonies not bagged were also taken to compare bag effects.

confirmation of specificity was demonstrated by the absence of detection in controls taken from the field when the water temperature was 26, 29 or 31 °C and laboratory temperature experiments (Fig. 9A and B). Only at Admiral reef where samples were taken in August 2000 was the target transcript detected (see Fig. 9C). Since the D25 probe detected its target transcript at this location, this would suggest that corals at Admiral reef were expressing the gene that was induced only when corals in the laboratory were exposed to dibrom. This transcript is not induced by high temperature, permethrin, lindane, naphthalene, copper or mercury exposures. There are a number of possibilities for explaining this response. One possibility is the corals on Admiral Reef had previously been exposed to dibrom or its breakdown product dichlorvos (DDVP) which is known to have a longer half-life than dibrom. DDVP has been detected in water samples offshore of Key Largo at concentrations of about 0.5 µg/l (Pierce, 1998). The second possibility is that there are other, as yet

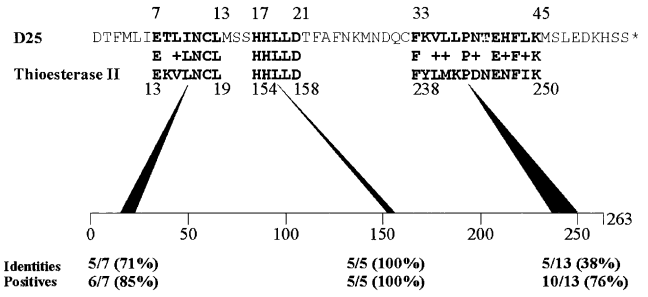

Fig. 11. Sequence similarities ($E = 0.0022$) of the dibrom probe D25 and the 263 amino acid Thioesterase II (medium chain) (Swiss-Prot accession number PO8635) (BEAUTY blastp 1.4.7MP, default options, versus 78,469 sequences in the CRSeqAnnot database on 26 February 2001). High scoring segment pairs represent those positions of compared sequences where identical amino acids are located (identities) or amino acids of comparable function are recognized (positives).

unidentified chemicals, that induced the same response elicited by dibrom in our laboratory experiments. There is a paucity of data linking pesticide application and coral responses in situ (Rawlins et al., 1998), however, the results presented in this study may represent a way to link organophosphate exposures in the field to changes in coral stress gene expression.

Results from the laboratory exposures indicate the expression of the D25 target transcript can be generated at dibrom concentrations of 0.5 μg/l, but when the concentration reaches 5.0 μg/l the response is shut down (Fig. 1). By comparison, results from Admiral Reef indicate that field controls and the bagged dibrom exposed samples were expressing the transcript (Fig. 9C).

The threshold dibrom concentration necessary to induce gene expression and the duration of a detectable response in not known. Although we have demonstrated that the D25 probe is capable of detecting responses to dibrom exposure at concentrations of 0.5 μg/l, it may also be capable of detecting dibrom concentrations that are below the current detection limits of analytical chemistry instruments. A full dose response of coral stress gene expression for dibrom would provide the data necessary for assessing the sensitivity of gene induction. Studies have examined cholinesterase activity in cnidarians (Talesa et al., 1996), however, there are no studies of cholinesterase inhibition of cnidarians in situ. Future studies should include collection of water quality data along with coral sampling at frequent intervals throughout the pesticide application season to correlate responses with pesticide applications.

The long dibrom probe D50 represents a transcript that is a more general stress response, capable of being induced by a number of stressors. This conclusion is supported by results of differential expression studies (Fig. 1), mini-array analysis (Fig. 6), and temperature experiments (Fig. 10B). Specifically, this target transcript is induced by dibrom exposures at both 0.5 and 5.0 μg/l as well as 300 μg/l naphthalene. In addition, changes in water temperature also induced the expression of this transcript and rapid, small changes in temperature in the laboratory can have the same effect as elevated temperatures in the field. Results from long probe hybridizations suggest that dose-response curves from exposure to many different stressors would be informative to further characterize the expression of this target transcript.

Probing Southern blots of azooxanthellate genomic sperm DNA from *A. formosa* by Morgan et al. (2001) with these dibrom probes suggests that dibrom induced genes are contained within the coral genome. Similar genes may also be present in the symbiotic zooxanthellae, but obtaining zooxanthellae DNA free of host coral contamination has not been possible (Gates et al., 1992).

4.2. Temporal periodicity of stress

The influence of dibrom and temperature on the expression of target transcripts examined in this study is consistent with the temporal nature of these stressors in field populations since expression was not continuous throughout all sampling periods. The transcript detected by the D25 probe was only present in August 2000 (Fig. 9). The D50 target transcript was also detected in August, but unlike the D25 transcript, its expression can be explained by elevated water temperature (Fig. 10). Although expression of the D25 transcript clearly signals the presence of another stressor besides temperature, we do not know whether this stressor is dibrom. Dibrom applications are not continuous throughout the year and non-point sources of pesticides are most likely aerial drift during application, tidal flushing after application, or terrestrial runoff after precipitation (Pierce, 1998). Numerous studies have determined the seasonality of water temperatures commonly experienced in the Florida Keys and the elevated temperatures experienced in August are consistent with conditions known to stress corals (Jokiel and Coles, 1990; Fitt et al., 1993). We also do not know if high temperatures modify coral sensitivity to dibrom. Our lab exposures were at 26 °C in developing the dibrom probes, whereas detection of the D25 target transcript in the field was at 31 °C.

4.3. Effects of multiple stressors

This study represents one of the first attempts to examine the effects of multiple stressors in corals using molecular responses as endpoints. 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), exposure to heavy metals (Harland and Brown, 1989; Jones, 1997) or cyanide (Jones and Steven, 1997) can all induce bleaching. It is also possible that a

single stressor may elicit a variety of responses that can only be detected using an assortment of different biomarkers (Downs et al., 2000). Elevated temperature for example can induce production of heat shock proteins (HSP) (Black et al., 1995; Fang et al., 1997; Sharp et al., 1997; Gates and Edmunds, 1999; Downs et al., 2000). Molecular responses such as HSP production represent individual components of a more generalized stress response. The two molecular responses examined in this study have one common characteristic, they are both up-regulated upon exposure to dibrom (see Figs. 1, 3, 4). The response detected by the D50 probe can also be induced by temperature (Fig. 10B). Consequently, if field samples had only been probed with D50, then it would have been impossible to differentiate whether observed responses were from temperature or another stressor. The combined effect of both stressors (Fig. 10C) was similar to the effect of temperature alone (Fig. 10A and B) for the expression of the D50 transcript. By including the D25 probe it is possible to detect the presence of a second stressor in field samples, reaffirming corals respond to different stressors by expressing specific genes. This conclusion is further supported by the fact that the probe H30 detected a stress-related transcript in the dibrom exposed laboratory samples (Fig. 8), demonstrating the expression of at least three different stress genes in corals exposed to dibrom.

4.4. Searching for similar sequences

The bioinformatic analysis of D25 represents a preliminary result of using nucleotide sequence information that was translated and compared to protein databases. The putative identification of D25 as an esterase would be consistent with gene products expected to be induced by organophosphate pesticide exposure. Subsequent BLAST analyses yielding lower (significant) *E* values will only be attained as additional gene sequence for the D25 transcript is acquired and compared to a greater number of esterase sequences. This result however, is not intended to confirm the identification of a particular gene product. It is important to remember that annotations in genomic (nucleotide sequence) databases are subject to limitations about protein functions especially within a particular biological context (Milkos and Maleszka, 2001). This bioinformatic result does suggest that further investigations are warranted regarding the role of esterase production in coral stress responses.

4.5. Reduction in fitness

Studies have shown that corals can develop of some degree of tolerance/resistance to a variety of stressors such as elevated temperature (Black et al., 1995; Sharp et al., 1997), hydrocarbons (Guzmán et al., 1994), heavy metals (Harland and Brown, 1989), and UV exposures (Gleason and Wellingtion, 1993). Resistance, however, sometimes comes at the cost of a reduction in fitness. In an examination of a Caribbean reef after a major oil spill, Guzmán et al. (1994) determined that corals were capable of faster regeneration rates after injuries on heavily oiled reefs, but the allocation of resources to regeneration decreased fecundity and growth. The physiological cost associated with overproduction of a specific gene product is likely the result of a trade off in resource allocation (Coustau et al., 2000). Such a tradeoff has been observed in other invertebrates overproducing esterases utilized to sequester and hydrolyze organophosphate insecticides (Devonshire and Field, 1991; Chevillon et al., 1999). Studies have shown that esterases can constitute up to 3% of the total cellular protein content in some invertebrates (Devonshire and Field, 1991). Clearly, the allocation of resources to overproduce a specific gene product is likely to impact the production of many other gene products. The detection of a putative esterase transcript in response to dibrom is consistent with the up-regulation of a stressor-induced enzyme. The D25 probe detected its target transcript in field samples taken in August 2000, overexpression of this transcript could have significant impacts on the fitness of adult *A. cervicornis* during this time of the year because this is their reproductive period (*A. cervicornis* in known to spawn during the month of August). Corals typically direct a greater proportion of their resources to production of offspring during this period. A substantial cost of allocating resources to elevated esterase production in August is likely since it would compete with reproductive effort.

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