EFFECTS OF STRESSORS ON DIFFERENTIAL GENE EXPRESSION AND SECONDARY METABOLITES BY *AXINELLA CORRUGATA*

by

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Shirley Pomponi, Department of Biological Sciences, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

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Title: Effects of stressors on differential gene expression and secondary metabolites by *Axinella corrugata*

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Sponges are an important source of bioactive marine natural products, or secondary metabolites. The common Caribbean reef sponge, *Axinella corrugata*, produces an antitumor and antibacterial chemical, stevensine. This study determined whether environmental stressors, such as elevated temperature and exposure to *Amphibalanus amphitrite* larvae, affect the production of stevensine by *A.corrugata* and if the stressors caused *A.corrugata* to exhibit differential gene expression. Temperature stress resulted in no significant change in the production of stevensine; only two genes were significantly differentially expressed, including hsp70. Larval stressed resulted in increased production of stevensine and significant differential gene expression (more than seventy genes). This study suggests that *A.corrugata* may be resilient to elevations in temperature and that one of stevensine's roles in nature is as an antifoulant.

DEDICATION

I dedicate this thesis to my loving family for their unconditional love and support.

EFFECTS OF STRESSORS ON DIFFERENTIAL GENE EXPRESSION AND SECONDARY METABOLITES BY *AXINELLA CORRUGATA*

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INTRODUCTION AND BACKGROUND

Sponges have been around for about 600 million years, as estimated from the fossil record (Srivastava et al. 2010). There are 7,000 identified extant species, and it is hypothesized that approximately 8,000 species are yet to be described (Hooper and Van Soest 2002). Sponges are a dominant part of many marine ecosystems with the ability to not only survive, but proliferate, in most benthic environments as well as some freshwater environments (Yalçın 2007; Bell 2008). Their resilient nature and the fact that they have not just survived for so long, but are a thriving and dominant part of marine ecosystems have made sponges an interesting phylum to study from an ecological perspective. Their role in the production of biologically active marine natural products (MNPs) has made them an invaluable resource for drug discovery. Investigation into the isolation and identification of MNPs is an area of research that has shown significant progress towards the potential development of many antifungal, antimicrobial, and antitumor pharmaceuticals (Yalçın 2007). Natural products, or secondary metabolites, are organic compounds that are not typically involved in the normal development, growth, or reproduction of organisms, but presumably confer an evolutionary advantage to the producing organisms. The majority of marine natural products (MNPs) that are currently in clinical trials or preclinical evaluation have been isolated from invertebrates such as sponges, tunicates, mollusks and bryozoans (Proksch et al. 2002) with sponges being the

most prolific source of new MNPs with bioactivity (Faulkner 2000a, 2000b, 2002; Blunt 2002, 2004).

Sustainable Supply of Marine-derived Chemicals

One of the problems with using natural products for drug development is obtaining the chemical in bulk amounts. Understanding the molecular biology of the marine organisms from which these natural products are derived could enhance the synthesis of secondary metabolites under controlled conditions and lead to *in vitro* production of sponge-derived drugs (Schippers et al. 2011). There are several approaches that have been used for supplying adequate amounts of marine natural products for drug discovery and development. These include chemical synthesis (Koehn and Carter 2005), wild harvest, microbial fermentation, and aquaculture (reviewed in Faulkner, 2000). *In vitro* production is another promising alternative (Pomponi and Willoughby 1994). There has been some success in optimization of sponge cell culture techniques (Pomponi 2006), and *in vitro* production of the antitumor compound stevensine has been demonstrated (Pomponi et al. 1997) but there has not yet been success in developing a sponge cell line for sustainable *in vitro* production.

Axinella corrugata (Porifera: Demospongiae: Halichondrida: Axinellidae) is a common reef sponge that has been used in many *in vitro* studies with some success in primary cell culture development. Increases in *A. corrugata* cell numbers have been achieved using enriched cell culture media consisting of Medium 199 in combination with other supplements and the lectin phytohemagglutinin (Pomponi and Willoughby 1994). The bioactive compound, stevensine, produced by *Axinella corrugata* (Albizati

and Faulkner 1985; Wright and Thompson 1988) was determined to be derived from the sponge and not its microbial symbionts (Andrade et al 1999). *A. corrugata* has also been used as a model sponge for over a decade in the Pomponi lab, proving to be abundant and easily collectible, as well as resilient and responsive when used for *in vitro* studies.

Roles of Secondary Metabolites

There are many theories as to why marine organisms produce secondary metabolites; these include defense against predation, protection against UV radiation, and inhibition of settlement and fouling, to name a few. One of the most ubiquitous theories is that they are synthesized to combat predation (reviewed in Wilson et al. 1999). Soft-bodied organisms with no obvious physical defense mechanisms, e.g., sponges, nudibranchs, and tunicates, are also the richest sources of secondary metabolites (Pawlik 1993).

The coral, *Acropora formosa,* produces mycosporine amino-acids that have absorbance maxima that include UV wavelengths. These compounds decrease with increases in depth suggesting that they may confer some protection against UV irradiation (reviewed in Pawlik 1993). The marine sponge *Dysidea herbacea* also produces mycosporine-like amino acids, which may confer UV protection and may be related to the reproductive state of the organism (Bandaranayake et al. 1996).

Since many of the marine organisms producing secondary metabolites are benthic and sessile, studies have been done on the activity of secondary metabolites as antifoulants. The ascidian, *Eudistoma olivacuem,* possesses eudistomin G and H, metabolites which prevent settlement of the bryozoan, *Bugula neritina,* on the surface of the ascidian (Davis and Wright 1990). Bioactive compounds produced from epibiotic

bacteria found on the surface of the soft coral *Dendronephthya sp.* may contribute to the antifoulant properties of the coral against bacteria and invertebrate larvae (Dobretsov and Qian 2004). The Caribbean coral reef sponge *Axinella corrugata* produces stevensine, a chemical that has antitumor and antimicrobial activity (Wright and Thompson 1988; Yalçın 2007) and has been shown to deter predation by the Caribbean fish *Thalassoma bifasciatum* (Wilson et al. 1999).

Environmental Stressors: Elevated Temperature and Larval Settlement

This study aimed to test how exposure to elevated temperature and fouling organisms affected *A.corrugata's* gene expression and production of secondary metabolites. Different protection responses are produced by organisms that live in environments exposed to stressful conditions (reviewed in Agell et al. 2001). Increased temperature exposure was selected as a stressor due to predicted increases in seawater temperatures associated with climate change. Projections for terrestrial temperature increases are from 1.4°C to 5.8°C by 2100, with coastal marine system predictions being slightly lower (Harvell et al. 2002). Under elevated temperature conditions, organisms produce molecular chaperones, such as heat-shock proteins (hsp70, hsp90, GroEl, TCP1, hsp60) (Lindquist 1986; Sanders, 1993). In sponges, the most studied molecular response to changes in temperature is the expression of hsp70 (H. C. Schröder and Müller 2006), however, the response is variable between different sponges. For example, hsp70 is expressed in *Suberites domunica* as a reponse to thermal stress at 31°C (Bachinski et al. 1997), while the sponge *Cliona celata* showed no significant differences in hsp70 expression in response to temperature stress at 31°C (Duckworth and Peterson 2013).

Another study on *C. celata* revealed no significant loss of pigmentation in response to heat and salinity stress, indicating that the sponge may have a higher threshold to those stressors (Miller et al. 2010). Temperature stress not only affects expression of chaperone proteins, but can affect the production of secondary metabolites. When temperature stress was applied to the St. John's wort plant, the secondary metabolites hypericin and pseudohypericin increased in concentration in the shoots of the plant with increasing temperature (Zobayed and Kozai, 2005).

The second stressor tested in this study was the effect of fouling stress by cyprid larvae of the barnacle, *Amphibalanus amphitrite*, on *A. corrugata*. Many invertebrate larvae of different fouling organisms use α-adrenergic receptor-ligand signals for the induction of settlement (Wang et al. 2006). The catecholamine epinephrine induces metamorphosis and settlement in molluscs, which is potentially mediated through adrenergic receptors (Garcia-Lavandeira *et al* 2004). Cyprids of the barnacle *A. amphitrite* are also stimulated to settle by catecholamines, and certain adrenergic antagonists prevent settlement (Dahlström et al. 2000). The α-adrenergic antagonists idazoxan and phentolamine inhibit settlement of *A. amphitrite*, *Bugula neritina* larvae and *Hydroides elegans* larvae (Dahms *et al.* 2004). Stevensine, a MNP produced by *A.corrugata,* acts as an α1a adrenergic receptor antagonist (Wright et al. 1996) and may function as an antifoulant by displacing ligand binding to the α-adrenergic receptor of fouling larvae.

Gene-expression Analysis

The transcriptome is the complete set of RNA transcripts produced by the genome at any one time. The study of transcriptomes, or transcriptomics, differs from genomics in that the expression of the genes being observed can vary based on different conditions presented to the organism, whereas in the field of genomics, all of the genes present in the cells are studied regardless of circumstance. Transcriptomes can provide a general idea as to which proteins may be expressed in response to certain stimuli. Each condition presented to a sponge can result in expression of a different transcriptome. The goal of this study is to assess which transcriptomes are produced by *Axinella corrugata* in response to exposure to elevated temperature and larvae of an environmentally-relevant fouling organism. In order to study these transcriptomes, mRNA microarrays, a widely used technique to identify and analyze differential gene expression, was used. Microarray techniques have been utilized to investigate a variety of invertebrate organisms. For example, an anthozoan stress-focused microarray was used to diagnose the health of the stony coral *Montastraea cavernosa* in the field (Edge 2012); and *Axinella corragata* cDNA was cross-hybridized to mouse and human microarrays (Lopez et al. 2002). The results from these studies indicated that cross-species hybridization is an effective tool for gene expression profiling in non-model organisms, such as sponges (Willoughby 2002).

Hypotheses

Responses to environmental stress factors result in differential gene expression in sponges and affect the production of secondary metabolites.

1. Tissue explants of the sponge *Axinella corrugata* will respond to environmental stressors by up- or down-regulation of genes
2. Tissue explants of the sponge *A.corrugata* will respond to environmental stressors by changes in production of stevensine.

The specific aims of this research are to:

1. Identify gene expression profiles of *A.corrugata* tissue explants when exposed to different environmental stressors, specifically, elevated temperature and settlement of larvae of fouling organisms.
2. To understand if stress caused by fouling organisms is a driving force in the production of secondary metabolites, specifically, stevensine.

Identifying gene expression profiles will not only provide insight into the genes that are differentially expressed in response to environmental stressors, but could also lead to understanding how the differentially regulated genes correlate to the physiology and function of the sponge, i.e., why and how sponges produce marine natural products, which may contribute to the development of an *in vitro* production method for sponge-derived natural products.

METHODS AND MATERIALS

Field Collections

Six individuals of *Axinella corrugata*, approximately 15-20 cm by 8-12 cm, were collected during April 2012 by scuba from reefs off Dania Beach, Florida (26°10.90'N, 80°04.60'W), at a depth of 40-60 ft. At the time of collection, temperature and salinity were 23°C and 31ppt, respectively. A subsample, approximately 4 x 4 cm, of each individual was placed in 10 mls of Trizol® (Invitrogen) immediately upon return to the surface to serve as a control against any induced stress due to the transport of live samples to the laboratory at Harbor Branch Oceanographic Institute at Florida Atlantic University (HBOI). The remainder of each sponge was placed separately in gallon-sized ziplock bags in a cooler with ice packs for transport back to HBOI.

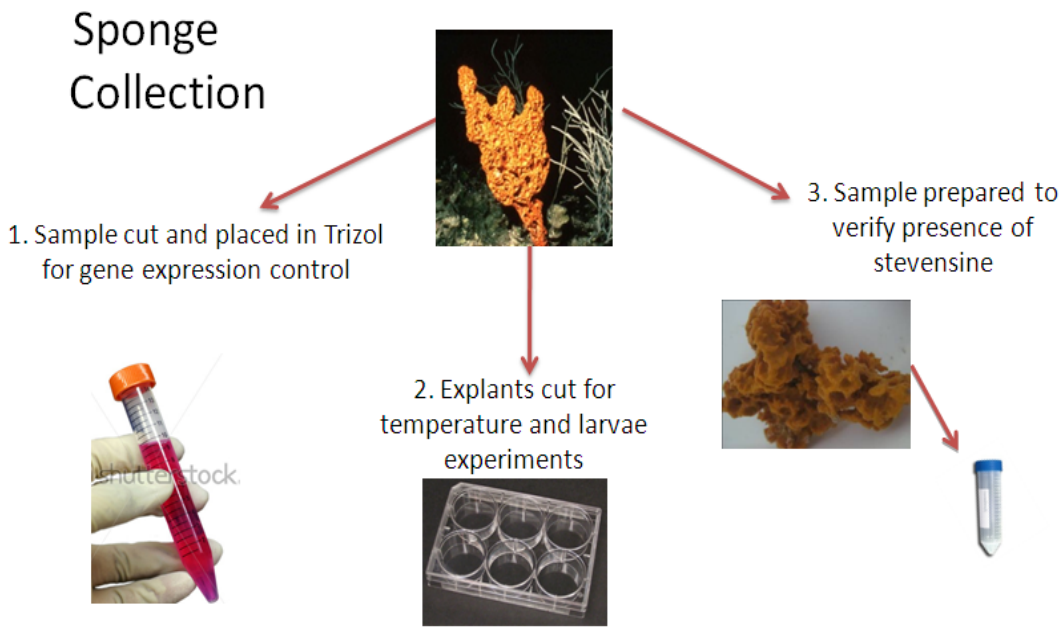

Figure 1. Processing of each replicate sample included dividing the sample into three subsamples. One subsample was cut and placed in Trizol to determine if explants serve as a proxy for environmental conditions. A second subsample was prepared for stress exposures. The final subsample was used as a control to confirm that each sample was producing stevensine.

Sponge Explant and Exposure Preparations

Sponge samples were sliced into 1 cm^3 fragments, or explants, after being transferred to HBOI. From each sponge, four 6-well plates were prepared as follows: six explants were cut from each sponge per plate used and placed into 3 wells of a six-well plate, with two explants per well. Each well was filled with 10 ml of filtered seawater (FSW). Plates with explants were then incubated at 23°C for 3 days with 12 hour light/dark cycles to allow the explants to adapt to the culture conditions. The FSW was changed on the third day before treatments were initiated.

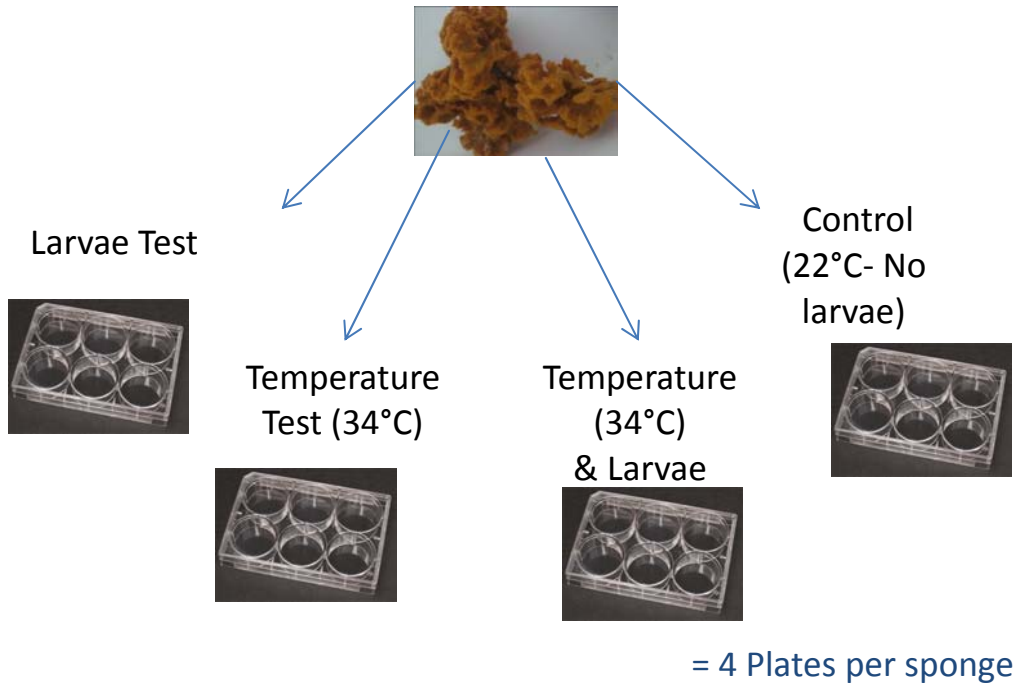

Figure 2. Explants from each replicate sample were distributed to four multiwell plates, one plate per stressor. There were four plates per replicate with a total of twenty-four plates for all six replicates. Exposures were conducted for twenty-four hours.

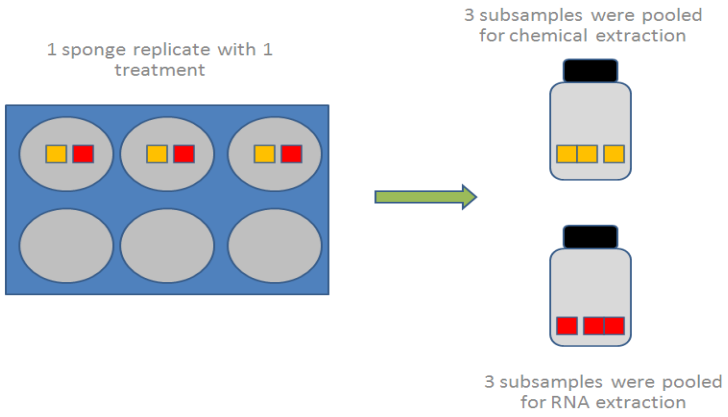

Figure 3. Separation of samples for chemical analysis and gene expression analysis. There were only three wells used per plate and two explants at 1cm^3 were placed in each well. The colors represent that half of the explants in each well were used for chemical anaylsis (yellow) while the other half were used for gene expression analysis (red). Explants were exposed to the stressors (temperature alone, larval challenge alone, both temperature and larvae together) for 24 hours.

Preparation of Fouling Larvae

One milliliter of seawater containing ~25-30 *Amphibalanus amphitrite* cyprids per ml was added directly to treatment wells via pipetting. The cyprids were provided by Dr. Dan Rittschof (Duke Marine Laboratory, Beaufort, North Carolina) and cultured based on details he provided. *Amphibalanus* cyprids settle at room temperature and do not actively swim under light. Cyprids were cultured at 6°C at a salinity of 35‰ in the dark for three days until ready for use.

Analysis of Stevensine Concentration

Extract Preparation. One subsample of each sponge individual was used for extraction and chemical analysis of stevensine concentration (Figs 1, 3). 10 – 20 g of each sample were placed into a stainless steel grinding cup. Ethanol (~20ml) was added to the cup and the sample was ground to a slurry using a single-bladed Virtis grinding apparatus. All contents were removed from the cup using a spatula, the cup was rinsed with ethanol, and the contents were transferred to a clean glass vial. After steeping overnight, the extract was vacuum-filtered and dried in a round-bottom flask using a Rotavapor R-114. A combination of methanol and ethyl acetate 1:1was used to remove the sample from the flask and transferred to a clean pre-tared glass vial and dried under a stream of nitrogen gas. The dried extract was then weighed and resuspended in methanol to a set concentration of 10mg/ml.

Calculation of Standard Curve. Quantification of stevensine was determined by comparison with a standard curve, obtained by HPLC analysis of pure stevensine (provided by Dr. Amy Wright, from the HBOI library of pure compounds). Serial

dilutions of stevensine in methanol, ranging from 31.25 µg/ml to 1mg/ml were prepared, and 20 µl of each dilution were analyzed by HPLC. The stevensine standard curve had a coefficient of determination of 0.9989 (Fig. 4). From this curve, an equation was derived (y=0.0348x-0.0003, where y= absorbance at 230nm) and used to quantify the total amount of stevensine in experimental samples.

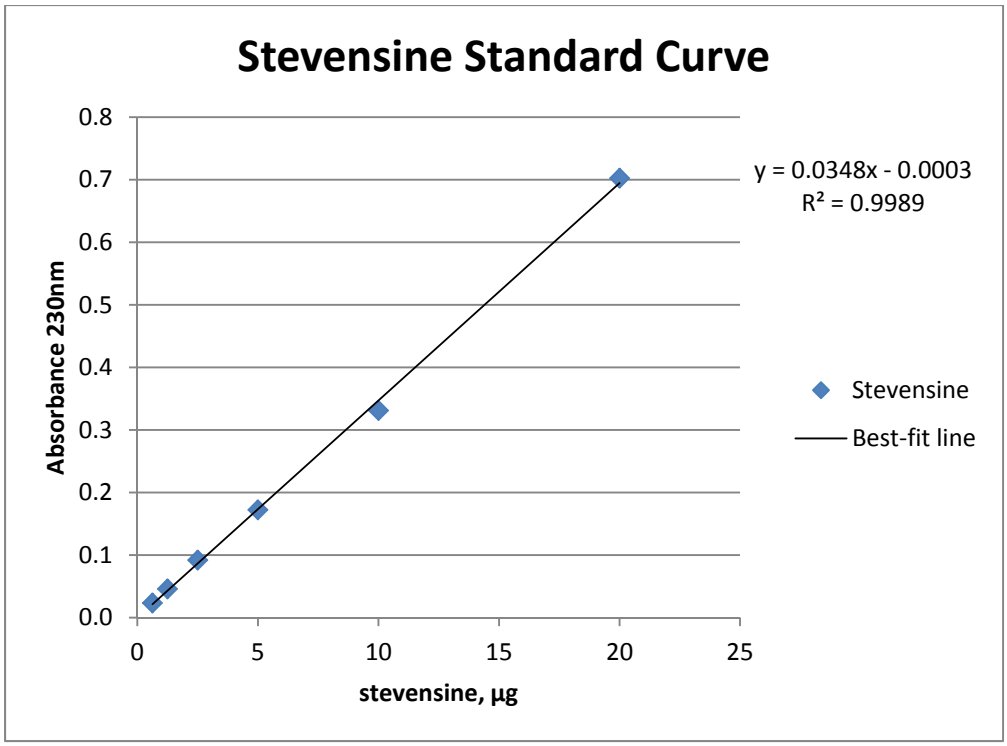

Figure 4. Serial dilutions of pure stevensine (31.25 – 1000 µg/ml) were used to derive a standard curve for quantification of test samples. Standard curve of pure stevensine analyzed at 230nm absorbance. Coefficient of determination = 0.9989.

HPLC Analysis. *Axinella corrugata* explants were extracted and analyzed for production and quantification of stevensine using HPLC (Pomponi *et al.* 1997). The samples were analyzed by HPLC using a Hitachi La Chrom HPLC system with autosampler. This system is comprised of a D-7000 interface, an L-7455 diode array detecter, an L-2700 AutoSampler, and an L-7100 pump. A Vydac Protein and Peptide

C18 analytical column (4.6 mm x 250 mm) was used for the analysis. Autosampler parameters are detailed: sample identification number, rack position, analytical method (Autosampleranalysis_30min), injection volume (20 µL), column equilibrium time (6 minutes), and a brief sample description. The parameters for the Autosampler analysis_30min program is as follows: Linear gradient flow maintained at 1 ml/minute: Solvent A: H_2O:CH_3CN:TFA (95:5:0.1% v/v); Solvent B: CH_3CN: 0.1%TFA; t=0 minutes A:B (9:1 v/v); t=20minutes 100% B; t= 30 minutes 100% B; t=32 minutes A:B (1:1 v/v). The analysis is detected using a photodiode array detector over the wavelengths 210 to 600 nm. The column was equilibrated 6 minutes between runs. Three methanol blanks were run prior to sample analysis to provide a good baseline before analysis. 20 µl of each extract were injected into the HPLC and their absorbance measured at 230 nm. This absorbance was entered into the equation obtained from the standard curve to determine total amount of stevensine contained in each experimental sample.

RNA Isolation, Purification, and Labeling

After stress exposure, one subsample of tissue from each sponge individual was used for RNA extraction and microarray analysis. The sponge pieces were placed in the commercially available guanidine-based preservative TRIzol (TRIzol®, Life Technologies) to stop gene expression. *Axinella corrugata* explants in 6-well plates were transferred to 15 ml tubes with 10 ml of TRIzol® preservative added to each tube. The samples were stored at -80°C until ready for RNA extraction. Total RNA was isolated

from a 2 ml aliquot of preserved sponge following the manufacturer's protocol (TRIzol® Life Technologies, Carlsbad, CA; based on (Chomczynski and Sacchi 2006). The protocol suggests using glycogen (5-10µg) when working with low tissue volumes. Due to low RNA yields (<100ng/µl), 5 µg glycogen was added as a carrier to the aqueous phase during the RNA precipitation step. Further purification was performed by adding an equal volume of 13.3 M lithium chloride to RNA pellets resuspended in nuclease-free water (Aglyamova et al. 2009). After purification, quantification and qualification of RNA were performed using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Inc., MA USA) and gel electrophoresis (Invitrogen, Carlsbad, CA; Cat#:G4010-01) High quality RNA samples from the coral *Eunicea fusca* were used for control lanes in E-gels. Five micrograms of each sample of purified total RNA were labeled with cyanine dye, Cy5, following the manufacturer's protocol (LabelIT®, Mirus Bio LLC). Labeled RNA was purified following the manufacturer's protocol using 5M sodium acetate and ethanol precipitation and then resuspended in 11 µl of nuclease-free water.

Microarray hybridization and data analysis

The microarray used in this study was created to detect anthozoan gene expression, printed by Ecogenomics and based on research by Dr. Sara Edge (Edge 2012). The array consists of anthozoan genes involved in cell functions ranging from metabolism and development, to regulation of apoptosis and the stress response (Edge 2007). There are 148 genes replicated three times on the array to generate 742 probes. The genes are divided into 27 categories based on primary cell function and then further

grouped to provide an overview of the response of the test species. The functional groups include normal cell function (NCF), multifunctional response (MF), stress response (SR) and symbiotic specific response (ZOOX) (Edge 2012).

Fluorescently labeled RNA (1 – 1.5 μg) from each sample was hybridized to replicate microarrays at 45°C for 14 hours, washed to removed excess fluorescent dye and then scanned according to the manufacturer's protocol (Combimatrix Inc.). A GenePix 4200A microarray scanner was used to detect fluorescence from each spot on the arrays and GenePix Pro 6.0 software was used to quantify signal intensities (Molecular Devices, LLC).

All arrays were visually inspected for hybridization irregularities and data from inconsistent spots, including blurs, smudges and false signals due to scratches, dust or mishandling were discarded (Edge 2007). Data were log base 2 transformed and normalized using a loess smoothing model. Analysis of variance (ANOVA) statistical F-test was performed to determine significant differences in gene expression (JMP Genomics, SAS Institute). The model incorporated treatments (control, larvae, temperature, and temperature plus larvae) as fixed effects, with a cut-off value for significance of $p<0.01$. Hierarchical clustering analysis of significant genes was performed between treatment groups resulting in clustering based on similarities in gene expression patterns (Edge 2007). Least squares profiles, based on normalized signal intensities, of significant genes generated by ANOVA were standardized to zero. Deviations from the standardized least squares means (SLSM) were compared between genes in different functional groups (Edge 2007).

RESULTS

Stevensine Analysis

The total amount of stevensine contained in each experimental sample is depicted in Figure 5, with amounts ranging from 1 to 9 mg of stevensine produced. Although samples were cut to $1cm^3$, there was variability among weights of the explants within each treatment. Since weights were not obtained prior to extraction, the values cannot be directly compared, but data were graphed to determine if there were trends in stevensine production. The graph shows a similar pattern in all samples in the presence of larvae, consistently resulting in an increase in stevensine production when compared to the control. Although changes in production of stevensine were observed with the other treatments, the results from temperature and the combination of temperature and larvae did not produce any consistent trends.

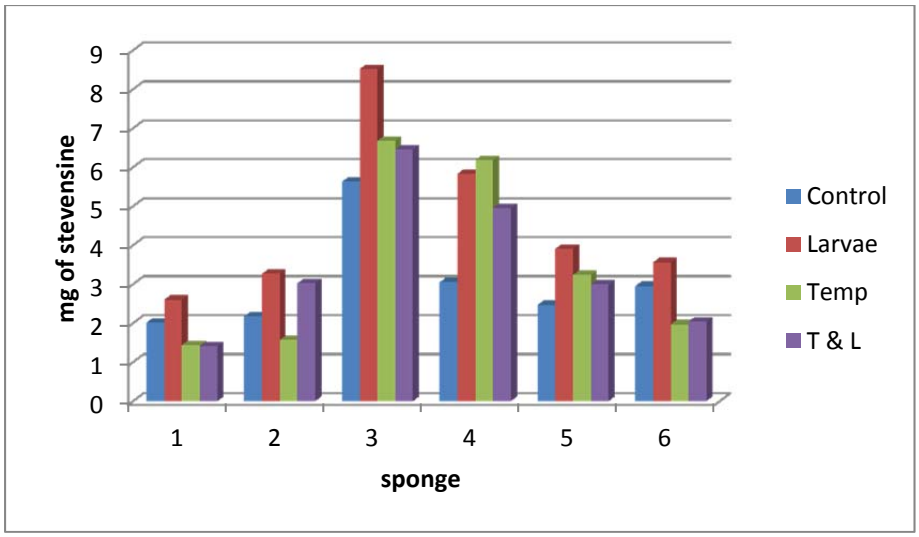

Figure 5. Total amount of stevensine in samples from each treatment. Exposure treatment groups are compared among the six replicate samples. Consistent trends of explants with larval stress show increases of stevensine when compared to the control; responses to temperature and the combination of temperature and larval stress (T & L) showed no consistent trends.

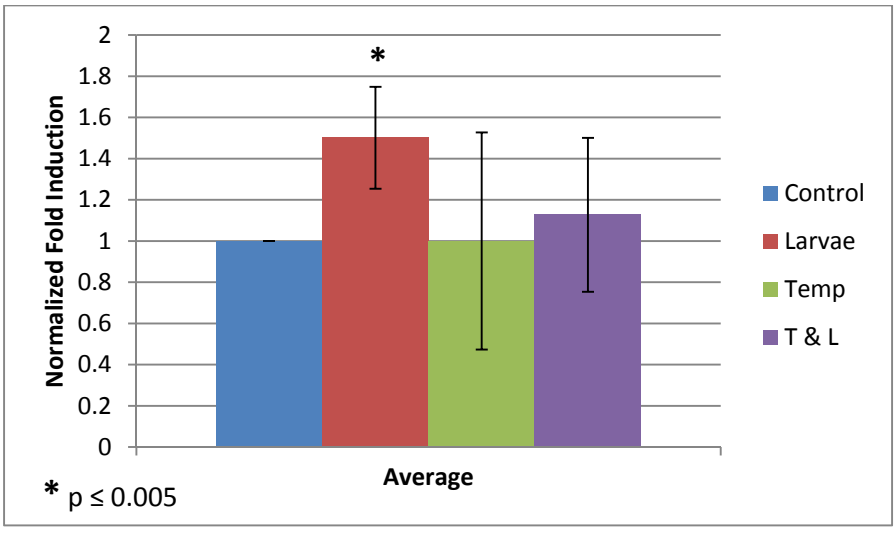

Figure 6. Normalized stevensine production per experimental treatment. A significant difference between larval stress and control was found, where larval stress on *A.corrugata* explants resulted in increased stevensine production when compared to the control. $P \leq 0.005$, n= 6.

To visualize trends, the average stevensine production per experimental treatment was calculated and normalized against the average of the control (Fig. 6). A student's t-Test was used to determine statistically significant difference between experimental treatments. There is a significant increase in stevensine production in response to larval stress compared to the control, with a P-value less than 0.005. The same response was not observed with the combined temperature and larval treatment. This is likely the result of elevated temperature killing the larvae. Upon exposure to the treatment temperature, the larvae were not moving, were presumed dead, and likely did not stimulate a stress response in the sponges.

Gene Expression Analysis

Samples were considered appropriate for labeling and hybridization after NanoDrop analysis and gel electrophoresis if two dark bands, representing 28S and 18S ribosomal RNA, appeared on the gel (Fig. 7).

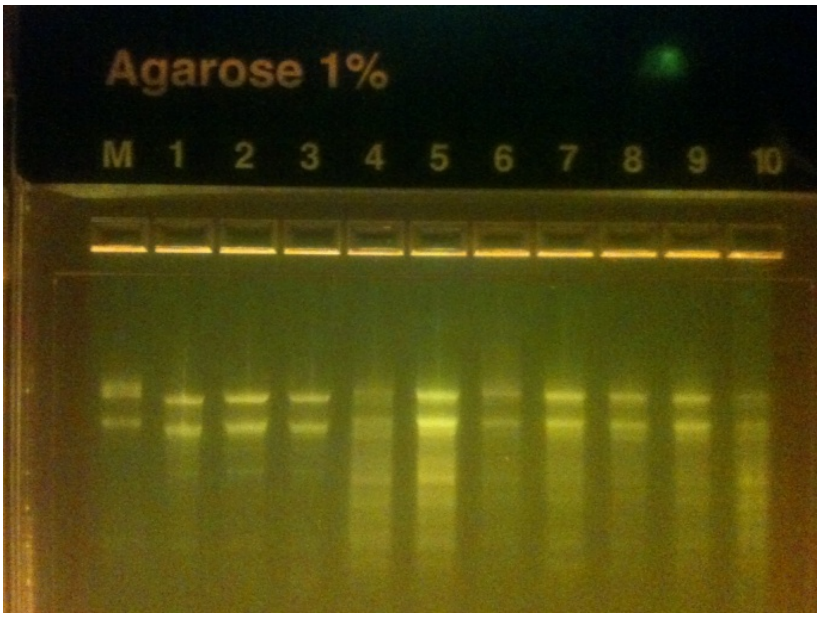

Figure 7. Electrophoresis gel testing for RNA quality. Quality RNA isolated from *Eunicea fusca* was used as a control (M lane), with the *A.corrugata* samples in lanes 1-10. The bands represent 28S and 18S RNA.

Based on the ANOVA test, exposure to larval stress resulted in the highest number of statistically different genes expressed when compared to control. Interestingly, very few changes were observed with exposure to increased temperature. The lack of significant changes in gene expression in the combination of temperature and larvae exposure is likely due to the larvae dying in the higher temperature (Table 2, Fig. 8). The treatment group labeled "extract" only differed from control groups by a total of six probes representing five different genes that are mostly involved with cell rearrangement.

Table 1. Number of Significant Probes**.** Number of probes whose responses had significant differences from the control, grouped according to experimental treatment based on ANOVA.

Treatments	Non-significant	Significant
Control vs. Extract	**730**	**6**
Control vs. Larvae	**646**	**90**
Control vs. Temperature	**734**	**2**
Control vs. Temp & Larvae	**736**	**0**

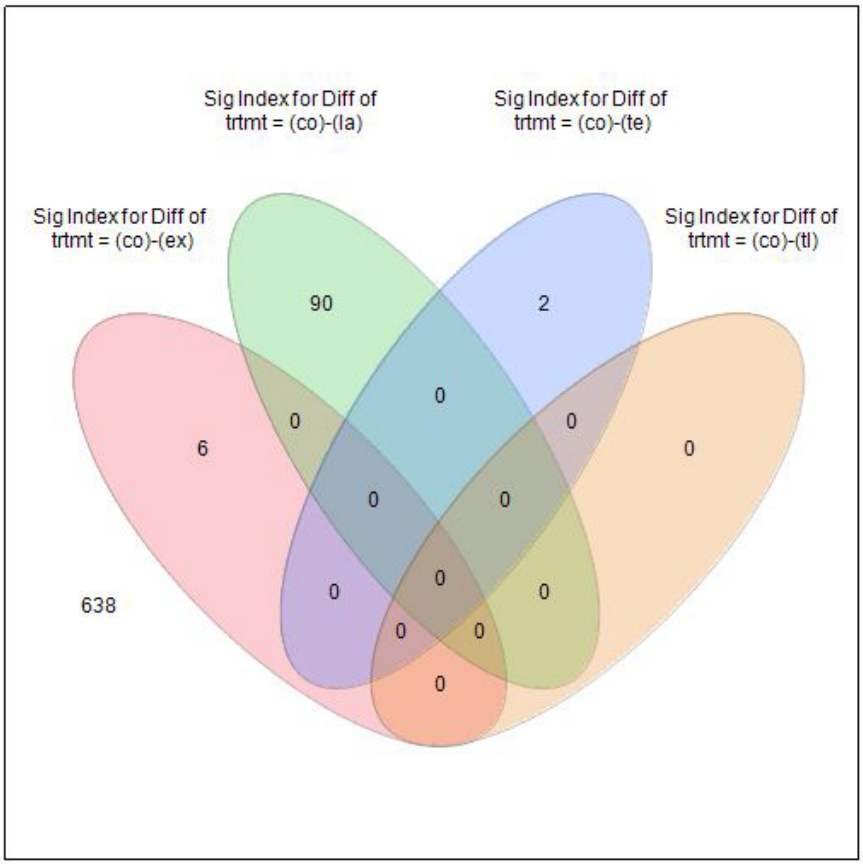

Figure 8. Significant differences of genes found when comparing each treatment to the untreated control. Left to right: comparisons of control (co) vs. untreated explants (ex) (i.e., subsample collected and preserved immediately after collection) with a difference of 6 significant genes (co-ex); comparison of control vs. larval exposure (la) with a difference of 90 genes (co-la); comparison of control vs. elevated temperature (te) with a difference of 2 significant genes (co-te); and comparison of control vs. temperature and larval exposure (tl) combined with no significant difference in genes found (co-tl). Of the genes that were found significant within each comparison, there was no overlapping of significant genes between treatment groups

Hierarchical clustering of the standard least squares means of genes among treatments is represented in the heat-map in Figure 9. Temperature stress and larval stress are grouped farthest away from the control, suggesting that gene responses in those treatments differed the most from control than the remaining experimental treatments. The combination of temperature and larval stress on *A.corrugata* was grouped closest to the control group, indicating the least amount of difference of gene expression. The heat map also suggests an inverse regulation of gene expression due to temperature exposure and to larval exposure: in one treatment the genes are up-regulated, and in the other treatment the same group of genes is down-regulated. Though the inverse relationship between temperature stress and larval stress is most apparent, the remaining treatments tend to follow the same gene expression trends as temperature stress, leaving larval stress to have the most opposing expression compared to the other groups (Fig. 10).

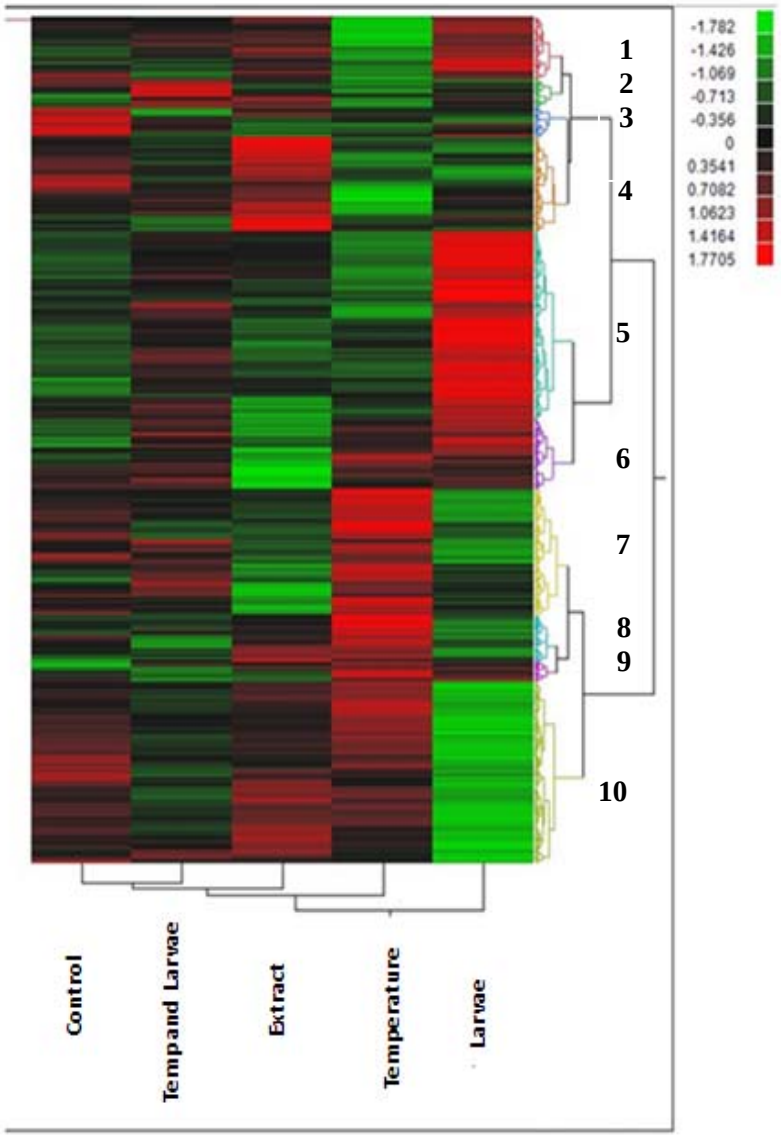

Figure 9. Heat map of relative fluorescence. Up- or down-regulation of gene expression based on relative fluorescence intensity of each gene on the microarray, as determined by SLSM. Deviations from the SLSM (standardized to zero) are represented by green (fluorescence intensities below zero) or red (fluorescence intensities above zero). Relative intensities are indicated in the legend. The y-axis represents different gene clusters which are represented by the different colored regions, where it shows ten different gene cluster groupings which are numbered.

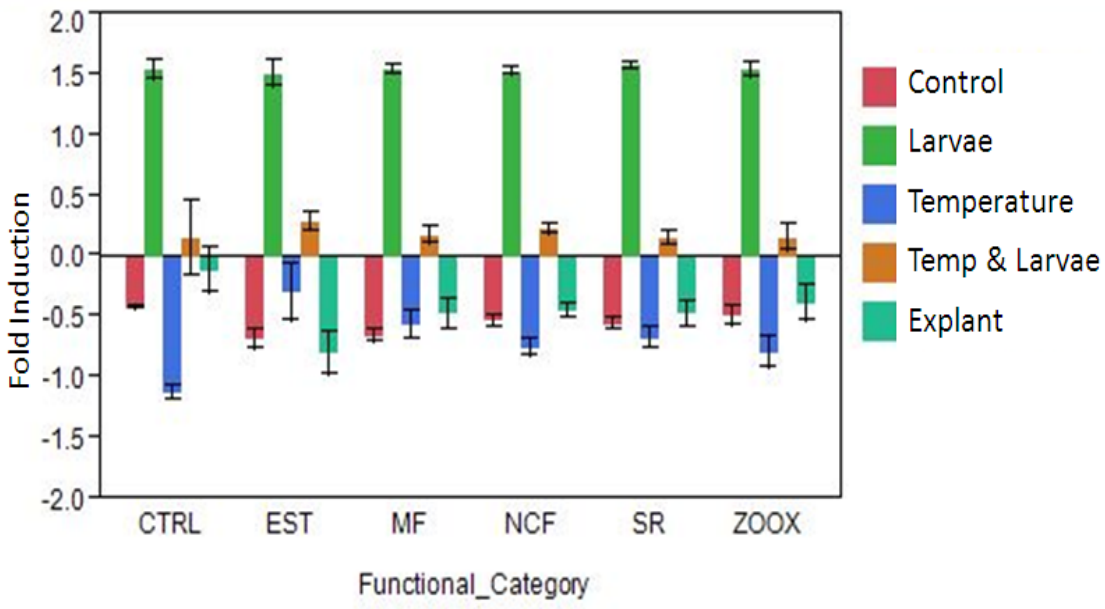

Figure 10. Graphical representation of hierarchical clustering. This chart represents the gene clusters 5 and 6 (on y-axis of Fig. 9) which were mostly up-regualted in larvae. Along the x-axis are the five different functional categories: Control (CTRL), Expressed Sequence Tag (EST), Mulitfunctional (MF), Normal Cell Function (NCF), Stress Response (SR), and Zooxanthellae. With the exception of the temperature and larval stress treatment group, all other treatment and control groups behaved similarly with a decrease in gene expression, while larval stress had large increases in up-regulation of gene expression.

Differential Gene Expression

In order to determine significant differential gene expression, ANOVA was used to indicate significance in differential expression by probes. Every probe represents one amino acid sequences of a particular gene and each probe is replicated 3-5 times on the array. Therefore, probes were averaged when ANOVA was conducted and significant probes do not have error bars when graphing. However, different sequences of the same gene can be represented by multiple probes and can therefore be represented with error bars. While undergoing statistical analysis of gene expression, two samples were excluded from analysis due to technical variability by samples not hybridizing properly to

the microarray, and in order to maintain a balanced design, four replicates of each sponge were used from each treatment for analysis. The standard least squares mean (SLSM) was determined in order to visualize differential gene expression and is a representation of ANOVA data comparable to fold change.

Responses to larval stress

The differences in gene expression at the level of functional groups between the control and the explants exposed to larvae stress were evaluated. The genes that were up- and down-regulated in response to larval stress compared to the control are listed in Table 3, and the total number of genes up- or down-regulated for each functional category is listed in Table 4 and displayed in Figure 10. The genes that cluster under the functional group "growth and development" showed the most up-regulation, with 7 genes in this group being up-regulated. The second functional group with the most genes up-regulated was oxidative stress, followed by cell respiration, metabolism, and regulation of transcription. In general, there was more up-regulation of gene expression than down-regulation due to this stressor. Genes in the functional groups "growth and development" and "response to xenobiotic" showed the most down-regulated genes with 4 genes in each.

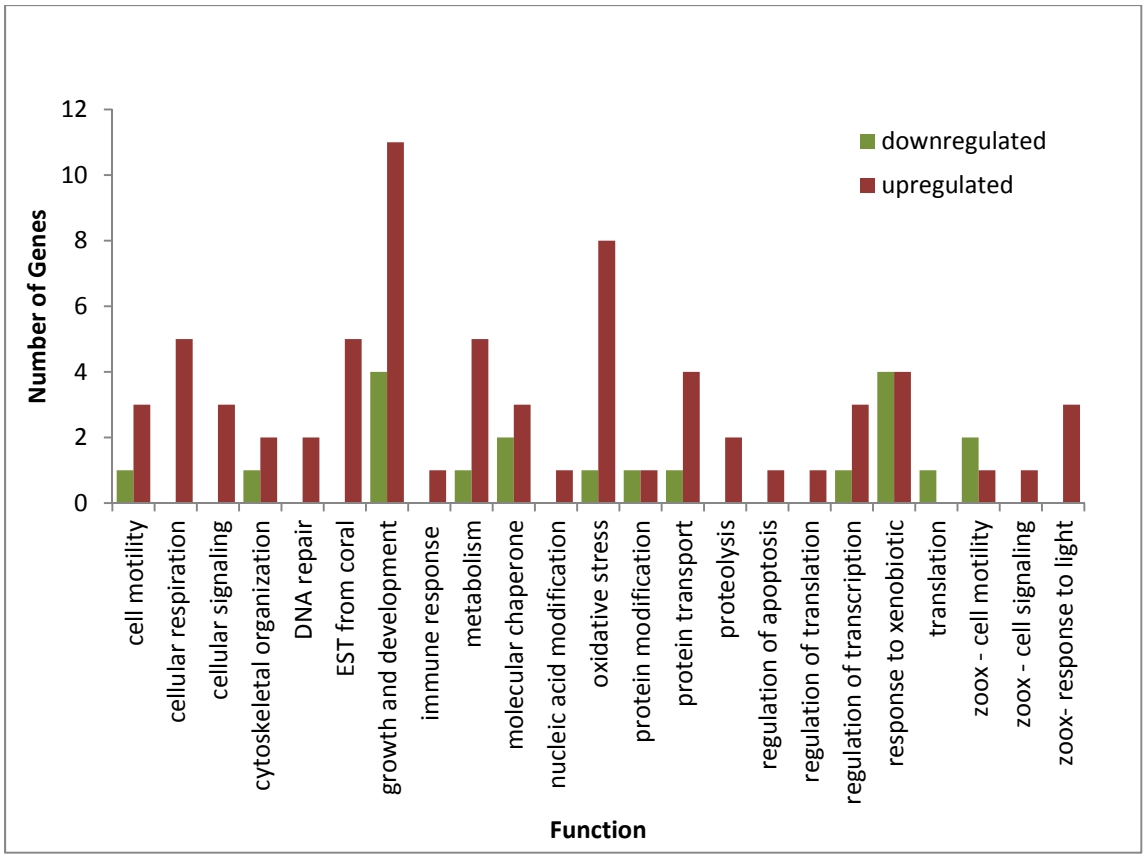

Figure 11. Functional gene groups regulated in response to larval exposure. There are 23 functional categories representing 70 genes differentially expressed.

Table 2. Genes up- and down-regulated in response to presence of *A.amphitrite* larvae vs. control. Genes highlighted red were up-regulated while those highlighted green were down-regulated. Genes that were expressed more on more than one probe were noted and their accession numbers were listed if different from first gene expressed. P-values for each gene sequence is listed. Some genes were found to be both significantly up- and down-regulated and are indicated in yellow.

Function	Gene	Accession_No	Accession_No: seq 2 and 3 if different from seq 1	seq 1,2,3: p-value
cell motility	tubulin, alpha	L13999		0.000659
	tubulin, beta	U60604		0.004888
	myosin heavy chain	EZ002271		0.004467, 0.001538
cellular respiration	NADH-ubiquinone dehydrogenase	EZ026766		0.008935
	Mitochondrial Cytochrome b (Cytb)	AB117374		0.006260, 0.001844
	succinate dehydrogenase	NM_001139616		0.000278
	cytochrome oxidase subunit I (COI)	AF013738		0.006392
cellular signaling	Calmodulin	EZ031544		0.002089
	calcium/calmodulin-dependent protein kinase IV-like (CAMK4)	DQ309544		0.002994
	Frizzled-8 (fzd8)	EZ019620		0.004272
cytoskeletal organization	Profilin	EZ023730		0.008494
	Integrin beta-2 (ITGB2)	AF005356		0.001853, 0.002158
DNA repair	DNA-3-methyladenine glycosylase (Mpg)	EZ033943		0.000089
	DNA-3-methyladenine glycosylase (Mpg)	EZ033943		0.005828
EST from coral	presettlement larvae	DY586756	DY585902	0.001072, 0.008641
	isolated from dark exposed *M. faveolata*	JK822210		0.007499, 0.002279
	isolated from dibrome exposed A. cervicornis	BI534456		0.002588
growth & development	Dystonin	JK845949		0.003469
	homeodomain protein cnox-2 (cnox-2)	AF245689		0.003382
	decapentaplegic (dpp)	AF285166		0.008856, 0.004432
	collagen	DQ309546		0.001986

	Galaxin	AB086183		0.003542
	troponin T (TnT)	JK822206		0.009319, 0.007485
	28 kDa heat- and acid-stable phosphoprotein (PDAP1)	EZ036134		0.001919, 0.005631, 0.001269
	spectrin alpha II chain (Spna2)	GQ502182		0.000325, 0.007938
	actin	AY360081		0.001447
	collagen	DQ309546		0.009244
immune response	ferritin	JK822205		0.000347
metabolism	beta-1,3-glucanase (acid glucanase)	XM_002165050		0.007636
	acyl-CoA thioesterase	DQ309539		0.007110
	arginine kinase	AY531316		0.002082, 0.000435
	Aldehyde dehydrogenase family 8 member A1 (aldh8a1)	EZ008165		0.000752
	Glycine amidinotransferase (GATM)	EZ002467		0.008303
molecular chaperone	HSP70 mRNA for heat shock protein 70	AB201749		0.002525, 0.009838
	Hsp70 interacting protein (suppression of tumorigenicity 13; ST13)	NM_001030757		0.005909, 0.001255
	hsp27	JK822212		0.003092
nucleic acid modification	regulator of nonsense transcripts	JK822213		0.001275
oxidative stress	Catalase (Cat)	DQ104435		0.005536
	heme binding protein 2 (HEBP2)	DQ213995		0.000620, 0.006059
	Glutaredoxin	EZ030451		0.000069
	Thioredoxin (Trx)	JK845950		0.005351
	copper/zinc SOD	AY164663	U27840, DQ309550	0.005817, 0.000496, 0.003094
	Glutaredoxin	EZ030451		0.001128
protein modification	polyubiquitin	JK822200		0.000843
	Cd36/Scavenger receptor	DQ309525		0.007560
protein transport	Importin-5 (IPO5)	EZ001642		0.009343
	Ribosome-binding protein 1 (Rrbp1)	EZ012511	EZ012511	0.004042, 0.001732
	TRAPD	JK822204		0.007980
	charged multivesicular body protein 2a (chmp2a)	JK822214		0.004224

proteolysis	Membrane-bound transcription factor site-1 protease (MBTPS1)	EZ004852	EZ004852	0.004286, 0.006659
regulation of apoptosis	caspase	DQ218058		0.009918
regulation of translation	Eukaryotic translation initiation factor 3 subunit A (Eif3a)	EZ002360		0.002797
regulation of transcription	nuclear receptor AmNR8	AF323688		0.003605
	Regulation of nuclear pre-mRNA domain-containing protein 1B (RPRD1B)	EZ013430		0.000114
	Coiled-coil domain-containing protein 58 (CCDC58)	EZ042546		0.006976
	Calreticulin (Calr)	EZ022703		0.000478
response to xenobiotic	Microsomal glutathione S-transferase 3 (MGST3)	EZ040771		0.003275, 0.007573
	multidrug resistance protein	XM_002154448		0.006667, 0.003869
	glutathione-s-transferase	EU747061		0.006083
	Selenium-binding protein 1 (selenbp1)	EZ043132		0.005569
	Tartrate-resistant acid phosphatase type 5 (ACP5)	EZ020317		0.002139
	naphthalene - Ceruloplasmin homolog	DN167139		0.006338
translation	28S ribosomal RNA gene	JK822201		0.003440
zoox - cell motility	Actin	AB086828		0.008941, 0.006227
	tubulin, beta	K03281		0.000418
zoox - cell signaling	calmodulin	AF007889		0.007587
zoox - response to light	peridinin chlorophyll-a binding protein	AY149139		0.001201, 0.000140
	peridinin chlorophyll-a binding protein apoprotein precursor (pcp) gene	AY149170		0.006646

Standardized least squares mean was used, as stated previously, to determine fluorescence intensity deviations among significant genes. Of the significant genes expressed, replicate gene expression occurrences were averaged and represented graphically with error bars in Figure 12.

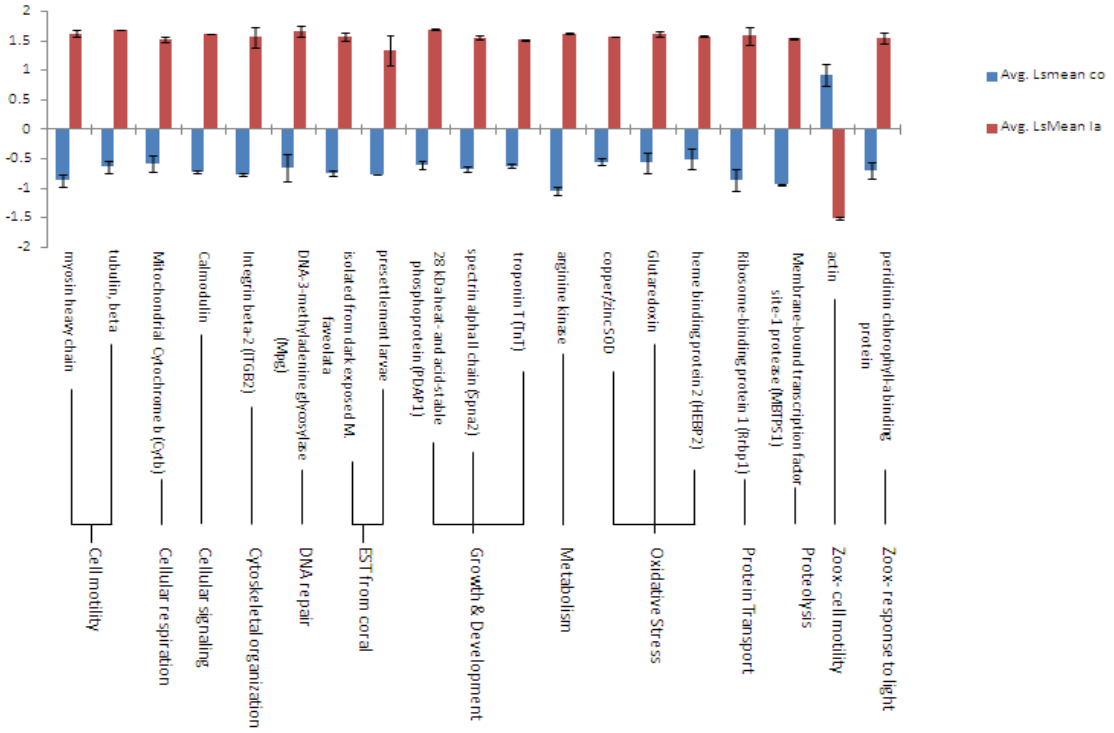

Figure 12. Deviation of significant genes (determined by ANOVA) from SLSM comparing control (blue) and larval stress treatment (red). SLSM = 0. Significant genes are further grouped by functional category.

Responses to temperature stress

Only two genes showed significant differences from the control in samples exposed to elevated temperature (Table 4). The gene leucine-rich repeat ribonuclease inhibitor family protein (NLRP4), whose function is in inflammation, was down-regulated. As expected, heat shock protein 70 (hsp 70), which is a molecular chaperone that serves as a marker for thermal stress, was up-regulated (Table 4).

Table 3. Genes significantly up- and down-regulated in response to elevated temperature stress vs. control. Genes highlighted red were up-regulated while those highlighted green were down-regulated.

Gene	Function	p-value
leucine-rich repeat ribonuclease inhibitor family protein (NLRP4)	inflammation	0.00444
hsp70	molecular chaperone	0.006954

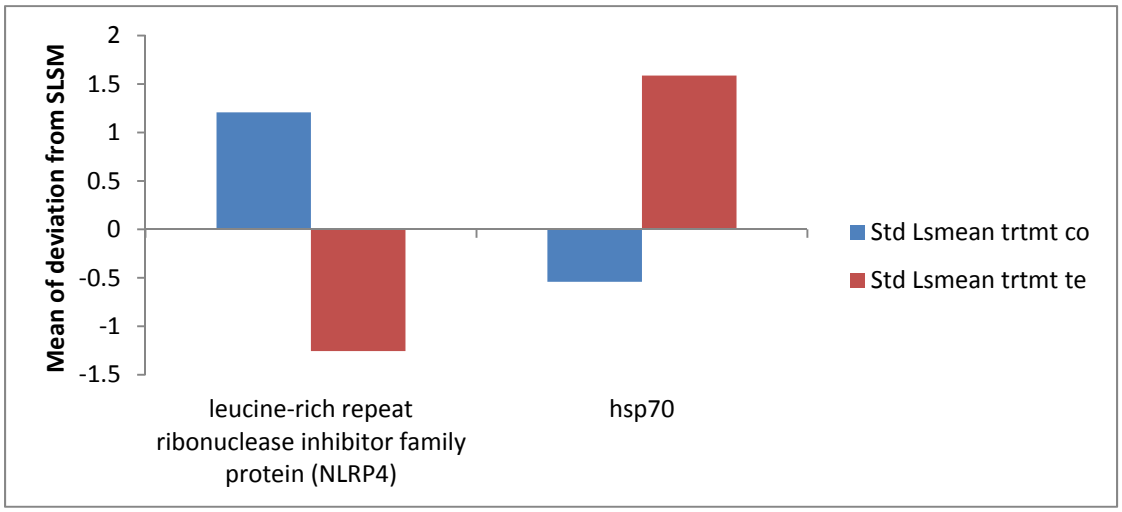

Figure 13. Deviation of significant genes (produced by ANOVA) from SLSM comparing the control (blue) and the temperature stress treatment (red). SLSM = 0.

Responses to the combination of temperature and larval stress

No statistically significant differences in gene expression were observed between *Axinella corrugata* controls and explants exposed to the combination of temperature and larval stress. This is likely the result of the death of the barnacle larvae. Further experiments are needed to determine what changes in gene expression are caused by exposure to a combination of these stressors.

DISCUSSION

The studies to determine the responses of *Axinella corrugata* explants to elevated temperatures and/or *Amphibalanus amphitrite* larvae exposure demonstrated significant responses. Analyses of stevensine concentration suggest that larval stress results in an increase in the production of this compound. Gene expression analyses demonstrated that *A.corrugata* explants were differentially impacted by temperature and larval stress.

The conditions in which the explants were maintained in the lab were chosen to mimic as much as possible environmental conditions. Due to a previously successful study of hybridization of *A.corrugata* to a human array to test for changes in sponge cell culture to phytohemagglutinin (Willoughby 2002), and the fact that many genes are highly conserved among phyla, it was hypothesized that a coral array would produce gene expression results in this experiment. Though some genes may be categorized under a functional group that pertains more to an anthozoan response, ex. ZOOX, they may serve a different function when expressed by sponges.

Responses to temperature stress

Water temperature fluctuations can be a common stressor for many marine organisms (Teoh 2010). Some studies have shown that sponges may be resilient to elevated temperature (Duckworth and Peterson 2013). A preliminary experiment in

which primary cell cultures of *Axinella corrugata* were exposed to higher temperatures resulted in an increase in cell growth (Grima 2011 unpublished data). There was no significant increase in stevensine production in explants exposed to elevated temperature.

Gene expression analyses in response to elevated temperature resulted in only two differentially expressed genes when compared to the control: the leucine-rich repeat ribonuclease inhibitor family protein (NLRP4), which is involved in inflammation, was significantly down-regulated. Hsp70 was significantly up-regulated in *A. corrugata* explants exposed to higher temperature. This response is to be expected since it is a thermal stress response gene, and most organisms display the same response when exposed to higher temperatures (Lindquist 1986; Müller et al. 1995; Bachinski et al. 1997).

Responses to stress by exposure to fouling larvae

Induction of metamorphosis of larvae of sessile marine invertebrates can be attributed to chemical cues from the environment (Kon-ya 1994). Catecholamines, such as epinephrine and norepinephrine, are ligands for α1 adrenergic receptors and induce settlement in marine larvae (Kon-ya 1994; Garcia-Lavandeira et al. 2005). The α-adrenergic blocker, phenotolamine, is an inhibitor of settlement by *Balanus* cyprids (Yamamoto et al. 1998). In this study, *Axinella corrugata* explants were exposed to *A. amphitrite* cyprids to test the hypothesis that such exposure would increase the production of the α-adrenergic antagonist, stevensine. Since sponge explant weights were not measured prior to extraction, the absolute change in concentration of stevensine could not be determined. However, distinct trends in stevensine concentration indicated an

increase in sponges exposed to larval stress (Fig. 6). This suggests that the presence of larvae elicits an increase in stevensine production in response to fouling. To confirm this hypothesis, the experiments need to be repeated, with initial weights measured.

Altered gene expression in response to the presence of *A. amphitrite* cyprids was greater than in other treatment groups. Differential gene expression involved cellular processes, described and categorized based on gene function such as: regulation of transcription, cell respiration, metabolism, bioluminescence, regulation of apoptosis, molecular chaperone, proteolysis, metal ion regulation, inflammation, DNA repair, oxidative, response to xenobiotic, wound healing, and zooxanthellae. Many of the genes significantly expressed have no functional description specific for sponges, but are common stress responses among a variety of organisms (Sanders 1993). For example, glutaredoxin is shown to be elevated in response to oxidative stress by *Saccharomyces cerevisiae* and *Brassica juncea* (Reddy and Sopory 1999; Pedrajas et al. 2002). Larval stress resulted in a considerable impact on gene expression; 70 genes were significantly expressed when compared to control explants. Differentially expressed gene functions were grouped into the following categories: Normal Cell Function (NCF), Multi-functional (MF), Stress Response (SR), and Symbiont-specific (ZOOX) (Edge et al. 2012).

Of the functional groupings of genes categorized under Normal Cell Function (NCF), genes involved in cell motility, cellular respiration, cell signaling, cytoskeletal organization, growth and development, metabolism, nucleic acid modification, protein modification, protein transport, regulation of transcription, regulation of translation, and translation showed significant differential expression from controls. Genes grouped under

cell respiration, cell signaling, nucleic acid modification, and regulation of translation were up-regulated, while the remaining categories had the majority of the genes expressed up-regulated as well. Many of the genes up-regulated are regulatory genes of the functions listed indicating that larval stress evokes a response in *A. corrugata* explants that disrupts normal cell activity. For example, myosin heavy chain and beta tubulin were up-regulated which could indicate possible cell reorganization in response to larval stress

Multifunctional (MF) categories of gene function that showed differential gene expression were molecular chaperones, proteolysis, and regulation of apoptosis. Of the six genes involved in regulation of apoptosis, only one gene, caspase-3/7, which has a role in the execution phase of apoptosis was up-regulated (www.genecards.org). This might suggest that apoptosis was not a main response to this stressor. No other analyses of caspase activity or apoptosis were conducted. Certain symbiont-specific (ZOOX) responses were differentially expressed under the gene function categories of zooxanthellae-cell motility, zooxanthellae-cell signaling, and zooxanthellae-response to light. Changes in these genes that are normally expressed by zooxanthellae are puzzling but may suggest that these genes serve the same function in sponges. The only potential exceptions are peridinin chlorophyll-a binding protein and peridinin chlorophyll-a-binding protein apoprotein precursor (pcp) gene. These genes are specific for plants and would require further investigation. There is the possibility that these genes serve different functions in sponges than plants, or that the sponges contain algal symbionts that also responded to the stressor.

Genes in the Stress Response (SR) functional category were differentially expressed in the DNA repair, immune response, oxidative stress, response to xenobiotic, and unknown functions (EST) in corals. The greatest number of differentially-expressed genes were in the oxidative stress group; most genes were up-regulated and most had more than one of the same gene up-regulated (glutaredoxin, copper/zinc SOD, heme binding protein 2, DNA-3-methyladenine glycosylase, multi-drug resistant protein, and microsomal glutathione S-transferase 3) (Fig. 11). Larval stress on *A. corrugata* explants appears to induce an oxidative stress response evident by the up-regulation of multiple oxidative stress response genes that are all involved in removing or breaking down oxygen radicals that are damaging to the cell (www.genecards.org).

Responses to stress by the combination of presence of larvae and elevated temperature

When two stressors were applied simultaneously, there was no significant change in stevensine concentration or gene expression. Since responses were observed with the stressors when tested separately, similar responses were expected in the combined treatment. Based on microscopic observations after treatment exposures were complete, *A. amphitrite* cyprids were not moving, had not metamorphosed when exposed to elevated temperature, and were presumed dead. Most likely, *A. corrugata* explants did not respond the same way to the combined treatment because the dead larvae posed no fouling threat to the sponges.

CONCLUSIONS

In their natural habitat, sponges are subjected to many forms of environmental pressures, including temperature stress, predation, fouling, and salinity stress. This study demonstrated that sponge explants can be useful alternatives to primary cell culture studies. Explants appear to be resilient to mechanical stress since there were only 6 differentially expressed genes when comparing the control explants to those retrieved immediately upon return to the surface. This study supports the validity of use of explants as a proxy for environmental studies of sponges.

Analyses of secondary metabolite production and differential gene expression demonstrated responses of *A. corrugata* to temperature stress, fouling stress, and the combination of both stressors. Chemical analysis of the production of stevensine by *A. corrugata* revealed that stress exerted by fouling organisms may induce an increase in production of the compound. This response was only observed with larvae stress and not elevated temperatures, suggesting it is specific for this stressor. This strongly suggests that one of stevensine's roles in nature is as an anti-foulant. This conclusion needs to be confirmed by repeating the experiment and measuring the initial sponge weights before chemical extraction to determine absolute concentration of stevensine.

This study confirmed that it is possible to observe differential gene expression of sponges when using an anthozoan-specific microarray. Temperature and larval stress on *A. corrugata* elicited significant differential gene expression responses by both stressors.

Only two significant probes, consisting of two genes, were differentially expressed when exposed to elevated temperature. This may indicate that while elevated water temperature exerts some stress in sponges, it is not an overwhelming effect for this species. The weak response to increased temperature may indicate that global warming may not be much of a threat to certain sponges.

Exposure to *A. amphitrite* larvae resulted in 70 differentially expressed genes by *Axinella corrugata*, suggesting that fouling by these larvae exerts a stronger stress response than elevated water temperatures. Increased production of secondary metabolites and differential gene expression observed in this study lays the foundation for future research on the role and biosynthesis of secondary metabolites in nature.

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